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Relationships between female quality, egg mass and eggshell blue-green colouration in southern rockhopper penguins : a test of the sexual signalling hypothesis

1 **Relationships between female quality, egg mass and eggshell blue-green colouration in**

2 **southern rockhopper penguins: a test of the sexual signalling hypothesis**

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5 Nina Dehnhard*, Rianne Pinxten, Laurent Demongin, Jeff Van Camp, Marcel Eens, Maud

6 Poisbleau

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8 *N. Dehnhard, R. Pinxten, L. Demongin, J. Van Camp, M. Eens and M. Poisbleau, Ethology*

9 *Research Group, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.*

10

11 *R. Pinxten, Department of Instructional and Educational Sciences, Research Group*

12 *Didactica, University of Antwerp, Venusstraat 35, 2000 Antwerp, Belgium.*

13

14 * corresponding author. email: nina.dehnhard@uantwerpen.be, Phone: + 32 (0)3 265.23.47

15 Fax: + 32 (0)3 820.22.71

Abstract

16

17 Eggshell blue-green colouration (BGC) is caused by the pigment biliverdin which has anti-

18 oxidant capacities. Eggshell BGC has therefore been interpreted as being costly for the

19 female, and therefore a signal of female quality ('sexual signalling hypothesis').

20   Southern rockhopper penguins *Eudyptes chrysocome* exhibit both a reversed hatching

21 asynchrony and a brood reduction strategy. First-laid (A-)eggs are smaller and hatch on

22 average one day after second-laid (B-)eggs, with B-eggs usually producing the only surviving

23 chick. According to the sexual signalling hypothesis, we predicted a positive relationship

24 between BGC and both female body mass and egg mass, and consequently within clutches a

25 stronger BGC in B-eggs than A-eggs. Furthermore, we expected a negative relationship

26 between BGC and clutch initiation date.

27   Contrasting these expectations, we found no effect of female body mass or egg mass

28 on BGC, and BGC in A-eggs increased with clutch initiation date, while there was no effect

29 in B-eggs. Within clutches, A-eggshells were more intensely blue-green coloured than B-

30 eggshells.

31   Concluding, our results appear to contradict the sexual signalling hypothesis. We,

32 however, did not measure pigment concentrations and solely relied on BGC from eggshell

33 photospectrometry, assuming that biliverdin concentrations were positively correlated with

34 BGC. We therefore caution that before to preclude the sexual signalling hypothesis, future

35 studies that include measurements of eggshell pigment concentrations in addition to BGC are

36 necessary. Altogether, a better understanding of the biological meaning of biliverdin, its

37 biochemical synthesis and anti-oxidative function in the avian body is urgently needed.

38

39 **Keywords:** Blue-green colouration, *Eudyptes chrysocome*, eggshell, hatching asynchrony,

40 sexual signalling hypothesis

Introduction

Avian eggshells exhibit a wide interspecific range of natural variation in both colouration and pigmentation, ranging from light blue to reddish-orange and from spotless to heavily spotted (Underwood and Sealy 2002; Kilner 2006; Cassey et al. 2012b). Potential explanations for this phenotypic diversity are as diverse as eggshell patterns and range from aposematism, crypsis, increased visibility in cavities, filtering solar radiation, thermal regulation, eggshell strength, egg recognition to sexual selection (reviewed in Underwood and Sealy 2002; Kilner 2006; Cherry and Gosler 2010; Riehl 2011).

Eggshell colouration and patterns are essentially determined by only two pigments, protoporphyrin (red-brown colouration) and biliverdin (blue-green colouration; hereafter BGC; Kennedy and Vevers 1976; Gorchein et al. 2009). Both pigments are derivatives of haemoglobin (Williams et al. 1994), and biliverdin has been shown to possess strong anti-oxidant capacities (Stocker et al. 1990; Kaur et al. 2003). By pigmenting their eggs with biliverdin, females are therefore removing a valuable anti-oxidant from their own body, and this should come at the cost of the females' health and survival (Moreno and Osorno 2003). The intensity of BGC has therefore been interpreted as being an honest signal of female quality to their male mates ('sexual signalling hypothesis'; Moreno and Osorno 2003). In fact, a range of both descriptive and experimental studies has demonstrated a positive relationship between the intensity of eggshell BGC and the physical condition, health state or antioxidant capabilities of females (Morales et al. 2006; Moreno et al. 2006; Siefferman et al. 2006; Krist and Grim 2007; Hanley et al. 2008; Morales et al. 2011; Navarro et al. 2011). Nevertheless, there are also several studies that did not support such associations (Cassey et al. 2008; Hargitai et al. 2008; Hanley and Doucet 2009; Honza et al. 2011; Johnsen et al. 2011), and eggshell BGC as a universal signal of female quality has thus been questioned (Riehl 2011; Cassey et al. 2012b).

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Southern rockhopper penguins *Eudyptes chrysocome chrysocome* are unique models to test

some predictions of the sexual signalling hypothesis because, like all the crested penguins

(genus *Eudyptes*), they display the unique combination of both an extreme egg size

dimorphism and a reversed hatching asynchrony. The second-laid B-eggs are about 28%

larger and heavier than the first-laid A-eggs, yet chicks from A-eggs hatch on average one

day after chicks from B-eggs (Poisbleau et al. 2008; Demongin et al. 2010). Due to the size-

dimorphism between siblings, A-chicks cannot compete for food and usually die from

starvation within few days after hatching (Gwynn 1953; Warham 1975; Poisbleau et al.

2008). However, A-eggs may serve as an insurance for the loss of the B-egg or -chick (St.

Clair and St. Clair 1996; Poisbleau et al. 2008; Dehnhard et al. 2014), and very rarely parents

manage to raise both chicks (Poisbleau et al. 2008).

Both A- and B-eggshells appear light blue-green coloured to humans. Based on this,

we used several measures of female quality to test some predictions of the sexual signalling

hypothesis: female body mass (standardized to A-egg laying date), egg mass and total clutch

mass as well as clutch initiation date (CID = A-egg laying date). Standardized female body

mass has been shown to be a reliable predictor of reproductive success in many penguin

species (Vleck and Vleck 2002; Robinson et al. 2005), including the southern rockhopper

penguin (Crawford et al. 2008). Egg mass (and size) and consequently total clutch mass in

birds is closely linked to female body mass (reviewed in Christians 2002) and a determinant

of hatchling size (reviewed in Krist 2011), which has also been shown in penguins (Reid and

Boersma 1990). Finally, CID in many bird species, including penguins (Moreno et al. 1997;

1998) is constrained by the females' ability to form eggs, with higher quality (often more

experienced) females being able to lay earlier and consequently having higher breeding

performances (Perrins 1973; Nisbet and Dann 2009; Polito et al. 2010).

91   If, as the sexual signalling hypothesis predicts, eggshell BGC is costly for females and

92   signals female quality in southern rockhopper penguins, we should observe an increase of

93   eggshell BGC with female body mass and A-egg mass, B-egg mass and consequently total

94   clutch masses. Within clutches, we therefore expect B-eggs to have a stronger eggshell BGC

95   than A-eggs since B-eggs are heavier and usually the egg that produces the only surviving

96   chick. We should furthermore observe a decrease of eggshell BGC with increasing (later)

97   CID.

Methods

Study species and study site

This study was carried out at the "Settlement Colony" on New Island, Falkland/Malvinas Islands (51°43' S, 61°18' W), from early October 2010 to mid-November 2010 (i.e. during the entire egg laying period). The colony held around 7500 pairs of breeding southern rockhopper penguins in December 2010. After the arrival of the first males (early October), we visited study sites daily, initially to mark active nests and subsequently to monitor egg laying dates. Laying period ranged from Oct 27th (first A-egg) until Nov 9th (last B-egg). Within clutches, A-eggs were laid on average four days (mean ± SD = 4.2 ± 0.5, N = 85 clutches) before B-eggs. A total of 170 eggs from 85 clutches were colour measured (details see below) and subsequently weighed with a digital pocket balance (CM 320-IN, Kern, Germany; accuracy of 0.1 g), all within 24 hours after laying.

Sixty out of the 85 study nest females were weighed to the nearest 10 g (digital spring balance) on the day they laid their first egg and their clutches were collected for the purpose of other studies after egg colouration was measured. The other 25 females were weighed two to three times between October 12th and November 22nd, but not on the day they laid their first egg. We therefore used linear regressions to extrapolate female body mass at A-egg laying. We corrected female body mass by removing A-egg mass for captures before A-egg laying and by adding B-egg mass for captures after B-egg laying. We then calculated linear regressions individually for every female (all $R^2 \geq 0.99$) and corrected body masses according to the individual slopes (average gradient -33.3 g ± 2.7 SD mass loss per day).

Reflectance spectrophotometry

Eggshell reflection was measured using a portable Ocean Optics JAZ Spectrophotometer (range 320−700 nm) connected to a bi-furcated encased fiber optic probe. Reflectance was

123 measured perpendicular to the surface while illuminated with a build-in pulsed xenon lamp

124 relative to a diffuse white standard (WS1-SL, Ocean Optics Inc.). To minimize measurement

125 error, dark and white standard reflectance calibration measures were taken regularly during

126 sampling. We took three measurements from the blue-green background eggshell colouration

127 at every area (blunt, equator and point) of the eggshell. Similar to previous studies (e.g.

128 Morales et al. 2006; Siefferman et al. 2006; Cassey et al. 2008), we calculated reflectance-

129 based eggshell colouration using an index of BGC as the proportion of total reflectance in the

130 blue-green wavelength region ($R_{410–575}$) across the total spectrum ($R_{410-575}/R_{320–700}$). This is

131 suggested to correspond to the region of highest reflectance of the pigment biliverdin

132 (Falchuk et al. 2002) and to be a useful metric of eggshell BGC (e.g. Moreno et al. 2006;

133 Siefferman et al. 2006).

134 Notably, we did not measure biliverdin concentrations in the eggshell but assumed

135 that the spectral measurement of eggshell BGC would reflect the biliverdin concentration.

136 This relationship has been shown previously for two bird species with immaculate and (light)

137 blue-green eggs (Moreno et al. 2006; López-Rull et al. 2008; Morales et al. 2013), and thus

138 eggs which in their appearance resemble those of rockhopper penguins.

139 All spectral measurements were performed by the same observer (JVC), and while

140 covered by a dark cloth to avoid direct sunlight. We calculated repeatabilities in eggshell

141 BGC between areas (blunt, point and equator) using REML-based linear mixed models as

142 described in Nakagawa and Schielzeth (2010), in the rptR package (Schielzeth and Nakagawa

143 2013) in the program R (see details below). BGC were repeatable between eggshell positions

144 for both A-eggs (R = 0.47 ± 0.03 SE, p < 0.001) and B-eggs (R = 0.68 ± 0.04 SE, p < 0.001).

145 Since it is easier to find a clean spot and because eggshell measurements are done faster (with

146 the same accuracy) in the area around the equator than on the blunt and pointy ends, we used

147 only the measurements taken at the equator. BGC of the three measurements at the egg

148   equator in each sampled egg were highly repeatable both in A-eggs (R = 0.79 ± 0.04 SE, p <

149   0.001) and B-eggs (R = 0.98 ± 0.00 SE, p < 0.001). We therefore used the average values of

150   the three measurements at the equator for the statistical analyses on eggshell BGC.

151

152

153   *Statistical analysis*

154   We fitted a linear mixed effect model (LMM) to test some predictions of the sexual signalling

155   hypothesis. We first tested for correlations between potential covariates, and found a

156   significant correlation between female body mass and CID (Pearson's R = -0.24, p = 0.030, N

157   = 85 clutches), but no significant relationship between either female body mass and total

158   clutch mass (Pearson's R = 0.10, p = 0.366, N = 85 clutches) or total clutch mass and CID

159   (Pearson's R = 0.13, p = 0.236, N = 85 clutches). We furthermore determined variance

160   inflation factors (VIFs) to rule out possible issues with collinearity in the models. VIFs were

161   calculated in the package car (Fox and Weisberg 2010) for the linear model with all main

162   effects (interactions not included). VIFs were ≤ 2.35 and therefore did not indicate issues

163   with collinearity (Zuur et al. 2010). The global LMM was run on eggshell BGC as dependent

164   variable with nest as random factor, egg type (fixed factor: A- or B-egg), female body mass

165   (covariate), total clutch mass (covariate) and CID (covariate) as explanatory variables. We

166   furthermore included all possible two-way interactions between egg type and the covariates

167   into this global model. We conducted backwards-stepwise model selection (for the fixed

168   effects only), removing those explanatory variables that were not significant, commencing

169   with the interaction terms. In case that an interaction term with egg type was significant, we

170   proceeded with separate linear models (LMs) for A- and B-eggs (and therefore without any

171   random factor). We used CID in models for both A- and B-eggs, as the laying date of the B-

172   egg was strictly linked to the laying date of the A-egg (= CID) (Pearson's R = 0.98, p <

173   0.001, N = 85 clutches). As total clutch mass might inadequately account for the differential

174   investment of females in A- and B-egg mass, we additionally ran LMs for the relationship

175   between BGC (dependent variable) and egg mass (explanatory variable), separately for A-

176   and B-eggs.

177   We furthermore preformed a paired t-test for the entire dataset (N = 85 clutches) to

178   test for differences between A- and B-eggs within clutches. All statistical analyses were

179   performed in R (version 3.1.1; R Development Core Team 2014). LMMs and LMs were fit

180   using restricted maximum likelihood (REML), and all models were performed in the package

181   lme4 (Bates et al. 2011). We present t-values from model summaries. P-values were obtained

182   by comparing the model with the variable in question with the model without this variable

183   (and models were fit with maximum likelihood for this procedure). We further present both

184   marginal R^2 values (based on the variance explained only by fixed effects) and conditional R^2

185   values (based on the variance explained by both fixed and random effects) for the final LMM,

186   calculated following Nakagawa & Schielzeth (2013).

Results

187 **Results**

188 The reflectance spectra of southern rockhopper penguin eggs have a bimodal shape: the

189 major peak of reflectance is found in the blue-green part of the spectrum (~520 nm) and a

190 minor peak is found in the UV part of the spectrum (~330 nm) (Fig. 1).

191

192 LMMs showed that eggshell BGC was neither affected by the interaction between egg type

193 and female body mass (LMM: $t = -0.459$, $p = 0.638$), nor by the interaction between egg type

194 and total clutch mass (LMM: $t = 0.895$, $p = 0.833$). Contrasting the sexual signalling

195 hypothesis, the effects of female body mass (LMM: $t = 0.087$, $p = 0.929$) and total clutch

196 mass (LMM: $t = -0.546$, $p = 0.580$) were also not significant. These interaction terms and

197 variables were stepwise removed from the global model. When tested separately in A- and B-

198 eggs, BGC did not correlate with individual egg mass either (LM: $t = -0.43$, $p = 0.668$ and $t =$

199 0.49 and $p = 0.626$ for A-eggs and B-eggs, respectively; Fig. 2), confirming the earlier result

200 that total clutch mass was not related to BGC. Nevertheless, within clutches, A-eggs had

201 eggshells with a significantly stronger BGC than B-eggs (paired t-test (one-tailed): $t_{84} = 9.91$,

202 $p < 0.001$; Fig. 3). This was observed in 76 out of 85 clutches.

203   The final LMM therefore included egg type, CID and the interaction between egg

204 type and CID as explanatory variables for eggshell BGC. These variables together explained

205 39.0% of the total variance in eggshell BGC (marginal R^2-value). In contrast, nest as a

206 random factor added comparatively little explanatory power (conditional R^2-value: 44.7%).

207 Because of the significant interaction between egg type and CID in this final model (LMM: t

208 $= -2.785$, $p = 0.006$), we continued with separate analyses for A- and B-eggs. In A-eggs,

209 BGC increased with CID (LM with CID as only explanatory variable: $t = 3.338$, $p = 0.001$;

210 Fig. 4), while there was no effect of CID on BGC in B-eggs ($t = -0.330$, $p = 0.743$; Fig. 4).

211

Discussion

The sexual signalling hypothesis states that eggshell BGC should be an honest signal of female quality to their mates, as depositing biliverdin into the eggs is costly for the females (Moreno and Osorno 2003; Moreno et al. 2005; Morales et al. 2006). We therefore tested whether eggshell BGC increased with measures of female quality and differed between A- and B-eggs in crested penguins. We predicted a positive effect of female body mass on BGC, and that BGC would further increase with egg mass and total clutch mass. Due to the egg-size dimorphism, we consequently expected to find a stronger BGC in B-eggs than A-eggs. However, we found no effect of female body mass, egg mass nor total clutch mass on eggshell BGC. Unexpectedly, within the same clutch, A-eggs had a stronger BGC than B-eggs. Finally, the intensity of BGC increased with CID in A-eggs, while there was no effect on B-eggs. Altogether, our results appear to contradict the sexual signalling hypothesis and rather provide evidence for a negative association between BGC and female quality in southern rockhopper penguins.

Our results are in line with several previous studies in other bird species that either found no relationship or the opposite effect as expected for eggshell BGC and either female body mass, egg mass/total clutch mass and laying dates (Cassey et al. 2008; Hargitai et al. 2008; Hanley and Doucet 2009; Johnsen et al. 2011; Cassey et al. 2012a). Similarly, the literature shows no consistent relationship between eggshell BGC and laying order: BGC either increased (Siefferman et al. 2006; Hargitai et al. 2008), or decreased with laying order (Krist and Grim 2007; Johnsen et al. 2011; Morales et al. 2011), and in one study the middle egg was the most chromatic one (Hanley and Doucet 2009). Therefore, our data agree with the overall literature and once more speak against the ubiquitous application of the sexual-signalling hypothesis (Riehl 2011). As female body mass, clutch mass and CID have been reliable indicators of female quality in other birds, including penguins (e.g. Perrins 1973;

237   Nisbet and Dann 2009; Polito et al. 2010), it appears unlikely that none of them would signal

238   female quality in southern rockhopper penguins. On the other hand, a female's antioxidant

239   capacities and thus ability to deposit biliverdin into her eggs might not be reflected by its

240   body mass but might necessitate the measurement of plasma antioxidants in the female's

241   blood (Morales et al. 2008). Our study is limited in this regard, as we did not measure the

242   females' antioxidant levels, and we are furthermore limited to correlative data. Nevertheless,

243   our data showed an increase in BGC with CID in A-eggs, and therefore the opposite effect as

244   expected under the sexual signalling hypothesis.

245   Finally, our observation that BGC was stronger in A-eggshells compared to B-

246   eggshells within the same clutch also appears to contradict the sexual signalling hypothesis.

247   Based on the egg mass differences between A- and B-eggs we had expected to find a stronger

248   BGC in B-eggs. Under the assumption that egg mass would not reflect female quality and this

249   would explain the lacking relationship between egg mass and BGC as discussed above, we

250   would therefore have expected to find no difference in BGC between A- and B-eggs. The

251   finding of a stronger BGC in A-eggshells than B-eggshells is therefore inconsistent in either

252   way. Moreover, considering that in most clutches only the chick originating from the B-egg

253   survives until fledging (Strange 1982; Poisbleau et al. 2008), it appears counterintuitive that

254   females apparently invest more of a costly pigment into those eggs that usually fail to

255   produce a chick (also see Poisbleau et al. 2011a, 2011b).

256

257   To the best of our knowledge, this has been the first study on BGC in penguin eggs. It would

258   be highly interesting to see whether there is evidence for or against the sexual signalling

259   hypotheses in those penguin genera that do not show a reversed hatching asynchrony. To

260   conclude, our results add to the growing amount of evidence against the sexual signalling

261   hypothesis and once more raise the question why female birds lay – to the human eye –

262   peculiarly blue-green coloured eggs. Importantly, however, the majority of publications that

263   tested the sexual signalling hypothesis, including ours, were based on the assumption that

264   spectrophotometric measurements of the BGC are positively correlated with biliverdin

265   concentrations. This relationship has indeed been shown in two species with spotless, blue-

266   green coloured eggs (Moreno et al. 2006; López-Rull et al. 2008; Morales et al. 2013), thus

267   eggs which resemble rockhopper penguin eggs in appearance. Recently, however, Cassey et

268   al. (2012a) have raised the concern that at least in spotted eggshells biliverdin concentrations

269   might not correlate well with spectral measurements of BGC. We therefore caution that

270   although our results appear to contradict the sexual signalling hypothesis, we cannot refute

271   this hypothesis with certainty. We therefore recommend that future studies on eggshell

272   colouration should include eggshell pigment concentrations in addition to spectrophotometric

273   measurements. In addition, a better understanding of the biochemical pathway of the

274   biliverdin synthesis and its anti-oxidative function in the avian body appears crucial to

275   interpret the costs and benefits for females to produce coloured eggs.

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449 **Fig. 1.** Mean spectral reflectance of southern rockhopper penguin eggs (displayed is the

450   average per clutch). Reflectance spectra were averaged at 10 nm intervals. Vertical bars

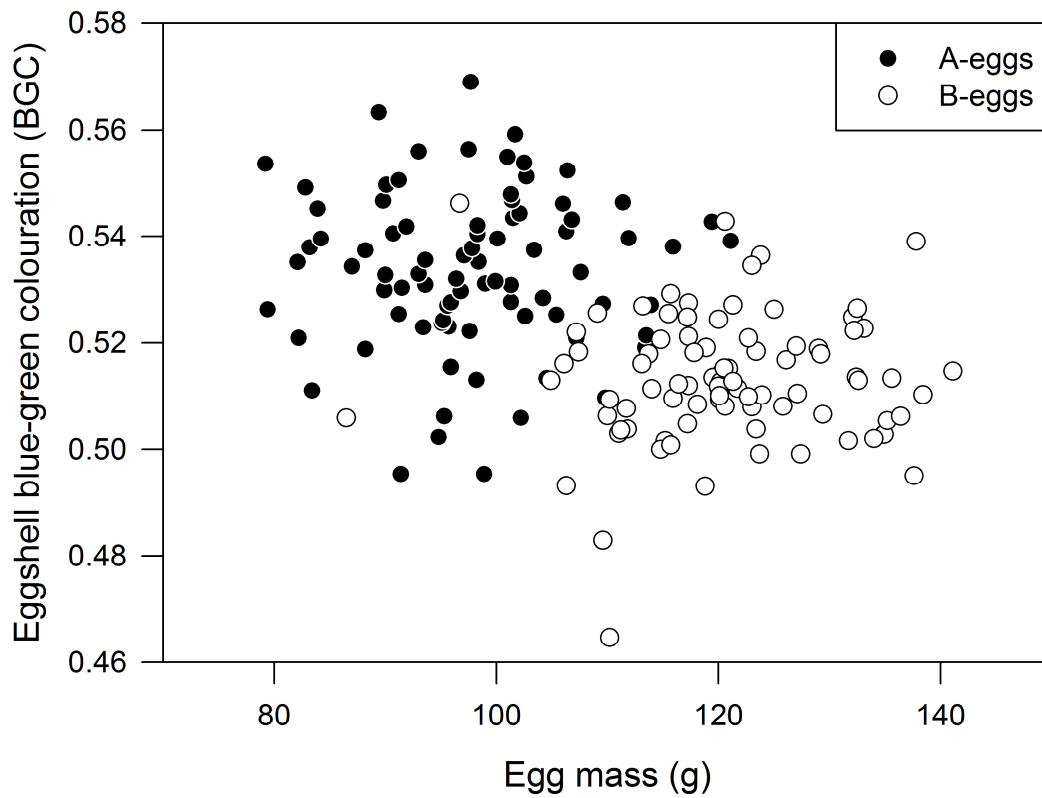451   denote ± 95% confidence intervals. Sample sizes were N = 85 clutches.

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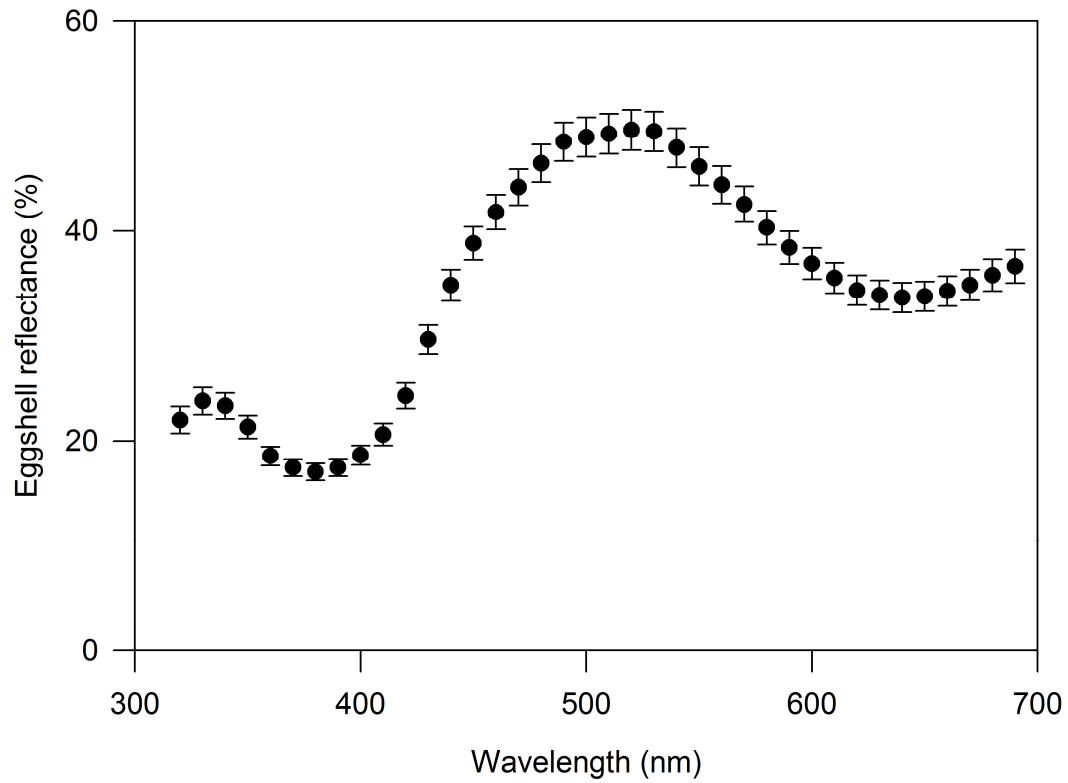
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455 **Fig. 2.** Relationship between individual egg mass and intensity of eggshell blue-green

456 colouration (BGC) in A- and B-eggs. Sample sizes were N = 85 for both A- and B-eggs.
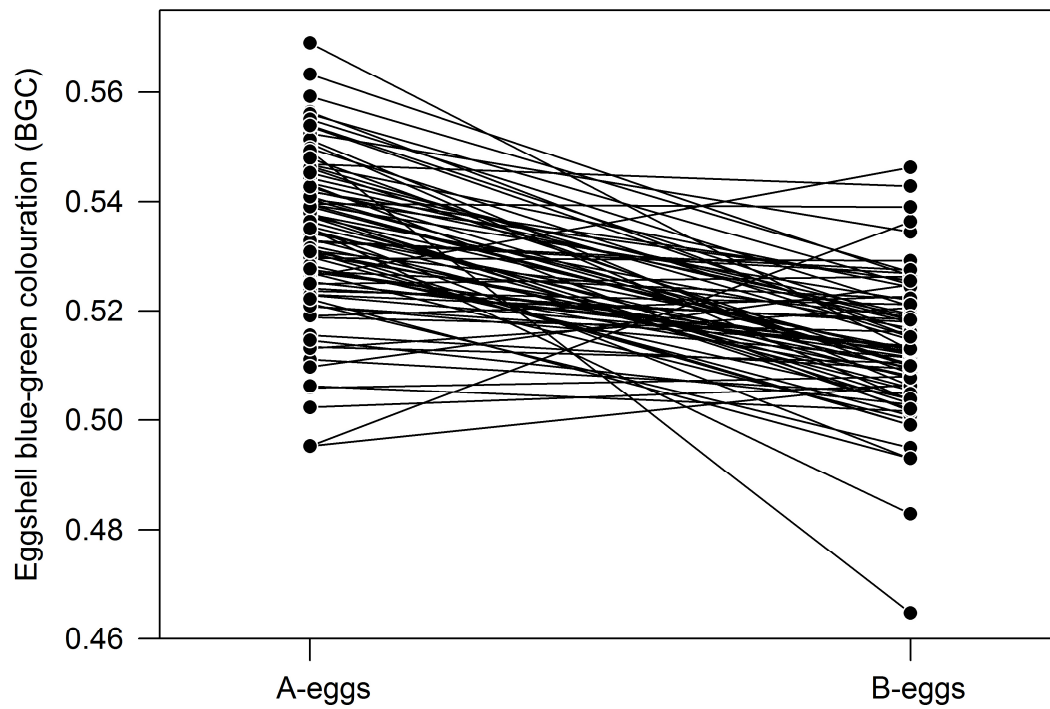
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460 **Fig. 3.** Intensity of eggshell blue-green colouration (BGC) in A- and B-eggs of southern

461 rockhopper penguins. Lines represent the connection between eggs from the same clutch.

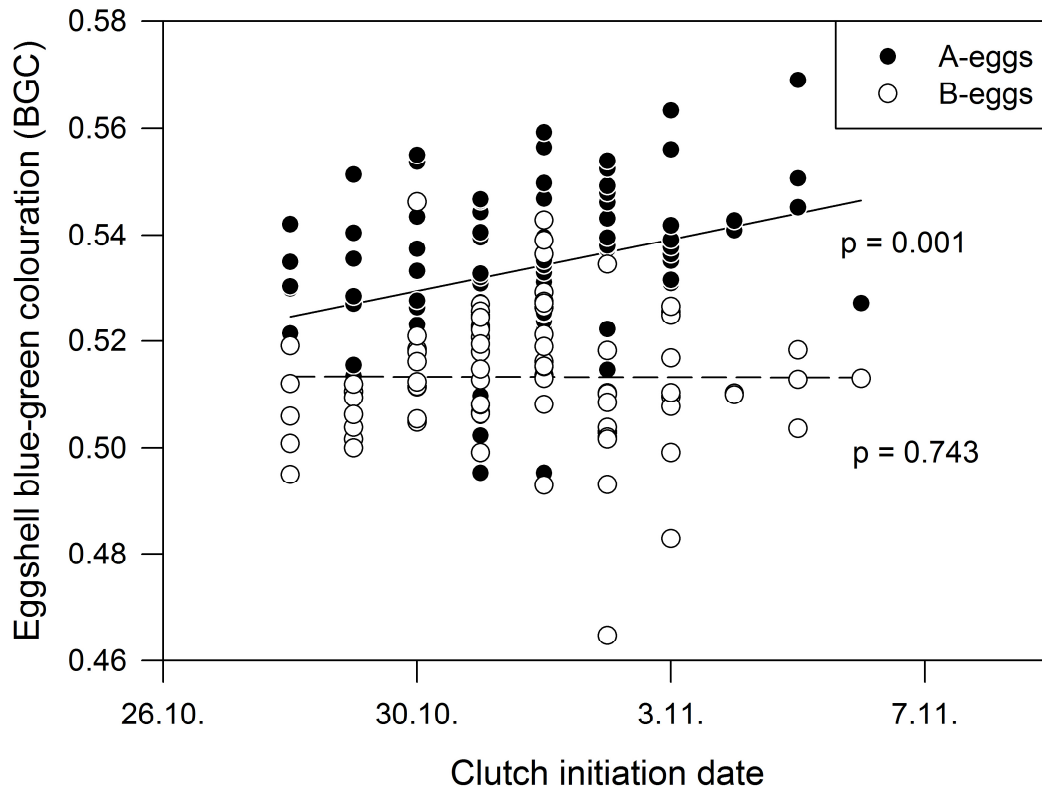462 Sample sizes were N = 85 for both A- and B-eggs.

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466   **Fig. 4.** Relationship between clutch initiation date (CID) and intensity of eggshell blue-green

467   colouration (BGC) in A- and B-eggs. We present linear regression lines to visualize the effect

468   of CID (even though not significant for B-eggs). P-values were obtained from linear models

469   conducted separately for A- and B-eggs. Sample sizes were N = 85 for both A- and B-eggs.

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