1   Running headline: Soler et al.: Nestling colour, detectability or quality?

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3   IS THE RELATIONSHIP BETWEEN COLOUR AND IMMUNE RESPONSE

4   MEDIATED BY NUTRITIONAL CONDITION IN SPOTLESS STARLING

5   NESTLINGS?

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27 The hypothesis that nestling colouration plays a central role in parent-offspring

28 communication, because it influences parental feeding decisions, has recently received

29 strong experimental support. In the European starlings (*Sturnus vulgaris*) and Alpine

30 swifts (*Apus melva*), manipulation of ultraviolet (UV) reflectance of nestling mouth and

31 skin affected amount of food provided by parents, and, furthermore, skin brightness of

32 starling nestlings predicted their T-cell mediated immune response. Therefore, a link

33 between nestling colouration and immunity, mediated by parental effort, was suggested.

34 Here, we further explore this hypothesis by experimentally feeding some spotless

35 starling (*Sturnus unicolor*) nestlings while leaving others in the same nest as control.

36 First, we found a significant effect of food supplementation on nestling immune

37 response, which is a requirement for the hypothesis. Secondly, we confirmed in spotless

38 starlings the association between skin brightness and ability to raise an immune

39 response. However, this correlation disappeared when controlling for between-nest

40 variation. These results suggest that parental feeding preference is not the only factor

41 explaining nestling immunity, and that covariation between mean brood nestling

42 colouration and parental quality, and/or intrinsic (i.e. genetic) quality of nestlings, may

43 explain the association between immunity and colouration of nestlings. Finally, within-

44 nest variation in nestling colouration partially explained immune responses because the

45 effect of experimental food-supply was larger for nestlings with high values of skin-

46 brightness. We discuss these results as a possible evidence of nestling colouration

47 partially reflecting intrinsic characteristics that affects both ability to produce efficient

48 immune responses and parental feeding preferences.

49

50 Key words: Parent-offspring communication, PHA, signals of need, T-cell mediated

51 immune response, UV colouration.

52   Introduction

53   Nestling colouration has received special attention during recent years mainly because it

54   may play an important role in parent-offspring communication. The most conspicuous

55   traits that unfeathered nestlings display to their parents are flanges and mouth cavity,

56   and parents may prefer to feed nestlings with the most conspicuous traits (e.g., Kilner &

57   Davies 1998). This preferential feeding by parents of the most conspicuous nestlings

58   has recently received experimental support (Heeb et al. 2003; Jourdie et al. 2004; but

59   see Tschirren et al. 2005), and can be predicted from parents preferentially feeding

60   nestlings that are more easily detectable, and/or of better phenotypic quality. For

61   instance, red colour may be attractive for parents because it could reflect nestling's level

62   of hunger, or because it could be carotenoid-based and thus indicate health status of

63   their offspring (Kilner 1997; Saino et al. 2000a; Saino & Møller 2002). However,

64   parental feeding decision in relation to phenotypic quality of their offspring may be

65   context dependent and, for instance, vary through the breeding the breeding season

66   (Bize et al. 2006).

67   Nestling colouration may also serve as a cue for locating chicks at the nest.

68   Depending on light and other environmental conditions (i.e. background colour) at the

69   nest, some colours are more easily detected than others. For instance, in dark conditions

70   such as those in hole-nests, yellow is more easily detected than red (Heeb et al. 2003).

71   Additionally, spectral irradiance of nest background is usually minimum at the

72   ultraviolet wavelength (300-400 nm) (Hunt et al. 2003; Jourdie et al. 2004) and, thus,

73   nestlings may reach maximum contrast (i.e., conspicuousness) by showing a peak of

74   reflectance at those wavelengths (Hunt et al. 2003). In accordance with the importance

75   of nestling's conspicuousness, reflectance spectra of nestling mouth and flanges in most

76   species studied to date show a peak in the ultraviolet (Hunt et al. 2003). More

77   importantly, the manipulation of UV reflectance in body skin and flanges of nestling

78   starlings (*Sturnus vulgaris*) resulted in a differential increase in body mass of UV-

79   reflecting nestlings just two hours after the experiment (Jourdie et al. 2004).

80   Whatever the reasons why parents prefer to feed nestlings with a particular

81   colouration (Kilner 1997), such preferential food allocation by parents predicts a

82   relationship between nestling colour and phenotypic quality of fledglings. In accordance

83   with this, Jourdie et al. (2004) found a positive relationship between T-cell mediated

84   immune response and brightness of skin reflectance including both ultraviolet and

85   visible light in starling (*Sturnus vulgaris*) nestlings. However, evidence for a causal link

86   between UV skin reflectance and condition in offspring is still lacking. Since nestling's

87   immune response is a trait that depends on nutritional condition (e.g., Saino et al. 1997;

88   Alonso-Alvarez & Tella 2001; De Neve et al. 2004), the above relationship between

89   brightness and immunity might be mediated by parents preferentially feeding nestlings

90   with more UV reflectance (Jourdie et al. 2004). In addition, the relationship between

91   nestling phenotypic quality and colouration can also be due to skin reflectance of

92   nestlings signalling nestling's immunity and parent feeding nestlings in relation to the

93   expression of the signal, which should in turn brighten skin further. A significant

94   genetic component of immune response has been detected for nestlings of several

95   species (Saino et al. 1997; see, Soler et al. 2003b, and references therein).

96   Consequently, a genetic correlation between immune response and nestling colouration

97   might explain not only the relationship between these two traits, but also feeding

98   preferences by parents. In any case, if parents differentially feed nestlings with a

99   particular colouration, and these nestlings experience an improvement in their immune

100   response, a direct link between nestling colouration and fitness can be established, since

101 immunocompetence is a major predictor of nestling survival and recruitment (Christe et

102 al. 2001; Møller & Saino 2004; Moreno et al. 2005; Cichon & Dubiec 2005).

103   Here, we explore the hypothesis that, because of the parental feeding

104 preferences, nestling colour influence nestling nutritional condition, and that this

105 preferential feeding are responsible for the relationship between colouration and T-cell

106 mediated immune response (hereafter PHA response) of nestlings. To test this

107 hypothesis we performed an experiment to test the effect of food supplementation on

108 the immune response of nestlings. The hypothesis predicts a positive relationship

109 between immune response and nestling colouration, as reported by Jourdie et al. (2004).

110 This relationship could be mediated exclusively by the nutritional condition of

111 nestlings, but there may also be some intrinsic genetically-determined potential for

112 immune system development that reflects the reproductive value of offspring (Kilner

113 1997; Saino et al. 2000b). We explore these possibilities by investigating the effects of

114 nestling colouration and experimental treatment on variation in immune response within

115 and between nests.

116

117 Material and Methods

118 The study was carried out in Guadix (37º18'N, 3º11'W), south-eastern Spain, during the

119 breeding season of 2005 (April – June), in nest-boxes recently (February 2005) installed

120 close to or within colonies of spotless starlings already established in old buildings of

121 the area. The species is polygynous (Veiga et al. 2001), clutch size typically of 4-5 eggs,

122 and with nestlings usually hatching asynchronously (last egg hatching up to 24 hours

123 after others) (Cramp 1998). Nestlings are fed mainly with insects (Motis et al. 1997) by

124 females and, sometimes, also by males (Veiga et al. 2002).

125

126   Experimental procedure

127   Three days after the first nestling hatched (i.e. when nestlings where 2-3 days old), each

128   hatchling was weighed and marked with a permanent-colour marker on the tarsus.

129   Hatchlings were ranked according to body mass within each nest. While the heaviest

130   nestling was randomly assigned to the food (experimental) or water (control)

131   treatments, treatment of the other chicks in the same nest alternated according to body

132   mass rank. The dose of the food treatment consisted of 0.2 ml of calorie-rich pasta,

133   loaded with essential micronutrients (minerals, vitamins, and amino acids; 5 calories per

134   gramme; Nutri-Calorías, Shering-Plough Animal Health), used as a strong calorie and

135   nutritional supplement by veterinarians. Water treatment simply consisted in 0.2 ml of

136   mineral water. Subsequently, nests were revisited every second day (5 visits in total),

137   and during each visit tarsi were re-coloured and nestlings consistently provided with

138   food or water treatment. One possible problem of this experimental approach was that

139   feeding experimentally some nestlings might provoke that these nestlings were

140   demanding less food from their parents and, consequently, the parents might allocate

141   more food to the rest of the brood. Thus, it would be possible that all nestlings in the

142   brood were effectively receiving additional food. However, this effect would be

143   conservative in the sense that it would reduce any difference between the treatment and

144   control. Therefore, although non-significant effects of this experiment on nestling traits

145   should be considered cautiously, a significant effect on a target trait will indicate that its

146   expression depends on the nutritional conditions experienced by nestlings during

147   development.

148   About four days before fledging, i.e. when they were 13-14 days old, nestlings

149   were ringed, weighed (with a Pesola spring balance, accuracy 0.5 g), and measured

150   (tarsus length with a digital calliper to the nearest 0.01 mm, wing and tail length with a

151   ruler to the nearest mm). Moreover, all nestlings were injected subcutaneously with

152   phytohemagglutinin-P (PHA-P, Sigma Chemical Co.) in the wing web to evaluate the *in*

153   *vivo* T-cell mediated immune response following standardized protocols (e.g., Cheng &

154   Lamont 1988; Lochmiller et al. 1993; Soler et al. 2003a). Briefly, after we measured

155   wing-web thickness (with a Mitutoyo digital pressure-sensitive micrometer, model ID-

156   CI012 BS to the nearest 0.01 mm), we injected fledglings subcutaneous in the right

157   wing web with 0.2 mg of PHA dissolved in 0.04 ml of physiological saline solution

158   (Bausch & Lomb Co.). The left wing web was injected with 0.04 ml of physiological

159   saline solution. We measured the thickness of each wing web at the injection site before

160   and 24 hours after the injection and estimated T-cell mediated immune response as the

161   change in thickness of the right wing web (PHA injection) minus the change in

162   thickness of the left wing web. We repeated measurements of thickness of each wing

163   web three times, which was highly repeatable (Repeatability = 97.2%, $F_{97,196}$ = 106.7, P

164   < 0.0001) and, thus, the mean value was used in subsequent analyses.

165   Experimental procedures were licensed by the "Consejería de Medio Ambiente,

166   Dirección General de Gestión del Medio Natural de la Junta de Andalucía". Our

167   experiment, as well as the visitation rate of nests, did not cause nest desertion because

168   no single nest in which we measured nestling colour was deserted. The PHA injection is

169   now a routine in studies of ecological immunology and it is assumed that it does not

170   affect nestling survival (Merino et al. 1999). In accordance, only one of the nestlings

171   that we injected with PHA died within the following 24 hours, but it was due to its very

172   bad physical condition (body mass of dead nestling: 41g; mean body mass of nestlings

173   of the same age in our studied population (SD): 75.8 (9.38)). Moreover, our food

174   provisioning affected positively the level of T-cell-mediated immune response of

175   experimental nestlings, which is a good predictor of recruitment (see Moreno et al.

176   2005). The treatment did not affect probability of survival (total nestlings died = 26

177   (17.8%) (experimental: 14, control 12), Chi-square = 0.26, df = 1, P = 0.61), which

178   result smaller than that reported by Cramp (1998) for natural cavities (29.0 %) and nest

179   boxes (minimum = 21.7%). Therefore, our study did not affect starling welfare.

180

181   Estimating colour of nestlings

182   Following the protocol of Jourdie et al. (2004), when nestlings were 4-5 days old

183   (just before performing the second experimental feeding), we measured nestling

184   coloration on mouth, the surrounding flanges, and head skin of all nestlings of a nest.

185   Reflectance spectra (300-700 nm) were recorded using an Ocean Optics equipment

186   [S2000 spectrometer connected to a deuterium-halogen light (D2-W, mini) by a coaxial

187   reflectance probe (QR-400-7-UV-vis) and the OOIBase32^{TM} operating software (Ocean

188   Optics, Inc. Dunedin, FL, USA)]. Reflectance was always measured with the probe

189   placed at a constant distance and reaching the object at 45º. Measurements were relative

190   and referred to a standard white reference (WS-2) and to the dark, which we calibrated

191   before measurement of each nestling.

192   We measured mouth colour by gently keeping the gape open and introducing the

193   probe to the centre of the upper mouthpart. Flanges, however, were measured

194   maintaining nestlings with the mouth almost closed, and placing the probe on the angle

195   of the mouth-flanges, thus, avoiding confusion with mouth colouration. We decided to

196   differentiate between mouth and flange colouration because different functions in

197   parent-offspring communication have been suggested (see, Kilner & Davies 1998).

198   Finally, skin colouration was measured at the head, close to the ear, trying to avoid

199   growing feathers. All colour measurements were repeated three times and variation

200 between nestlings was larger than variation within nestlings (Repeatability > 55%, $F_{162,}$

201 $_{325}$ > 4.7, P < 0.0001), justifying the use of mean values per nestling.

202   For each nestling we calculated the average values of their spectra for mouth,

203 flanges and skin. From these spectra, and following methodology described in Jourdie

204 et al. (2004), we calculated median maximal value of UV reflectance (M1: median (320

205 and 360 nm)), median baseline reflectance value (M2: median (440 and 480 nm)) and

206 the median reflectance in the visible spectrum (M3: median (540 and 700 nm)). Spectral

207 brightness of mouths and flanges was then obtained as (M1 – M2) + (M3 – M2), while

208 spectral brightness of head skin was calculated as (M1 – M2) + (M3 – M2) + (M1 –

209 M3). In addition, we also estimated the percentage of UV (300-400 nm) reflectance in

210 relation to that of the complete spectrum (300-700 nm), which significantly correlated

211 with estimates from Jourdie et al. (2004) methodology (R > 0.35, N = 106, P < 0.0001).

212 Alternative approaches to measuring nestling colouration have been used in other

213 studies (Hunt et al. 2003; Bize et al. 2006) but we have followed Jourdie at al.'s (2004)

214 methods in order to replicate their results and resolve the underlying mechanism.

215

216   Statistical tests

217 The frequency distributions of morphological (body mass, and tarsus and wing lengths)

218 and coloration variables did not differ significantly from normal distribution, and, thus,

219 we used parametric statistical tests. Specifically, in order to analyse variation in nestling

220 immune response we used general linear models (GLM) with experimental treatment as

221 a fixed factor and nest identity as a random factor. Nestling colouration was introduced

222 as a covariate in the model when trying to explore its relationship with immune

223 response after controlling for the effect of the experiment and nest identity. Moreover

224 we also introduced in the model the interaction between nest identity and treatment,

225   which is a random factor that tests for differences in treatment effects between nests.

226   Finally, the interaction between nestling colouration and treatment effect was also

227   introduced in the model to test for a possible differential effect of food-supplementation

228   depending on nestling colouration.

229   To evaluate whether the relationship between nestling colouration and immune

230   response was mainly due to between- or within-nest covariation of these two variables,

231   we run GLMs with type I and III decomposition of sums of squares. While in type III

232   decomposition of sums of squares (orthogonal estimated effects) the order in which the

233   factors are introduced in the model does not affect the estimation of their effects on the

234   dependent variable, the use of type I implies that the effect of a target factor is estimated

235   after controlling for the effect of previous factors on the dependent variable (e.g.,

236   Statsoft 2001). Therefore, if the effect of nestling colouration on immune response

237   varied depending on either the use of type I or III decomposition error, or position of

238   factors in the models (i.e. before or after nest identity), this would suggest that the

239   relationship between nestling colouration and immune response was mainly due to

240   covariation between these variables at the nest level.

241   Information of all studied variables was collected for 106 nestlings from 39

242   nests. All statistical tests were performed with the software Statistica 6.0 (Stafsoft

243   2001).

244

245   Results

246

247   Reflectance spectra of skin, mouth and flanges of spotless starlings are shown in Fig. 1,

248   being very similar to those previously published for the starling *Sturnus vulgaris*

249   (Jourdie et al. 2004). In agreement with previous work on the closely related starling,

250   we found a positive relationship between brightness of skin and level of T-cell mediated

251   immune response in spotless starling nestlings (using mean brood values as independent

252   data points; regression analysis, beta(SE) = 0.36(0.15), t_{38} = 2.37, P = 0.023). However,

253   no other colour variable (spectral brightness of flanges and mouth, see Material and

254   Methods) explained significant amounts of variation in nestling immune response

255   (mean brood values as independent data points; $0.05 < R < 0.14$, $P > 0.37$). Moreover,

256   morphological variables of nestlings (body mass, and wing and tarsus length) were not

257   significantly correlated with any of the nestling colour variables used (body mass: 0.03

258    $< R < 0.26$, $P > 0.10$; tarsus length: $-0.14 < R < -0.03$, $P > 0.4$; wing length: $-0.20 < R <$

259   0.16, $P > 0.28$). Therefore, in subsequent analyses we only used skin brightness as a

260   measure of nestling colouration, and level of T-cell-mediated immune response as a

261   measure of nestling phenotypic quality.

262

263   FIG. 1 AND 2 ABOUT HERE

264

265   Experimental food supplementation significantly affected nestling immune

266   response (GLM, type III decomposition of sums of squares; nest identity as a random

267   factor and food-supply treatment as a fixed factor; the interaction between nest identity

268   and treatment was also included in the model) ($F_{1,37.9}$ = 4.92, P = 0.033), but not other

269   traits such as body mass ($F_{1,34.6}$ = 0.12, P = 0.73), and tarsus ($F_{1,37.6}$ = 0.93, P = 0.34)

270   and wing length ($F_{1,29.3}$ = 1.17, P = 0.29). Food-supplemented nestlings showed larger

271   immune response (mean = 0.66 mm, SE = 0.03) than control nestlings (mean = 0.59

272   mm; SE = 0.03). Moreover, between-nest variation in the level of immune response was

273   larger than within-nest variation ($F_{38,34}$ = 3.13, P = 0.0005). The effect of the experiment

274   was similar in most starling nests (interaction between nest identity and experimental

275 treatment, $F_{32,34} = 0.84$, $P = 0.68$), which validates our experimental approach. Finally,

276 these results were independent of brood size since the effect of our experiment did not

277 vary in relation to brood size (GLM, similar to that explained before but including the

278 interaction between food treatment and brood size in the model, $F_{2,40.5} = 1.96$, $P = 0.15$).

279 When brightness of nestling skin was introduced as a covariate in the previous

280 model, it did not explain a significant proportion of variance in nestling immune

281 response (Table 1). This seemed to contradict the significant association between these

282 two variables reported above. However, in this last analysis, between-nest variation in

283 nestling colouration was statistically controlled by including nest identity in the model,

284 and brightness of nestling skin significantly varied among nests ($F_{38,67} = 3.35$, $P <$

285 0.0001). This means that a particular nestling was more similar in colouration to its

286 nest-mates than to nestlings from other nests. Therefore, it is possible that between-nest

287 covariation in nestling colouration and immunity explained the detected association

288 between these two variables. In accordance with this interpretation, when running the

289 above model but using a type I decomposition of sums of squares (this approach

290 estimates the contribution of all factors in the model taking into account the order of the

291 factors) and introducing skin brightness before nest identity in the model (i.e., the

292 covariate was not controlled for between-nest variation; see Material and Methods), all

293 variables explained a significant proportion of residual variance in nestling immune

294 response (Table 1). However, when nest identity was the first variable introduced in the

295 model (and thus all other factors were controlled for nest identity), the effect of skin

296 brightness was no longer significant (Table 1). These results suggest that the

297 relationship between skin colouration and nestling immune response was mainly due to

298 between-nest covariation of the two variables, while within-nest variation in immune

299 response is better explained by experimental treatment.

300

301   TABLE 1 ABOUT HERE

302

303   Finally, in accordance with the importance of within-nest variation of skin

304   brightness in explaining immune response of nestlings, we found that the interaction

305   between experimental treatment and nestling-skin brightness explained a significant

306   proportion of the variance in nestling T-cell mediated immune response (Table 1). The

307   effect of the experiment was larger in nestlings with greater brightness of the skin (Fig.

308   2).

309

310   Discussion

311

312   We found support for the hypothesis that the level of T-cell mediated immune response

313   in spotless starling nestlings is a nutritional-dependent trait, because experimental food-

314   supplemented nestlings developed a stronger immune response than control ones (see

315   Results). This result suggests that a biased parental investment in some nestlings

316   showing traits attractive for parents would result in a relationship between the

317   expression of those traits (that affect parental investment) and nestling immunity. Such

318   a relationship may have important implications, because the level of T-cell mediated

319   immune response is a good predictor of nestling survival and recruitment in at least

320   some species (Christe et al. 2001; Møller & Saino 2004; Moreno et al. 2005; Cichon &

321   Dubiec 2005). For example, a direct link between the elaboration of traits attractive for

322   parents and reproductive value can be established. This link, however, could be

323   mediated not only by parents feeding the most detectable nestlings, but also by parents

324   adaptively and preferentially feeding nestlings with the highest reproductive value.

325   Nestling mouth colouration affects parental investment (e.g., Gotmark &

326   Ahlstrom 1997; Saino et al. 2000a; Heeb et al. 2003; Jourdie et al. 2004) and,

327   consequently, a relationship between this trait and nestling immune response can be

328   predicted. Furthermore, it has been experimentally demonstrated that UV reflectance of

329   both skin and mouth parts in starling and Alpine swift (*Apus melva*) nestlings affects

330   parental food provisioning (Jourdie et al. 2004; Bize et al. 2006), a result that also

331   predicts a relationship between skin colouration and immunity of nestlings. In

332   accordance with this scenario, Jourdie et al. (2004) found a positive relationship

333   between skin brightness and the level of T-cell mediated immune response in starling

334   nestlings. Here, we have also found such a relationship in spotless starling nestlings (see

335   Fig. 2). In addition, the within-nest variation in both nestling colour and immune

336   response was lower than between-nest variation. If the relationship between nestling

337   colouration and immunity was due to differential parental investment in the most

338   brightly coloured nestlings within a brood as hypothesised by Jourdie et al. (2004), the

339   relationship should still hold after controlling for variation due to nest identity.

340   However, when this variation was controlled for, nestling colouration no longer

341   explained significantly the level of T-cell mediated immune response of nestlings (see

342   Results). We can conclude that the relationship between nestling colouration and

343   immunity was mainly due to between-nest differences in nestling colour that covaried

344   with differences in parental quality and/or genetic quality of nestlings.

345   Within-nest variation in nestling colouration explained nestling immune

346   response, because the effect of food-supplementation on immune response was stronger

347   in nestlings with bright skin colour (see Fig. 2). The opposite result would be predicted

348   if nestling colour did not reflect immunocompetence of nestlings, but only feedings by

349   parents through nestling detectability (see, Gotmark & Ahlstrom 1997; Heeb et al.

350 2003; Jourdie et al. 2004). This would be caused by extra food having a differential

351 positive effect on nestlings of low nutritional condition (i.e., low value of skin

352 brightness). Nestling colouration was measured during the second experimental feeding

353 (i.e., visit), but the experiment did not affect nestling colouration significantly, because

354 food supplemented and control nestlings did not differ in skin brightness (results not

355 shown). Therefore, the significant interaction between nestling colouration and

356 experimental treatment cannot be explained by the experiment affecting both nestling

357 colour and immunity. Instead, this interaction suggests that nestlings with bright skin

358 used extra food to improve their ability to produce a strong immune response in a more

359 efficient way than pale nestlings. These results are consistent with nestling colouration

360 being a signal, not only of condition (e.g., Kilner 1997), but also of intrinsic nestling

361 characteristics that predict PHA immune response at fledging, signal that parents

362 adaptively use to make feeding investment decisions (Saino et al. 2000a; Saino et al.

363 2000b; Hunt et al. 2003).

364 To conclude, we suggest that the relationship between nestling immunity and

365 colouration could be explained not only by parents preferentially feeding the most

366 detectable nestlings (see Jourdie et al. 2004), but also by the existence of intrinsic

367 characteristics of nestlings that are signalled by their colouration and that predict their

368 ability to produce a strong cell-mediated immune response.

369

375 the field was granted by the Dirección General de Gestión del Medio Natural of the

376 Consejería de Medio Ambiente of Junta de Andalucía.

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463 Table 1: General linear models explaining PHA response of nestlings (dependent

464 variable) with skin brightness of nestlings as a covariate, food treatment as a fixed

465 factor, and nest identity as a random factor. The interaction between experimental

466 treatment and nest identity was maintained in the model to conservatively adjust

467 degrees of freedom to approximately the number of nests with nestlings of both

468 treatments (food supplemented and control). Sums of squares were decomposed by

469 using type III (orthogonal) and type I (hierarchical) methodologies. Results from

470 introducing nest identity as the first or the third variable in the model are shown.

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	MS / Error	df	F	P
Type III decomposition				
Skin brightness (fixed) (1)	0.049 / 0.030	1, 32	1.65	0.21
Food treatment (fixed) (2)	0.096 / 0.030	1, 34.0	3.21	0.08
Nest identity (random) (3)	3.101 / 0.028	38, 33.5	2.93	0.001
(1)*(2) (fixed)	0.148 / 0.030	1, 32	4.95	0.033
(2)*(3) (fixed)	0.028 / 0.030	32, 32	0.93	0.58
Error	0.030			
Type I decomposition				
Skin brightness (fixed) (1)	0.378 / 0.074	1, 43.9	5.14	0.028
Food treatment (fixed) (2)	0.152 / 0.033	1, 30.2	4.59	0.04
Nest identity (random) (3)	0.084 / 0.028	38, 24.6	3.029	0.0025
(1)*(2) (fixed)	0.125 / 0.028	1, 35.6	4.46	0.042
(2)*(3) (fixed)	0.028 / 0.030	32, 32	0.93	0.58
Error	0.030			
Type I decomposition				
Nest identity (random) (3)	0.092 / 0.028	1, 23.8	3.33	0.001
Skin brightness (fixed) (1)	0.034 / 0.029	38, 63.9	1.17	0.28
Food treatment (fixed) (2)	0.186 / 0.028	1, 23.4	6.74	0.016
(1)*(2) (fixed)	0.125 / 0.028	1, 35.6	4.46	0.042
(2)*(3) (fixed)	0.028 / 0.028	32, 32	0.93	0.58
Error	0.030			

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477 Figure 1: Reflectance spectra (median values) from mouth, flanges and body skin of
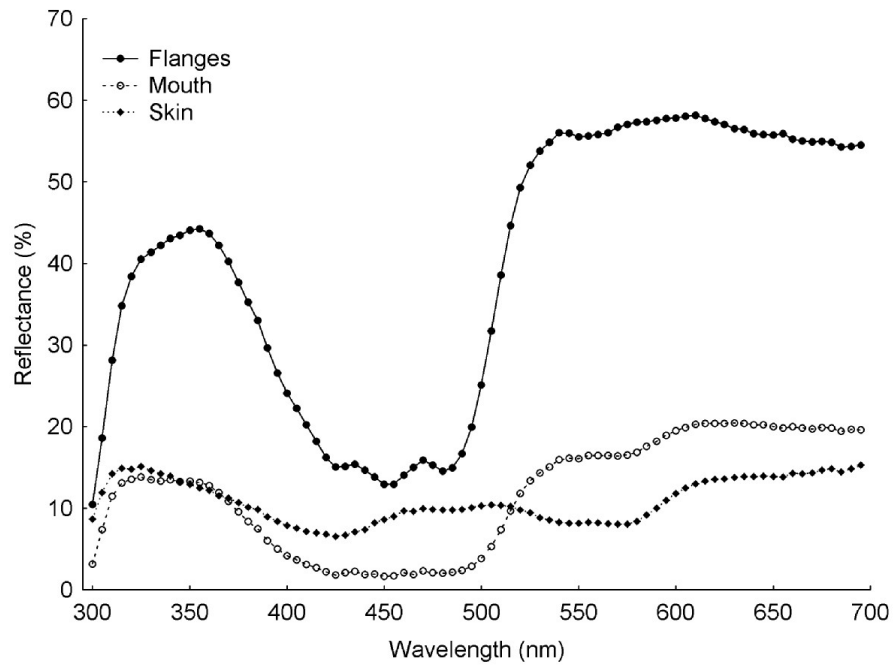
478 spotless starling nestlings.

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480 Figure 2: Relationships between T-cell mediated immune response (mm) (dependent

481 variable) and skin brightness in food supplemented and control spotless starling

482 nestlings. Regression equations are: $Y(control) = 0.499 + 0.008X$, and $Y(experimental)$

483 $= 0.457 + 0.019X$.
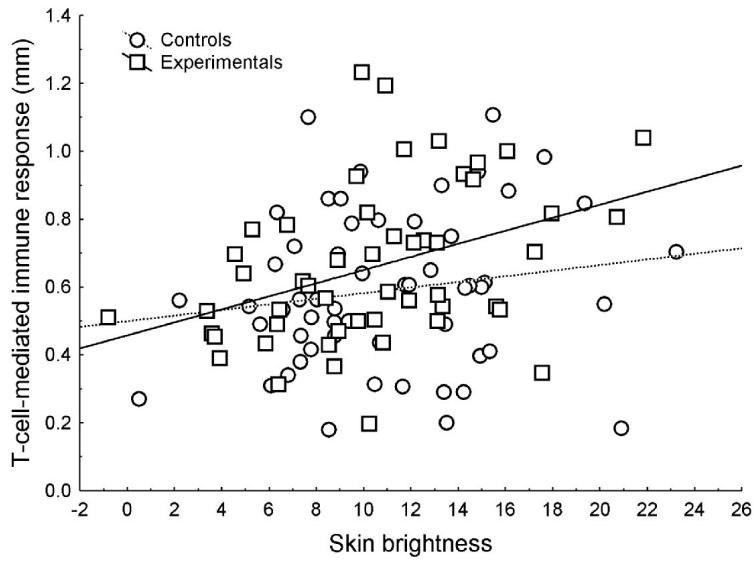
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485   Fig. 1

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