Sexual signal reliability in male zebra finches: food intake explains the impact of immune activation on carotenoid-based coloration

Alejandro Cantarero[1], Rafael Mateo[2,3], Pablo Camarero[2], Pedro Andrade[4], Miguel Carneiro[4], and Carlos Alonso-Alvarez[5,6*]

[1]Department of Physiology, Veterinary School, Complutense University of Madrid, Avenida Puerta de Hierro s/n, 28040 Madrid, Spain.

[2]Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC - UCLM - JCCM). Ronda de Toledo 12, 13005 Ciudad Real, Spain.

[3]Institute of Environmental Assessment and Water Research, IDAEA (CSIC). Jordi Girona 18-26, 08034 Barcelona, Spain.

[4]CIBIO/InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal

[5]Departamento de Ecología Evolutiva. Museo Nacional de Ciencias Naturales - CSIC. C/ José Gutiérrez Abascal 2, 28006 Madrid, Spain

[6] Instituto Pirenaico de Ecología (IPE-CSIC), Avda. Nuestra Señora de la Victoria 16, 22700 Jaca, Spain

*Corresponding author: carlos.alonso@csic.es

ABSTRACT

Theory predicts that traits evolving as animal signals must transmit reliable information about the bearer's quality. Two hypotheses are often invoked to explain how carotenoid-based colorations function as reliable individual quality signals. The first hypothesis suggests that carotenoid allocation trade-offs between signaling and survival-related functions maintain signal reliability. Only high-quality individuals could resolve these trade-offs without incurring a fitness loss. Such signals are plastic, and the signaler can strategically manage their expression level to increase fitness returns. The second hypothesis posits that certain carotenoid-based signals are strongly constrained in their expression, not allowing strategic manipulations or cheating. We challenged captive male zebra finches (*Taeniopygia guttata*) with simulated infections by injecting lipopolysaccharides (LPS) derived from *Escherichia coli* and quantified food intake, carotenoid and vitamin levels in several tissues. Compared to controls, LPS-treated birds reduced the bill redness, a sexual signal. However, this reduction was largely explained by decreased food intake caused by LPS. Bill redness negatively correlated with body temperature and spleen mass but positively associated with blood, spleen and subcutaneous fat carotenoid levels. Overall, our findings suggest that carotenoid-based bill coloration predominantly reflects the recent physiological condition of individuals and resource allocation trade-offs rather than directly signaling individual quality.

Keywords: carotenoids, fever, inflammatory response, sexual selection, splenic tissue.

INTRODUCTION

Conspicuous traits in animals often function as signals in social and sexual selection contexts. For these traits to evolve as signals, they must reliably convey information that benefits both the signaler and the receiver (Laidre & Johnstone, 2013; Biernaskie et al., 2014). Typically, the information content of the signal reflects the individual quality of the signal bearer, thereby influencing the receiver's mating and reproductive decisions. Among the traits commonly involved in sexual signaling are colorations, particularly those produced by pigments ranging from yellow to red. In vertebrates, many of these colorations are produced by carotenoids (e.g. Grether, 2004; McGraw, 2006). Carotenoids are pigments that must be obtained through food, and some authors suggested that they may be scarce in the environment, requiring specific foraging efforts to acquire them (Endler, 1980; Kodric-Brown, 1985; Hill, 1990). Moreover, carotenoids have important physiological functions as immune enhancers and antioxidants (Pérez-Rodríguez, 2009; Svensson & Wong, 2011). It has been suggested that carotenoid-based colorations are reliable individual quality signals as their production could incur costs (e.g., Weaver et al. 2017) or involve resource allocation trade-offs that require optimization (Szamado et al., 2022, 2023a). Both investing extra time and energy in foraging carotenoids, and the allocation of the ingested carotenoids to signals instead of homeostasis, may impose fitness costs or give place to suboptimal fitness returns (Møller et al., 2000; Alonso-Alvarez et al., 2008; but see Koch & Hill 2018). High-quality individuals are expected to better cope with these challenges, thereby expressing more intense coloration than low-quality individuals. In this scenario, theory suggests that the signal production mechanism should be enough plastic to allow animals to strategically make allocation decisions that optimize their fitness returns and potentially deceive signal receivers (e.g., Maynard Smith & Harper, 2003; Szamado et al., 2023b).

Nonetheless, according to animal signaling theory, some signals may be tightly constrained in their expression, exhibiting low plasticity and being strongly determined by individual quality (e.g. Maynard Smith and Harper, 2003). Such signals are theoretically unfalsifiable and are commonly referred to as "index signals" (Maynard Smith & Harper,

2003; Laidre & Johnstone, 2013; Biernaskie et al., 2014). It has been proposed that traits coloured by red ketocarotenoids (e.g. astaxanthin, α-doradexanthin, adonirubin, echinenone), which are enzymatically synthesized by some animals from dietary yellow carotenoids (xanthophylls such as lutein, zeaxanthin or β-cryptoxanthin), may serve as index signals (Hill, 2011; Lopes et al., 2016; Mundy et al., 2016; Weaver et al., 2017; Toomey et al., 2022). According to this hypothesis, the enzymes responsible for carotenoid conversion should be placed in the inner mitochondrial membrane and are tightly linked to cellular respiration processes (Johnson & Hill, 2013; Hill et al., 2019). This close association is thought to limit the potential for signal dishonesty, as the ability to produce red ketocarotenoids would inherently reflect the individual's metabolic efficiency (Weaver et al., 2018; but see also Koch et al., 2025).

In birds, experimental immune activation, such as exposure to antigens, has been shown to reduce the expression of carotenoid-based signals (e.g., Fitze et al., 2007; Merrill et al., 2016; George et al., 2017). Among the most commonly used antigens in such studies are lipopolysaccharides (LPS) of the bacterial cell wall typically derived from gram-negative bacteria such as *Escherichia coli* or *Salmonella* spp. (e.g. Buras et al. 2005). LPS antigens trigger an innate immune response involving the cell and humoral immune system (Bryant et al., 2010; Rathinam et al., 2019; Scalf et al., 2019). In poultry, LPS exposure has been shown to induce inflammation and increase the mass of key immune and metabolic organs, particularly the liver (e.g., Xie et al. 2000; Liebolt et al., 2016; Bai et al., 2019) and spleen (e.g., Koutsos et al., 2003; Rajput et al., 2013; Liebolt et al., 2016; Bai et al., 2019; but see also Li et al., 2017). Moreover, experimental studies have consistently confirmed a negative impact of LPS in the carotenoid-based coloration and circulating carotenoid levels in avian species (Alonso-Alvarez et al., 2004; Torres & Velando, 2007; Gautier et al., 2008; Cote et al., 2010; Romano et al., 2011; Sild et al., 2011; Rosenthal et al., 2012; Csernus et al., 2020). Similar declines in carotenoid-based coloration have also been observed in reptiles (López et al., 2009; Ibañez et al., 2014) and even in humans (Henderson et al., 2017).

103 Here, we investigate the hypotheses concerning the reliability of carotenoid-based
104 signals by analyzing carotenoid concentrations across multiple tissues (the bill, liver,
105 subcutaneous fat, and spleen) of males of zebra finches (*Taeniopygia guttata*). This
106 species possesses a sexually selected trait, the red bill, which is involved in mate choice
107 (Simons & Verhulst, 2011) and is pigmented by endogenously converted ketocarotenoids
108 (McGraw & Toomey, 2010). We used captive birds exposed to a simulated one-month
109 infection via repeated LPS injections. Our experimental design was similar to that of a
110 study conducted twenty years ago (the exact LPS dosage and very similar time-lapse; i.e.,
111 Alonso-Alvarez et al., 2004). In that study, birds were supplemented with carotenoids.
112 Here, we decided to simplify the design, avoiding pigment supplementation as
113 carotenoids are usually abundant in commercial diets (see here Supplementary Material;
114 SM), and pigment addition has been shown to not proportionally increase signal
115 expression (Koch et al., 2016). Alonso-Alvarez et al. (2004) reported significant declines in
116 both bill coloration and blood carotenoid levels in LPS-treated zebra finches compared to
117 controls. The same findings have been replicated two additional independent studies on
118 this species (i.e., Gautier et al., 2008 and Cote et al., 2010). Here, however, we tested the
119 influence of resource acquisition on signal expression by quantifying the ingestion of food
120 (seed mix) containing yellow substrate carotenoids. We should note that LPS often inhibit
121 food ingestion in different bird species (Baert et al., 2005; Rosenthal et al., 2012; Teemer
122 & Hawley, 2024; but see Koch et al., 2018), including zebra finches (Burness et al., 2010;
123 Sköld-Chiriac et al., 2015; Tapper et al., 2021). Therefore, the reduction in blood
124 carotenoid levels and coloration previously described in LPS-treated birds (above) may be
125 partly due to LPS-induced hypophagia, leading to decrease carotenoid intake. Here, we
126 specifically address this point by evaluating the relationship between food consumption
127 and the signaling outcomes.

128 We predict that individuals facing an immune challenge should prioritize self-
129 maintenance by allocating carotenoids to vital organs, such as the liver to protect it
130 against immune-induced damage (e.g., Kaulmann and Bohn 2014; Elvira-Torales et al.,
131 2019; Abou Elazab et al., 2022) and spleen for favoring its immune activity (e.g., Milani et

al., 2017; Csernus 2020, 2023), rather than investing them on coloration. Thus, mammalian studies suggest that inflammatory responses sequestrate retinol in the hepatic tissue (Rubin et al., 2017), and we may infer that something similar could occur with carotenoids as closely related compounds (Tang & Rusell, 2009). Alternatively, liver carotenoids might be consumed to counteract the oxidative stress linked to LPS-induced inflammation (e.g. Meriwether et al., 2010; Kaulmann & Bohn, 2014). Concerning the spleen, carotenoids have been shown to enhance spleen antibody production in mammals (reviewed, e.g., Milani et al., 2017) and to reduce LPS-induced spleen inflammation in chickens (Csernus 2020, 2023). Therefore, increasing spleen carotenoid levels should be an optimal strategy for combating an infection. For subcutaneous fat, we may predict that carotenoid levels may decrease in LPS-treated birds as a consequence of oxidative stress in adipose tissues (e.g., Bonet et al., 2015; Mounien & Tourniare, 2019). Finally, we hypothesize that food intake will be positively associated with signal intensity and carotenoid accumulation in ornaments and other tissues. However, the food intake and the immune challenge could also interact. In such a case, we predict that the relationship between food intake and signal intensity may be weaker in LPS-treated birds due to increased carotenoid demands for homeostasis.

Interpreting the results within the framework of the trade-off versus index signal hypotheses is challenging. We must first assume that individuals exhibiting the reddest bills at the start of the experiment represent high-quality individuals. Accordingly, low-quality individuals should invest more in self-maintenance and less in signal expression when enduring the immune challenge. In this case, initial redness should interact with treatment on the response variables. If such an interaction is not observed, it may suggest the occurrence of signal dishonesty or 'cheating', which would align more closely with the plastic resource allocation strategies proposed by the trade-off hypothesis.

Material and Methods

159 Adult male zebra finches (*n* = 70) were individually housed in cages (0.6 m × 0.4 m × 0.4 m)
160 placed in an indoor aviary (FIEB Foundation, Casarrubios del Monte, Spain) under
161 controlled conditions (see SM). All birds received *ad libitum* water, grit, and a commercial
162 seed mix (KIKI® exotic birds, Spain; composition in SM). After a minimum 14-day
163 quarantine and acclimation period, body mass and bill redness were measured, and a
164 blood sample was taken (Chronogram in Figure S1 of SM). Two days later, each feeder was
165 replenished and then weighed. The feeders (ref. M051, STA Soluzioni, Italy) were
166 equipped with a shell collector at the bottom, and an additional collector was placed at
167 the entrance. As no seeds or shells were found on the floor of the aviary or cage, we are
168 confident that all consumed grain was accurately quantified. The feeders were
169 replenished and weighed every three days with the content of both collectors included in
170 each measurement. The difference between two consecutive weights represented the
171 amount of grain consumed during that period. The first three-day period served to test for
172 a difference in food intake before the experiment began (i.e., before the first injection;
173 below). The total grain mass consumed by each bird from the first injection day until two
174 days before the last injection was considered the individual food intake over the 27-day
175 experimental period. This value was corrected for individual body mass at the first
176 injection day (i.e., "initial body mass"; Figure S2). Water expenditure (mL) was estimated
177 by weighing water dispensers one day after the second injection and again three days
178 later. Note that this measure may be partially influenced by water loss due to behaviors
179 other than drinking.

180 Five days after blood sampling, all birds were assigned to treatments. Thirty-five
181 birds received intraperitoneal injections of 0.01 mg of lipopolysaccharide (LPS) from the
182 cell wall of *Escherichia coli* (serotype O55:B5; SIGMA, St. Louis) diluted in 0.1 mL of PBS
183 (concentration = 0.1 mg/mL; i.e., LPS-treated birds). The remaining birds (n = 35) were
184 injected with the same volume (0.1 mL) of PBS alone (control birds). The treatments were
185 randomly distributed across aviary cages (rows and columns). No significant pre-treatment
186 differences were found in body mass, body size (tarsus length), size-corrected body mass
187 (body mass controlled for tarsus length, bill color measures (hue, saturation or brightness)

188 or initial (pre-experimental) food intake (all *p*-values > 0.38). To maintain the immune
189 challenge, birds were injected once per week for five consecutive weeks. Bill coloration
190 and body mass measures were repeated 28 days after the first injection and the birds
191 were assigned to one of three sampling blocks (n = 23, 23 and 24; treatment balanced
192 among blocks: χ^2 = 0.09, df = 2, p = 0.957). The blocks were necessary due to time
193 limitations during tissue extraction. On day 28, birds in the first block were injected (fifth
194 dose; Figure S1). The following day, a (second) blood sample was collected from these
195 birds, after which the birds were euthanized via cervical dislocation. This procedure was
196 repeated for the second and third blocks on subsequent days. Since the duration of the
197 experiment differed among blocks (24-48h difference; Figure S1), the block was included
198 as a random effect in the statistical models (see below).

199 Body temperature was measured immediately after euthanasia using an infrared
200 thermometer. For the first nine birds, temperature was measured individuals at the
201 midpoint of the breast after blowing feathers apart. However, these readings yielded
202 unexpectedly low values (mean ± SD: 37.72 ± 1.46 °C, range: 34.7-39.9 °C). For the
203 following birds, we directed the thermometer to pectoral muscles after being feather
204 plucked (41.45 ± 0.94 °C; 39.6-42.9 °C). We standardized the values to obtain mean = 0
205 and SD = 1 in both groups. Std-values were then used in the statistical analyses. Similar
206 results were obtained when analyses were restricted to the raw data from the latter
207 group alone (n = 61; SM). Following euthanasia, tissues including the bill (ramphoteca,
208 epidermis, and dermis), liver, spleen, intestines and subcutaneous fat (visible adipose
209 tissue connected to the skin) were collected. An aliquot was obtained from the bill, liver,
210 spleen, and fat for carotenoid and vitamin quantification using HPLC (see below). The
211 samples were immediately snap-frozen in dry ice and stored at −80°C. The experiment
212 was approved by the animal ethics government office (i.e., Consejería de Agricultura,
213 Junta de Castilla La Mancha; approval ref. JCCM 27-2021) in agreement with Directive
214 2010/63/EU.

215

216 *Color measurements*

217 Digital photography (Nikon® D300) was used to measure bill redness. Each bird was
218 photographed laterally, taking images from both sides of the head (see SM for details).
219 Digital photographs were standardized and analyzed using 'SpotEgg' software (Gómez &
220 Liñán-Cembrano, 2017). This image-processing tool for automatized analysis solves the
221 need for linearizing the camera's response to subtle light intensity variations (Stevens et
222 al., 2007). Mean red, green, and blue (RGB) values measured from the lateral area of the
223 bill (upper and lower mandibles) were used to calculate hue and brightness values
224 following Foley & van Dam (1982). The exact measured area was recorded. Values from
225 both sides of the bill were combined to obtain a single global measure. Measured
226 repeatability (Lessells and Boag 1987), calculated on a subset (n = 30) of digital
227 photographs measured twice was high ($r > 0.90$, $p < 0.001$). As lower hue values
228 correspond to redder coloration, hue values were reversed by multiplying by -1. This
229 transformed value was defined as "bill redness" and used in subsequent analyses.

230

231 *Carotenoid, retinoid, and tocopherol analyses*

232 Carotenoids, retinoids, and tocopherols were extracted following the protocols described
233 by García-de Blas et al. (2013) and Rodríguez-Estival et al. (2010) (see SM for additional
234 details on the carotenoid extraction). Identification and quantification were performed
235 using a high-performance liquid chromatography system (HPLC) (Agilent Technologies
236 1200 series) equipped with diode array and fluorescence detectors and a C30 column
237 (ProntoSIL 200-3-C30 3µm 250x4,6mm, Sharlab) (García-de Blas et al. 2011., 2013; Alonso-
238 Alvarez et al., 2018). Standards of lutein, zeaxanthin, canthaxanthin, astaxanthin, 3HOE, β-
239 cryptoxanthin, and β-carotene were obtained from CaroteNature (Lupsingen,
240 Switzerland). Saponification with KOH did not yield additional carotenoid recovery (see
241 saponification method in García-de Blas et al. 2011). Retinyl acetate was used as an
242 internal standard (IS) for carotenoid and retinol quantification, whereas tocopheryl
243 acetate was used as IS for tocopherol quantification. Concentrations were calculated with

244 standard calibration curves (ranging from 0.5 to 15 nmol/tube) controlling for the peak
245 area of the respective IS. Compounds were identified by comparing their retention times
246 and UV-Vis spectra with those of standards and literature-reported spectra. Lutein
247 derivatives detected in plasma samples were quantified using the lutein calibration curve,
248 assuming identical response factors. Carotenoid concentrations were expressed in
249 nmol/mL in the plasma and nmol/g in the liver, fat, spleen, and bill. Total carotenoid levels
250 were calculated as the sum of all identified carotenoid concentrations within each sample.
251 Blood carotenoid and vitamin levels (initial and final values) of the same bird were
252 analyzed in the same laboratory session. Spleen carotenoid and vitamin levels could only
253 be measured in a subset of samples (30%), corresponding to those that reached the
254 required minimum mass (> 13 mg; n = 21; 10 controls and 11 LPS-treated birds). We
255 should note that another aliquot of this tissue was reserved for RNA assays (unpublished
256 material). The spleen carotenoid analyses were performed in a single laboratory session,
257 with an even distribution of individuals across sampling blocks (χ^2 = 0.04, df= 2, p = 0.979).
258 As a result, these factors were not included as random effects in the statistical models.

259

260 *Statistics*

261 The values of bill redness (reversed hue), carotenoid and vitamin levels, organ mass and
262 body temperature were tested as dependent variables in separate models to disentangle
263 resource allocation strategies. ANCOVAs were used for bill redness, organ mass, and body
264 temperature, while Generalized Linear Mixed Models [GLMM] were applied to most
265 molecule concentration variables (i.e. PROC MIXED in SAS version 29). The laboratory
266 session and the sacrifice block were added as random terms in the GLMMs; however,
267 their influence was consistently non-significant (p > 0.08 for all tests). For fat and spleen
268 compounds, no influence of the random terms was detected (p > 0.90), and we used
269 Generalized Linear Models (GLMs).

270 Dependent variables were log- or square-root transformed when necessary to
271 meet normality assumptions. When transformed data still violated normality criteria non-

272 parametric statistical tests were applied. The distribution of subcutaneous fat retinol and
273 carotenoid levels was zero-inflated (i.e., 24% and 34% zero values, respectively). To
274 account for this, we applied zero-inflated negative binomial (ZINB) generalized linear
275 models (GLMs) (PROC GENMOD in SAS v29). The ZINB GLMs separately analyze the normal
276 and zero-inflated parts of the distribution (Ridout et al., 1998; Cantarero et al., 2020).

277 In all models, we followed a stepwise approach. First, we tested the effect of the
278 treatment alone. In the second step, we included treatment, initial bill redness (covariate),
279 and their interaction. In the third step, we tested treatment, food intake (as a covariate),
280 and its interaction with treatment. The "initial bill redness" covariate was the
281 standardized residual of a linear regression testing the initial bill hue (reversed) on two
282 confounding covariates: initial bill brightness (slope ± SD: = -8.28 ± 0.96, t = 8.59, p <
283 0.001) and the bill area selected for the measurement (slope ± SD: 1.58 ± 0.499, t = 3.15, p
284 = 0.002). The "food intake" covariate was the standardized residual of a linear regression
285 testing total food intake on initial body mass (slope ± SD: 2.29 ± 0.55, R^2 = 0.20, p < 0.001;
286 Figure S2 in SM). The results remained qualitatively unchanged if food intake values were
287 uncorrected by body mass when tested as a covariate. The "final bill redness" term was
288 used to define reversed hue corrected for bill hue and bill measured area at the last
289 sampling, i.e., not corrected for initial variability.

290 To account for potential confounding effects, additional covariates were included
291 in specific models. For the bill redness model, we included bill brightness and bill area
292 (both measured at the final sampling) along with the initial bill redness covariate. The
293 model testing plasma carotenoids included the "initial carotenoid values" that were, in
294 turn, the standardized residuals from a GLMM testing initial values as the dependent
295 variable and the laboratory session and the block of sacrifice as random factors.

296 The correlation coefficients for the association between bill redness and total
297 carotenoid levels in each tissue were calculated by testing the standardized residuals of
298 each parameter obtained from GLMMs (i.e., excluding the treatment but maintaining
299 confounding covariates and random factors). For the spleen, where measurements were

300 available only for a reduced sample (n = 21), simpler statistical tests were applied
301 depending on data distribution (Student's t-test, ANCOVA, or Mann–Whitney U tests).

302 Two-tailed statistics were used for most *p*-value calculations as the direction of the
303 effect was not well-established by previous studies. However, one-tailed *p*-values were
304 reported when assessing the effect of LPS treatment (not its interaction) on bill color and
305 plasma total carotenoid levels, given the substantial prior evidence supporting a clear
306 directional prediction (i.e., an LPS-induced decrease in their values; see Introduction; see
307 also Rice and Gaines 1994; Ruxton & Neuhaüser, 2010; Cho & Abe, 2013). Satterthwaite's
308 correction was used to calculate degrees of freedom in GLMMs. Effect sizes are reported
309 as Cohen's *d* for group comparisons with *p*-values < 0.12, while Pearson's correlation
310 coefficients are used to represent effect sizes in correlational analyses (Cohen, 1992 In
311 instances where carotenoid levels could not be quantified due to extraction issues (see
312 sample sizes in Table S1), the covariates (food intake or initial bill redness residuals) were
313 recalculated from the dataset that only included values for the dependent variable.

314

315 **RESULTS**

316 *Water, food intake and body mass*

317 The LPS-treated birds exhibited significantly higher (log-transformed) water expenditure
318 than controls (Student's *t*: 2.01, df = 54.7, $p = 0.049$, $n = 69$; Figure 1A for raw values). LPS-
319 treated birds also showed lower food intake ($F_{1,67} = 4.49$, $p = 0.038$, $n = 70$; initial body
320 mass: $F_{1,67} = 18{,}01$, $p < 0.001$, estimate ± SE: 2.29 ± 0.540). The immune challenge did not
321 influence final body mass (treatment: $F_{1,67} = 0.28$, $p = 0.597$; initial body mass: $F_{1,67} = 1088$,
322 $p < 0.001$; estimate ± SE: 1.04 ± 0.032). Including food intake as an additional covariate did
323 not significantly explain final body mass variability ($p = 0.434$). Neither the initial bill
324 redness ($p = 0.433$) nor its interaction with treatment ($p = 0.802$) significantly influenced
325 food intake.

326

Bill color

At the end of the experiment, LPS-treated birds exhibited lower bill redness compared to controls (treatment: $F_{1,65}$ = 3.21, p = 0.039; brightness: $F_{1,65}$ = 28.4, p < 0.001, estimate ± SE: -4.45 ± 0.835; area measured: $F_{1,65}$ = 15.29, p < 0.001, estimate ± SE: 1.86 ± 0.475; initial redness: $F_{1,65}$ = 13.81, p < 0.001, estimate ± SE: -1.043 ± 0.281; see Figure 1C). The interaction between initial bill redness and treatment was non-significant when added to the same model (Table 1). However, when the food intake covariate was also added, it showed a significant positive correlation to bill redness (Figure 1D) without interaction with treatment (Table 1), whereas the latter became non-significant (Table 1; Figure 1E). The correlation between the treatment and food intake remained significant when the interaction was removed (i.e., $F_{1,64}$ = 11.35, p = 0.001; estimate ± SE: 0.923 ± 0.274).

Table 1. Models testing the impact of the immune challenge on bill redness (n = 70), and bill (n = 63) and plasma (n = 67) carotenoid levels. The bill redness models included bill brightness and measured area as covariates (both p < 0.001). The initial bill redness covariate was the residual of a model testing bill redness at the start of the experiment corrected by bill brightness and measured area at the same time. One-tailed p-values for the treatment factor on bill redness and plasma carotenoids (see Statistical analyses).

Bill redness	Estimate	SE	*F*	*df*	*P*
Treatment (control)	0.989	0.543	3.24	1,64	0.039
Initial bill redness	0.820	0.391	14.04	1,64	<0.001
Treatment x Initial bill redness	0.470	0.575	0.67	1,64	0.416
Treatment (control)	0.536	0.532	1.02	1,63	0.159
Initial bill redness	1.149	0.265	18.77	1,63	<0.001
Food intake	0.938	0.448	10.52	1,63	0.002
Treatment x food intake	-0.024	0.561	0.01	1,63	0.966
Bill carotenoids					
Treatment (control)	34.633	21.504	2.59	57.3	0.112
Initial bill redness	9.665	14.690	0.03	58.4	0.855
Treatment x Initial bill redness	-23.425	22.222	1.11	57.4	0.296
Treatment (control)	35.313	22.166	2.54	57.3	0.117
Food intake	7.470	18.120	0.03	59	0.865
Treatment x food intake	-18.924	23.033	0.67	57.3	0.415
Plasma carotenoids					
Initial value	0.038	0.003	135.42	23.6	<0.001

Treatment (control)	0.581	0.303	3.7	57.5	0.029
Initial bill redness	-0.213	0.219	0.22	57.7	0.637
Treatment x Initial bill redness	0.279	0.329	0.72	59.2	0.400
Initial value	0.038	0.003	132.9	57.5	<0.001
Treatment (control)	0.517	0.307	2.84	59.7	0.097
Food intake	0.353	0.255	0.46	57.1	0.498
Treatment x food intake	-0.474	0.315	2.26	24.7	0.138

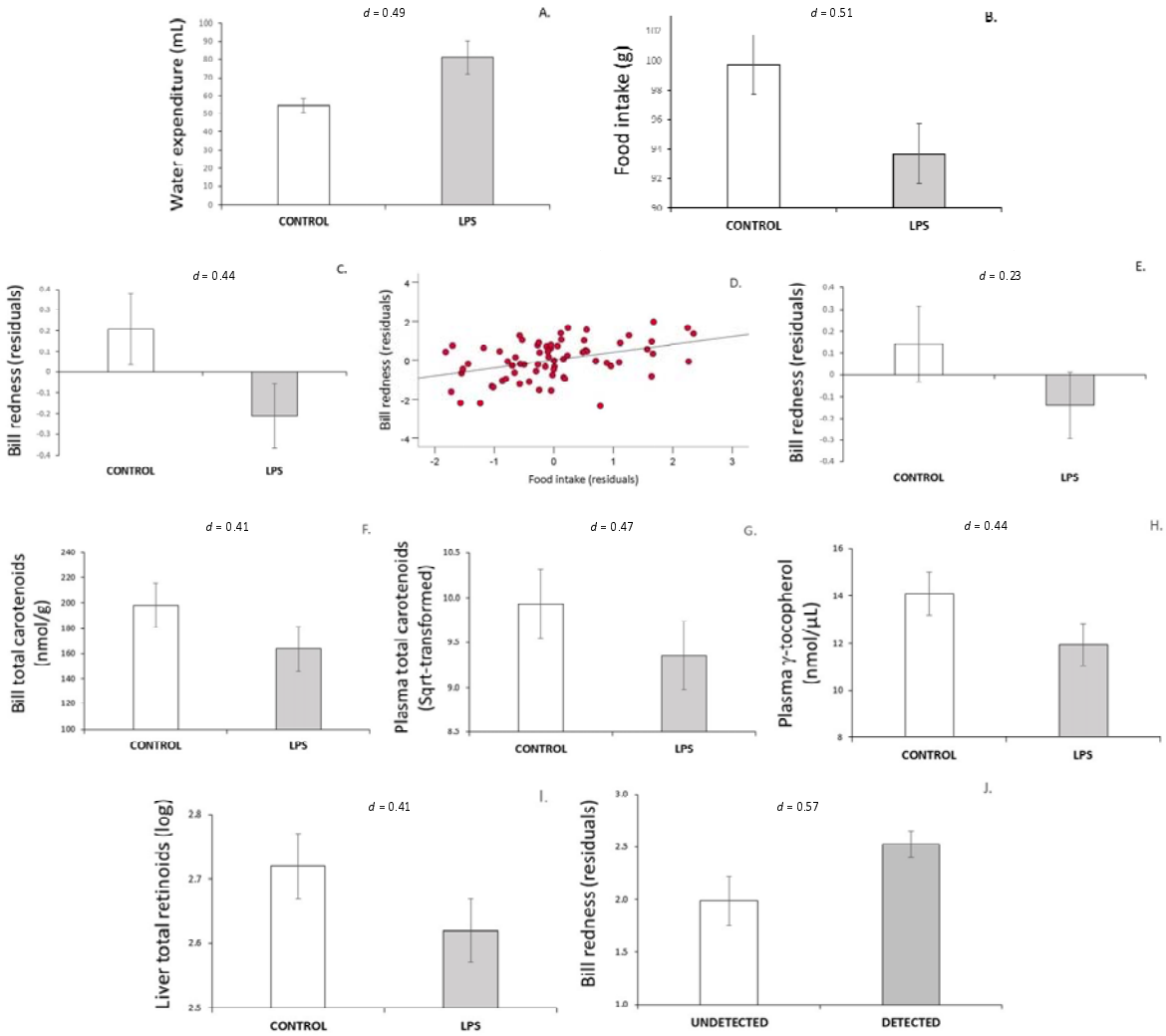


Figure 1. LPS impact on water expenditure (A), food intake (B) and bill redness (C; i.e., residuals of a mixed model controlling for bill brightness, bill measured area, initial bill redness and sacrifice session). Relationship between bill redness (those residuals) and food intake controlled for initial body mass (residuals) (D). LPS impact on bill redness after controlling for food intake residuals (E), bill total carotenoid levels (F), plasma total carotenoid (G) , plasma γ-tocopherol (H) and liver total retinoid levels (I). Difference in bill redness (Table 1 model's residuals, see Results) in birds with undetectable or detectable carotenoids in their subcutaneous fat (L). Least squared means ± SE from mixed models (other comparisons: means ± SE). Cohen's *d* effect sizes are shown.

367 *Bill composition*

368 In the bill, we detected retinol, astaxanthin, α-doradexanthin, adonixanthin, zeaxanthin,
369 α- and γ- tocopherols, as well as two unidentified carotenoids (Table S1). LPS-treated birds
370 exhibited lower mean total carotenoid levels than controls (Figure 1F); however, this
371 treatment effect only showed a weak trend toward significance ($F_{1,59.3}$ = 2.63, p = 0.110).
372 The inclusion of the initial bill redness or food intake covariates and their interactions with
373 treatment did not yield significant effects (Table 1). Retinol was detected in nine birds,
374 evenly distributed across treatments (χ^2 = 2.13, df= 1, p = 0.144), with no significant
375 difference between groups (Mann-Whitney U = 519, p = 0.219). The two tocopherols did
376 not differ between treatments (F's p-values > 0.18). Bill total carotenoid levels (residuals
377 from the Table 1 model without treatment) were positively correlated to bill redness
378 when assessed as reversed hue at the end of the experiment (Pearson's r = 0.27, p =
379 0.033, n = 63; Figure S3).

380

381 *Blood plasma*

382 In plasma samples, we detected retinol, lutein, zeaxanthin, four lutein derivatives,
383 echinenone (in only two samples), and α- and γ–tocopherols (Table S1). LPS-treated birds
384 showed significantly lower total carotenoid levels (square-root transformed) than controls
385 (treatment: $F_{59.4}$= 3.67, p = 0.030; initial value: $F_{1,23.5}$ = 140.12, p < 0.001, estimate ± SE: -
386 0.038 ± 0.003; Figure 1G). Neither initial bill redness, food intake, nor their interaction
387 with treatment significantly explain variation in plasma total carotenoid levels (Table 1).

388 Given the rapid turnover of circulating carotenoids, we further tested food intake
389 recorded exclusively during the week prior to the last blood sampling (mean ± SD: 21.14 ±
390 3.02 g). In this case, a significant interaction with treatment emerged (treatment: $F_{1.57.2}$ =
391 7.73, p = 0.004; initial value: $F_{1,25.5}$ = 135.28, p < 0.001; food intake: $F_{1.57.7}$ = 0.54, p = 0.465;
392 interaction: $F_{1.57.3}$ = 6.48, p = 0.014). Specifically, a positive relationship between food
393 intake and plasma carotenoid levels was found in LPS-treated birds (slope ± SE: 0.164 ±

394 0.055; $F_{1.24.9}$ = 8.77, p = 0.007; r = 0.49) but not in controls (-0.105 ± 0.085; $F_{1.29.6}$ = 1.54, p =
395 0.225; r = -0.09).

396 When each carotenoid was tested individually, all showed at least a statistical
397 trend towards reduced levels in LPS-treated birds (all p < 0.10), except zeaxanthin (i.e., p =
398 0.675). Furthermore, total plasma carotenoid levels at the last blood sampling (residuals
399 from a mixed model only controlling for random terms) were positively correlated with
400 final bill redness (Pearson's r = 0.33, p = 0.006, n = 70). Retinol or α-tocopherol levels did
401 not differ significantly between treatments (p-values > 0.66). However, LPS-treated birds
402 showed a statistical trend toward lower plasma γ-tocopherol levels compared to controls
403 ($F_{1.61.9}$= 3.22. p = 0.077; initial value: $F_{1.28.1}$ = 1975. p < 0.001; estimate ± SE: 0.986 ± 0.022;
404 Figure 1H). The addition of the food intake covariate to this model did not report a
405 significant effect (p = 0.461).

406

407 *Liver*

408 The relative liver mass (i.e., controlled for body mass) did not differ significantly between
409 treatments (ANCOVA: $F_{1.67}$ = 1.85, p = 0.178, n = 70; body mass at sacrifice: $F_{1.67}$ = 27.57, p <
410 0.001). The initial bill redness and its interaction with treatment had no significant effect
411 on relative liver mass (all p-values > 0.60). Similarly, treatment had no significant effect on
412 total carotenoid levels ($F_{1.63}$ = 0.01, p = 0.996). Neither the initial bill redness nor its
413 interaction with treatment influenced liver total carotenoid levels (Table 2). The
414 interaction between food intake and treatment was also non-significant; however, food
415 intake alone showed a trend toward a positive association with liver carotenoid levels
416 (Table 2), suggesting that birds consuming more food tended to accumulate higher
417 carotenoid levels in the liver (Figure S4).

418 In terms of specific compounds, lutein, three lutein derivatives, zeaxanthin, and β-
419 cryptoxanthin were detected in the liver. None of them showed significant differences
420 between treatments when analyzed separately (all p's > 0.30). Retinol, four unidentified
421 retinoids, α- and γ-tocopherol were also detected (Table S1). Total retinoid levels (log-

422 transformed) showed a weak trend toward a decrease in LPS-treated birds ($F_{1.62.5}$ =2.76, p
423 = 0.101; Figure 1-I). α- and γ-tocopherol did not differ significantly between treatment (p-
424 values > 0.59). Finally, liver total carotenoids or retinoids (i.e., residuals from their mixed
425 models without treatment) were not significantly correlated with bill redness (i.e., Table 1
426 model without treatment residuals; r = 0.132, p = 0.286, and r = -0.170, p = 0.169,
427 respectively).

428

429 **Table 2.** Models testing the impact of the immune challenge on the liver, subcutaneous fat, and
430 spleen total carotenoid levels. Alternative models testing the influence of initial bill redness and
431 food intake are shown.

Liver (Normal)	**Estimate**	**SE**	***F***	***df***	***P***
Treatment (control)	-0.002	0.282	0.00	61	0.993
Initial bill redness	-0.121	0.209	0.19	61.1	0.669
Treatment x Initial bill redness	0.118	0.290	0.17	61.1	0.684
Treatment (control)	-0.153	0.285	0.29	61.2	0.593
Food intake	0.388	0.250	3.85	61.7	0.054
Treatment x food intake	-0.166	0.303	0.30	61.1	0.586
Subcutaneous fat (ZINB)	**Estimate**	**SE**	χ^2	***df***	***P***
Treatment (control)	-0.217	0.212	1.04	1	0.307
Initial bill redness	0.105	0.128	0.25	1	0.616
Treatment x Initial bill redness	-0.111	0.199	0.32	1	0.573
Treatment (control)	-0.221	0.210	1.11	1	0.292
Food intake	0.328	0.163	0.49	1	0.482
Treatment x food intake	-0.488	0.238	4.22	1	0.040
Spleen (Normal)	**Estimate**	**SE**	***F***	***df***	***P***
Treatment (control)	-2.850	4.578	0.388	1.17	0.542
Initial bill redness	1.664	3.974	0.004	1.17	0.948
Treatment x Initial bill redness	2.995	5.068	0.349	1.17	0.562
Treatment (control)	-3.954	4.330	0.834	1.17	0.374
Food intake	4.913	4.034	1.563	1.17	0.228
Treatment x food intake	-3.727	4.878	0.584	1.17	0.455

432

433

434 *Subcutaneous fat*

435 We detected retinol, zeaxanthin, lutein, a lutein derivative, and α- and γ- tocopherols in
436 fat(Table S1). Total carotenoid levels were not significantly affected by treatment (χ^2 =
437 1.07, p = 0.301; zero-inflated part of the model: χ^2 = 0.18, p = 0.675). Similarly, the initial
438 bill redness and its interaction with treatment had no significant effect (Table 2).
439 However, food intake showed a significant interaction with treatment in the continuous
440 part of the model (Table 2). When analyzed separately, no significant association between
441 food intake and fat carotenoids was found in controls (slope ± SE: -0.148 ± 0.141; χ^2 =
442 1.10, p = 0.294), but LPS-treated birds exhibited a trend toward a significant positive
443 relationship (slope ± SE: 0.331 ± 0.182; χ^2 = 3.32, p = 0.068). The zero-inflated part of
444 these models consistently reported non-significant results (p > 0.46). The analyses of each
445 parameter separately did not show a significant treatment effect (all *p-values* > 0.20).
446 Additionally, we tested whether the presence or absence of fat carotenoids (as a binary
447 factor) predicted bill redness in the model from Table 1. Birds with detectable fat
448 carotenoid levels showed significantly redder bills ($F_{1.64}$ = 5.76, p = 0.019; d = 0.57; Figure
449 1-J). The inclusion of this binary factor did not alter the treatment effect on bill color (p =
450 0.027) and showed no significant interaction with treatment (p = 0.980).

451

452 *Spleen*

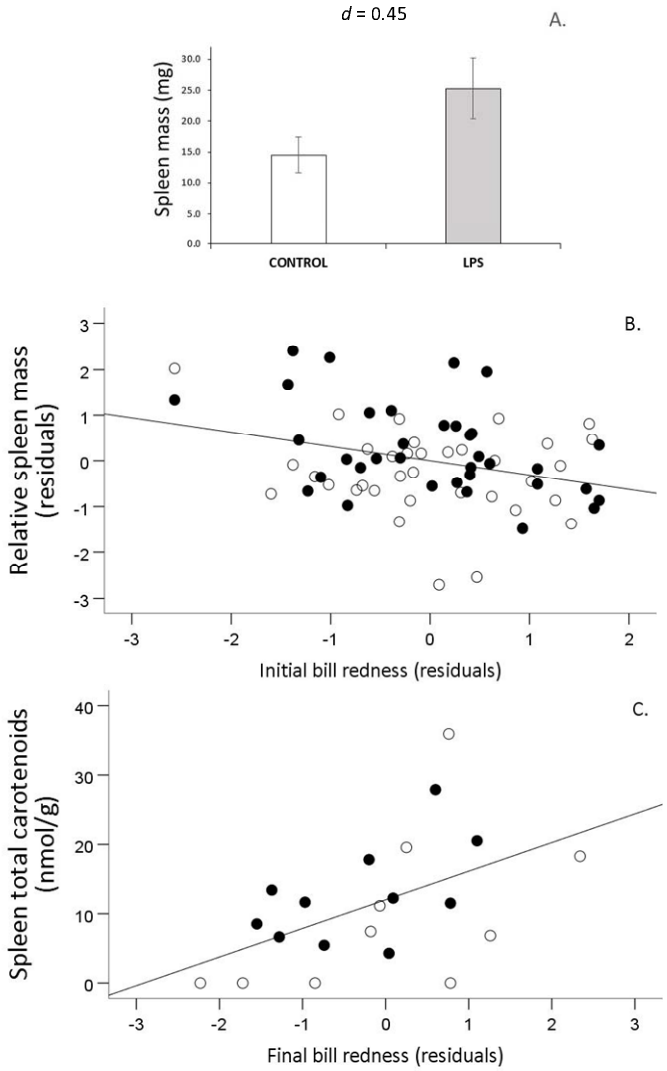
453 LPS-treated birds were characterized by significantly heavier spleens (i.e., "relative mass":
454 log-transformed spleen mass controlled for body mass) compared to controls (ANCOVA:
455 $F_{1.67}$ = 4.82, p = 0.032, n = 70; body mass at sacrifice: $F_{1.67}$ = 11.04, p = 0.001, d = 0.52; least
456 squared means ± SE: 1.01 ± 0.06 and 1.19 ± 0.06, respectively; Figure 2A for raw values).
457 Initial bill redness was negatively associated with relative spleen mass ($F_{1.65}$ = 7.41, p =
458 0.008, slope ± SD: -0.162 ± 0.059), with no significant interaction with treatment ($F_{1.65}$ =
459 1.12, p = 0.293). The negative correlation persisted after removing the interaction and
460 treatment factor ($F_{1.67}$ = 7.30, p = 0.009; r = -0.31, n = 70), indicating that birds with redder
461 bills at the start of the study exhibited lighter spleens than paler individuals (Figure 2B).

462 Food intake did not influence spleen mass nor interact with treatment (both *p-values* >
463 0.55). Additionally, relative spleen mass (residuals) had no significant effect when added
464 as a covariate to the Table 1 bill redness model ($F_{1.64}$ = 2.10, p = 0.152).

465 Carotenoids in spleen tissue could only be analyzed in 10 controls and 11 LPS-
466 treated birds that provided enough tissue mass (> 13 mg). These birds were significantly
467 heavier at both the start and end of the experiment compared to the remaining
468 individuals (Student's t both: p < 0.010, d = 0.70 and 0.71, respectively). They also showed
469 a trend to significantly lower initial bill redness compared to non-analyzed birds ($F_{1.66}$ =
470 3.65, p = 0.060, d = 0.47; means ±SE: -0.32 ± 0.25 and 0.14 ± 0.13, respectively). Note that
471 birds with heavier spleens were associated with paler initial bill coloration in the whole of
472 the sample (Figure 2B), a relationship also present in the subsample (r = -0.46, P = 0.035;
473 n = 21; Figure S5).

474 Within this subsample, LPS-treated birds had higher average spleen masses than
475 controls (mean, median, and rank: 43, 33, 96 mg and 30, 21, 86 mg, respectively),
476 although the difference was non-significant (Mann-Whitnney's U = 36, p = 0.181). The
477 treatment did not affect initial bill redness ($F_{1.17}$ = 1.54, p = 0.232) or bill redness
478 controlled for initial color ($F_{1.16}$ = 0.01, p = 0.936). β-cryptoxanthin, zeaxanthin, lutein,
479 three lutein derivatives, retinol, three unidentified retinoids, and α- and γ-tocopherol
480 were detected in spleen tissue (Table S1). Total carotenoid levels were unaffected by
481 treatment (t = 0.67, df = 19, p = 0.512), initial bill redness, or food intake (Table 2). When
482 testing individual compounds, only lutein showed a trend towards a treatment effect (t =
483 1.78, df = 19, p = 0.091, d = 0.77), with LPS-treated birds showing higher lutein values
484 (means ± SE: 2.06 ± 1.90 and 1.21 ± 0.46 nmol/g, respectively). Interestingly, lutein was
485 detected in all LPS-treated birds, whereas five control birds showed zero values (χ^2 = 7.22,
486 df=1, p = 0.007). A similar pattern was observed for total carotenoid levels, with four
487 control birds lacking detectable carotenoids, compared to none in the LPS-treated group
488 (χ^2 = 5.44, df =1, p = 0.020).

489 Lastly, we tested whether spleen carotenoid content could be revealed by the
490 concurrent bill redness measure. In a model analyzing spleen total carotenoids
491 (dependent variable), final bill redness showed a significant positive association ($F_{1.17}$ =
492 5.88, p = 0.027; slope ± SE: 4.12 ± 2.93; r = 0.50; Figure 2C) but no interaction with
493 treatment (treatment: $F_{1.17}$ = 1.23, p = 0.283; interaction: $F_{1.17}$ = 0.02, p = 0.895). This
494 positive relationship remained significant (i.e., $F_{1.16}$ = 7.29, p = 0.016, slope ± SE: 4.27 ±
495 1.51) when adding the spleen mass as a covariate ($F_{1.16}$ = 3.29, p = 0.089, slope ± SE: 0.12
496 ± 0.06) to test the absolute amount of spleen carotenoids.



497

498 Figure 2. (A) Treatment effect on spleen mass (raw values; means ± SE). (B) Relationship between
499 spleen mass controlled for body mass (residuals) and initial bill redness and (C) between spleen total

carotenoid levels and final bill redness. Bill redness were residuals from models with bill brightness and measured bill area at the first (B) and second (C) sampling. Empty and filled dots for controls and LPS-treated birds, respectively. The treatment did not interact with bill redness (see Results).

Body temperature

The body temperature was significantly higher in LPS-treated birds compared to controls (t = 2.18, df = 68, p = 0.032; Figure 3A). When the initial bill redness was included as a covariate in an ANCOVA model testing body temperature, a trend toward a significant interaction with treatment was observed ($F_{1.66}$ = 0.03, p = 0.861; interaction: $F_{1.66}$ = 3.47, p = 0.067). The sign of the slope differed between LPS-treated and control birds (slope ± SE: 0.197 ± 0.158 and -0.238 ± 0.172, respectively), but neither slope significantly deviated from zero (p > 0.175). When we instead tested the final bill redness, this covariate was negatively correlated ($F_{1.66}$ = 4.37, p = 0.041; slope ± SE: -0.151 ± 0.178; r = 0.32; Figure 3B; interaction: $F_{1.66}$ = 0.66, p = 0.419). Food intake did not affect body temperature nor interact with treatment (both *p-values* > 0.25). No significant associations between body temperature and carotenoid levels in the bill, plasma, spleen or fat were detected (all p-values > 0.22). Body temperature was also unrelated to relative spleen mass (r = 0.03, p = 0.820, n = 70).

Nonetheless, when testing liver total carotenoid levels (residuals from a mixed model with only the random terms), we found a trend toward a significant interaction between body temperature and liver carotenoids (covariate: $F_{1.63}$ = 0.37, p = 0.545; interaction: $F_{1.63}$ = 3.46, p = 0.067). Control birds showed a negative relationship ($F_{1.33}$ = 4.69, p = 0.038; slope ± SE: -0.276 ± 0.128; r = -0.35), but LPS-treated birds did not ($F_{1.30}$ = 0.57, p = 0.457; slope ± SE: 0.140 ± 0.186, r = 0.14; Figure 3C).

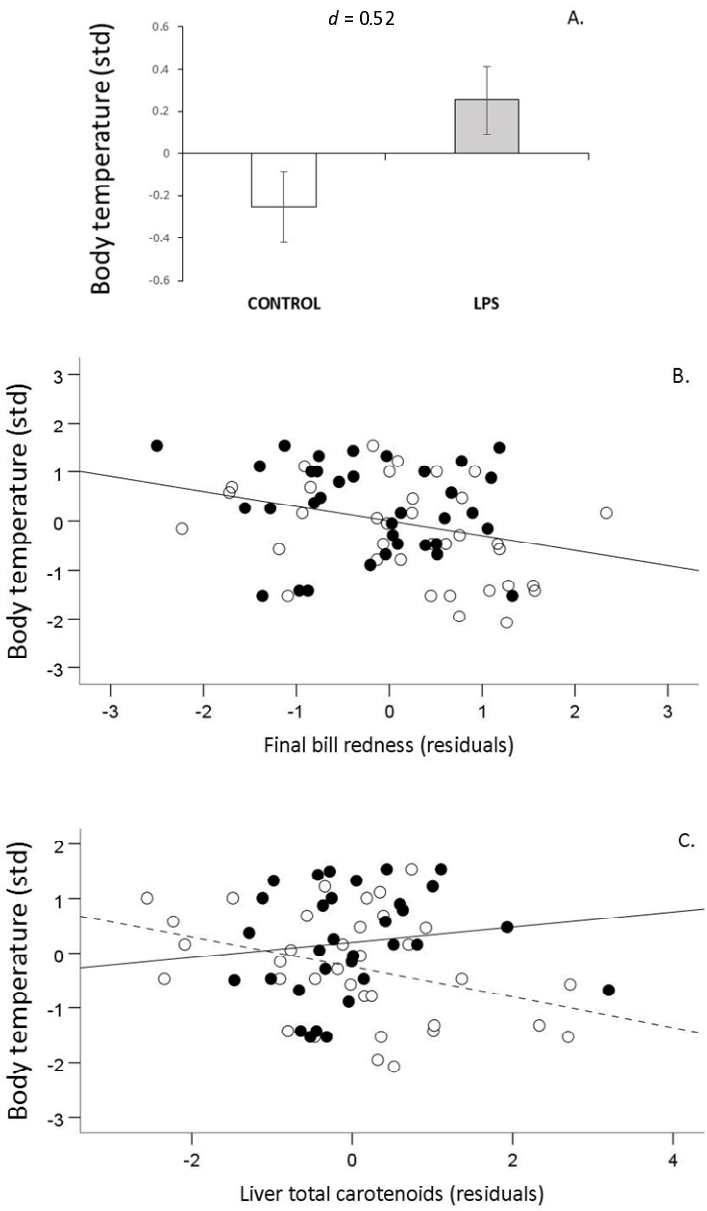


Figure 3. LPS effect on body temperature (A; means ± SE), relationship between body temperature and final bill redness (B) or liver total carotenoids (C). Temperature are standardized values and final bill redness were residuals from a mixed model including bill brightness and measured area at the last sampling event (see Methods). Controls: empty dots and dotted line. LPS-treated birds: filled dots and continuous line.

534 **DISCUSSION**

535 Our experimental immune challenge decreased bill redness and circulating carotenoid
536 levels in male zebra finches, agreeing with results from previously published data in this
537 and other species (see Introduction). However, we detected no significant interaction
538 between initial bill redness and treatment effects, suggesting that the birds' response to
539 the immune challenge was not influenced by their initial signal expression. This implies
540 that the strategy followed by the birds when investing in signaling under pressure
541 (immune challenge) is independent of their previous condition. Thus, the link between
542 signal level and individual quality could be weaker than expected for an index signal.
543 Moreover, the amount of food intake strongly influenced the signal expression level,
544 highlighting the high plasticity of the ketocarotenoid-based ornament.

545 The high plasticity of the zebra finch red bill can also be deduced from four
546 experimental studies in this species reporting quick changes in the trait. Thus, an increase
547 in bill redness was found in birds exposed to handling stress during four weeks (McGraw
548 et al., 2011). A similar bill color increment was found in males housed with females during
549 three weeks compared to those housed with males (Gautier et al., 2008). Even quicker,
550 testosterone implants increased male bill redness in three days (Ardia et al., 2010) and
551 certain immune activation (complete Freund's adjuvant injection) declined bill color in
552 only 48 hours (George et al., 2017).

553 Four findings support the presence of systemic inflammation in our LPS-treated
554 birds. First, there was a trend towards a significant decrease in blood γ-tocopherol values.
555 This could be attributed to its consumption when fighting the reactive nitrogen species
556 (e.g., nitric oxide) released by inflammation (e.g., Christen et al., 1997; Jiang et al., 2022).
557 Second, hepatic retinoids also showed a tendency to decline, which could also be due to
558 their use as antioxidants (Tesoriere et al., 1993; Palace et al., 1999) or as a substrate for
559 retinoic acid production, essential to mount the cellular immune response (Cifelli & Rose,
560 2006; Broadhurst et al., 2012; Rubin et al., 2017). Third, LPS-treated birds exhibited
561 heavier spleens, consistent with previous results in poultry (Koutsos et al., 2003; Rajput et
562 al., 2013; Bai et al., 2019). Fourth, LPS-treated birds showed higher body temperatures

563 than controls, in agreement with findings in different passerine species, including zebra
564 finches (Sköld-Chiriac et al., 2015; Tapper et al., 2021; but see Burness et al., 2010).

565 The LPS-induced reduction in food intake seems to have limited carotenoid
566 allocation to sexual coloration. The potential inhibitory effect of LPS on food intake is
567 currently well-known (see Introduction). We should highlight that an LPS-induced decline
568 in carotenoid-based colorations was interpreted in this and other animal species as
569 directly due to carotenoid being allocated to immune function stimulation or combat
570 reactive oxygen species released by inflammation (e.g., Alonso-Alvarez et al., 2004; Torres
571 & Velando, 2007; López et al., 2009; Cote et al., 2010; Romano et al., 2011; Rosenthal et
572 al., 2012). However, our result highlights the necessity of controlling birds' behavior when
573 testing the impact of immune challenges on carotenoid-based ornaments. The possibility
574 of a food intake influence on carotenoid-based signaling under an immune challenge or
575 stress was previously argued (McGraw et al., 2011; Rosenthal et al., 2012) but has not
576 been directly demonstrated until now.

577 The observed influence of food intake on bill redness challenges the notion that
578 red ketocarotenoid-based signals depend on intricate production mechanisms (involving
579 cell respiration) that are supposedly independent of resource allocation strategies
580 (Johnson & Hill, 2013; Hill et al., 2019, 2023). While it is theoretically expected that such
581 complex mechanisms ensure signal reliability through constraints (Podos, 2022), our
582 findings suggest that resource acquisition can deeply influence carotenoid-based
583 colorations, a possibility early suggested (Endler, 1980; Kodric-Brown, 1985; Hill, 1990;
584 Møller et al., 2000). Specifically, male zebra finches with redder bills appear to be those
585 better able to acquire sufficient carotenoid-rich food.

586 However, food intake itself was not directly correlated with bill carotenoid levels.
587 Thus, the link between food intake and bill redness (Figure 1D) may not be uniquely
588 attributed to dietary carotenoids acquired from the food. Perhaps, the overall nutritional
589 state could have caused changes in the tissue microstructure affecting coloration (e.g.,
590 Siefferman & Hill 2005; D'Alba et al., 2014). Additionally, limitations in carotenoid

591 extraction from the bill, which is a highly keratinized tissue, may contribute to the
592 absence of significant correlations. For instance, in a similar trait (i.e., the bill of red-
593 legged partridges, *Alectoris rufa*), we found a carotenoid recovery rate of 87% ± 3% (mean
594 ± CV; García de Blas et al., 2013). This means that 10-15% of the bill pigments could not
595 be extracted, adding error to the measure and complicating the detection of real effects.

596 Several findings support the reliability of red bill coloration as an indicator of
597 individual condition in our study. First, intriguingly, the birds with the reddest bills at the
598 start of the experiment had smaller spleens when sacrificed one month later, regardless
599 of treatment (Figure 2A). A larger spleen is often interpreted as a sign of better immune
600 competence (e.g., in birds: Møller & Erritzøe, 2002; Ardia, 2005; in mammals: Corbin et
601 al., 2008). In that scenario, the red keto-carotenoid-based signal could cheat those
602 interpreting it as redder bill individuals would have smaller spleens and, hence, poorer
603 immune competence. Alternatively, the reddest bill birds could be the healthiest
604 individuals if we consider that the spleen may become augmented during active
605 infections (e.g., Smith & Hunt, 2004; Figuerola et al., 2005). Our birds previously passed a
606 quarantine, and their behavior did not suggest health problems. In any case, we cannot
607 discard the presence of subtle infectious processes.

608 More intuitive support for red bill signal reliability was that the birds with the
609 reddest bills also had the lowest body temperatures. Here, we assume that the higher
610 body temperatures in the reported range (e.g., > 42°C) indicate underlying inflammatory
611 processes (raw values in Figure S7B, C). Body temperatures were also negatively
612 correlated to liver total carotenoid levels but only among controls (Figure 3C). Here, we
613 may deduce that the exposure to LPS forced the birds with high liver carotenoid
614 concentrations to sustain high body temperatures, flattening the regression slope (Figure
615 3C). However, this does not explain the lack of significant interaction in other tissues.

616 Among birds where the spleen carotenoids could be quantified (i.e., those with
617 heavier spleens and paler bills), LPS-treated individuals had a higher carotenoid detection
618 rate and a trend toward significantly higher spleen lutein levels. Despite the low sample

size, both results suggest enhanced carotenoid investment in the spleen during immune activation. This supports the presence of a carotenoid allocation trade-off between immune-competence and ketocarotenoid-based signaling if we consider the LPS-induced color decline in the full sample. However, that color decrease was undetected in the reduced subsample, perhaps due to its lower size. Further evidence for strategic carotenoid allocation comes from the significant positive correlations between recent food intake and carotenoid levels in plasma and fat of LPS-treated birds, but not controls. These immune-challenged individuals may have increased carotenoid absorption and storage from food, but not the carotenoid allocation to the signal (LPS-treated birds showed paler signals).

The positive relationship between final bill redness and spleen carotenoid levels further supports the reliability of bill color as an indicator of condition. Likewise, birds with measurable carotenoid concentrations in subcutaneous fat displayed redder bills at the end of the experiment. Carotenoid storage in fat is unlikely to serve as a reservoir for re-circulation. For example, in the Greylag geese (*Anser anser*), carotenoids stored in subcutaneous fat do not seem to be released back into circulation (Negro et al., 2001). Instead, mammalian studies suggest that carotenoids are relevant in lipid reserves, functioning as antioxidants supporting lipid catabolism pathways (Bonet et al., 2015; Mounien & Tourniare, 2019). Consequently, birds with redder bills may not only indicate their ability to invest carotenoids in their immune system but also their capacity to maintain efficient lipid metabolism.

In summary, in male zebra finches, the red keto-carotenoid-based bill signal and several parameters related to its production mechanism (blood, liver and fat carotenoid levels) were associated with food intake. The link to quality is mostly related to final bill redness, suggesting that the signal informs about the recent physiological condition (see also Gautier et al., 2008, Ardia et al., 2010, McGraw et al., 2011, George et al., 2017). The reddest birds at the end of the study were also those with lower body temperatures and detectable subcutaneous fat carotenoids. Taken together, our findings support the view that plastic resource allocation trade-offs underlie the reliability of red bill coloration in

male zebra finches, suggesting potential for strategic adjustment and even cheating strategies. Therefore, they do not seem to support a tight association of bill redness to individual quality, which should be expected for red keto-carotenoid ornaments more generally if they are rigidly constrained and unfalsifiable index signals (e.g. Weaver et al., 2017; Koch & Hill, 2018; Cantarero et al., 2020).

Ethics note: This experiment was approved by the animal ethics government office (i.e., Consejería de Agricultura, Junta de Castilla La Mancha; approval ref. JCCM 27-2021). All the authors consent to participate.

Acknowledgements: We thank María de los Santos Fernandez and Marta Garrido for their help in laboratory analyses.

Funding: Financial support was obtained from the project PID2019-109303GB-I00 from Ministerio de Ciencia e Innovación (MICINN; Spanish Government). Grant RYC2022-035559-I funded by MCIN/AEI/10.13039/501100011033 and by "ESF+" for AC.

Authors contribution: AC and CAA designed and performed the experiment. RA and PC performed chromatography analyses and datasets on carotenoids and vitamin levels. CAA obtained funds for the project. CAA performed the stats and wrote the first version of the text. MC and PA contributed to the experimental design. AC, RM, PC and MC revised the drafts of the text.

REFERENCES

Abou Elazab, M. F., Nasr, N. E., Ahmed, M. S., Alrashdi, B. M., Dahran, N., Alblihed, M. A., & Elmahallawy, E. K. (2022). The effects of bacterial lipopolysaccharide (LPS) on

670 turkey poults: assessment of biochemical parameters and histopathological changes.
671 *Veterinary Sciences*, *9*, 240. doi:10.3390/vetsci9050240

672 Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B., & Sorci, G.
673 (2004). An experimental test of the dose-dependent effect of carotenoids and
674 immune activation on sexual signals and antioxidant activity. *American Naturalist*,
675 *164*(5), 651–9. doi:10.1086/424971

676 Alonso-Alvarez, C., Blas, E. G.-D., & Mateo, R. (2018). Dietary canthaxanthin reduces
677 xanthophyll uptake and red coloration in adult red-legged partridges. *Journal of*
678 *Experimental Biology*, *221*(22), jeb185074. doi:10.1242/jeb.185074

679 Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O., & Viñuela, J. (2008). The
680 oxidation handicap hypothesis and the carotenoid allocation trade-off. *Journal of*
681 *Evolutionary Biology*, *21*(6), 1789–97. doi:10.1111/j.1420-9101.2008.01591.x

682 Ardia, D. R. (2005). Cross-fostering reveals an effect of spleen size and nest temperatures
683 on immune responses in nestling European starlings. *Oecologia*, *145*(2), 327–334.
684 doi:10.1007/s00442-005-0120-6

685 Ardia, D. R., Broughton, D. R., & Gleicher, M. J. (2010). Short-term exposure to
686 testosterone propionate leads to rapid bill color and dominance changes in zebra
687 finches. *Hormones and Behavior*, *58*(3), 526–532. doi:10.1016/j.yhbeh.2010.04.004

688 Baert, K., De Boever, S., Duchateau, L., & De Backer, P. (2005). Sodium salicylate
689 attenuates lipopolysaccharide (LPS)-induced adipsia, but not hypophagia, in broiler
690 chickens. *British Poultry Science*, *46*(2), 144–148. doi:10.1080/00071660500065243

691 Bai, W. Q., Zhang, K. Y., Ding, X. M., Bai, S. P., Wang, J. P., Peng, H. W., & Zeng, Q. F.
692 (2019). High dietary energy content increases inflammatory markers after
693 lipopolysaccharide challenge in meat ducks. *Poultry Science*, *98*(1), 164–171.
694 doi:10.3382/ps/pey380

695 Biernaskie, J. M., Grafen, A., & Perry, J. C. (2014). The evolution of index signals to avoid
696 the cost of dishonesty. *Proceedings of the Royal Society B*, *281*, 20140876.
697 doi:10.1098/rspb.2014.0876

698 Bonet, M. L., Canas, J. A., Ribot, J., & Palou, A. (2015). Carotenoids and their conversion
699 products in the control of adipocyte function, adiposity and obesity. *Archives of*
700 *Biochemistry and Biophysics*, *572*, 112–125. doi:10.1016/j.abb.2015.02.022

701 Broadhurst, M. J., Leung, J. M., Lim, K. C., Girgis, N. M., Gundra, U. M., Fallon, P. G., …
702 Loke, P. (2012). Upregulation of Retinal Dehydrogenase 2 in Alternatively Activated
703 Macrophages during Retinoid-dependent Type-2 Immunity to Helminth Infection in
704 Mice. *PLoS Pathogens*, *8*(8), e1002883. doi:10.1371/journal.ppat.1002883

705 Bryant, C. E., Spring, D. R., Gangloff, M., & Gay, N. J. (2010). The molecular basis of the
706 host response to lipopolysaccharide. *Nature Reviews Microbiology*, *8*(1), 8–14.
707 doi:10.1038/nrmicro2266

708 Buras, J. A., Holzmann, B., & Sitkovsky, M. (2005). Model organisms: Animal models of
709 sepsis: Setting the stage. *Nature Reviews Drug Discovery*, *4*(10), 854–865.
710 doi:10.1038/nrd1854

711 Butler, M. W., & McGraw, K. J. (2010). Relationships between dietary carotenoids, body
712 tissue carotenoids, parasite burden, and health state in wild mallard (Anas
713 platyrhynchos) ducklings. *Archives of Biochemistry and Biophysics*, *504*(1), 154–60.
714 doi:10.1016/j.abb.2010.07.003

715 Cantarero, A., Fernandez-eslava, B., Mateo, R., Camarero, P. R., Alonso, D., & Alonso-
716 alvarez, C. (2020). Testing the shared-pathway hypothesis in the carotenoid-based
717 coloration of red crossbills. *Evolution*, *74*, 2348–2364. doi:10.1111/evo.14073

718 Cho, H. C., & Abe, S. (2013). Is two-tailed testing for directional research hypotheses tests
719 legitimate? *Journal of Business Research*, *66*(9), 1261–1266.
720 doi:10.1016/j.jbusres.2012.02.023

721 Christen, S., Woodall, A. A., Shigenaga, M. K., Southwell-Keely, P. T., Duncan, M. W., &
722 Ames, B. N. (1997). γ-Tocopherol traps mutagenic electrophiles such as NOx and
723 complements α-tocopherol: Physiological implications. *Proceedings of the National*
724 *Academy of Sciences USA*, *94*(7), 3217–3222. doi:10.1073/pnas.94.7.3217

725 Cifelli, C. J., & Ross, A. C. (2006). All-trans-retinoic acid distribution and metabolism in
726 vitamin A-marginal rats. *American Journal of Physiology - Gastrointestinal and Liver*
727 *Physiology*, *291*, G195–G202. doi:10.1152/ajpgi.00011.2006

728 Cohen, J. (1992). A power primer. *Psychological Bulletin*, *112*(1), 155–159.

729 Corbin, E., Vicente, J., Martin-Hernando, M. P., Acevedo, P., Pérez-Rodríguez, L., &
730 Gortazar, C. (2008). Spleen mass as a measure of immune strength in mammals.
731 *Mammal Review*, *38*(1), 108–115. doi:10.1111/j.1365-2907.2007.00112.x

732 Cote, J., Arnoux, E., Sorci, G., Gaillard, M., & Faivre, B. (2010). Age-dependent allocation of
733 carotenoids to coloration versus antioxidant defences. *Journal of Experimental*
734 *Biology*, *213*(2), 271–277. doi:10.1242/jeb.035188

735 Csernus, B., Szabó, C., Knop, R., Kidane, R. G., Ndunguru, S. F., Gulyás, G., … Czeglédi, L.
736 (2023). Capsanthin supplementation modulates the immune response in broiler
737 chickens under Escherichia coli lipopolysaccharide challenge. *Archives Animal*
738 *Breeding*, *66*(1), 103–111. doi:10.5194/aab-66-103-2023

739 Csernus, B.; Biró, S.; Babinszky, L.; Komlósi, I.; Jávor, A.; Stündl, L.; Remenyik, J.; Bai, P.;
740 Oláh, J.; Pesti-Asbóth, G.; Levente Czeglédi. 2020. Effect of carotenoids,
741 oligosaccharides and anthocyanins on growth performance, immunological

742 parameters and intestinal morphology in broiler chickens challenged with *Escherichia*
743 *coli* lipopolysaccharide. *Animals*, 10, 347. https://doi.org/10.3390/ani10020347

744 D'Alba, L., Van Hemert, C., Spencer, K. A., Heidinger, B. J., Gill, L., Evans, N. P., … Shawkey,
745 M. D. (2014). Melanin-based color of plumage: role of condition and of feathers'
746 microstructure. *Integrative and Comparative Biology*, 54(4), 633–644.
747 doi:10.1093/icb/icu094

748 Elvira-Torales, L. I., García-Alonso, J., & Periago-Castón, M. J. (2019). Nutritional
749 importance of carotenoids and their effect on liver health: A review. *Antioxidants*,
750 *8*(7), 229. doi:10.3390/antiox8070229

751 Endler, J. A. (1980). Natural Selection on Color Patterns in *Poecilia reticulata*. *Evolution*,
752 *34*(1), 76–91.

753 Figuerola, J., Torres, J., Garrido, J., Green, A. J., & Negro, J. J. (2005). Do carotenoids and
754 spleen size vary with helminth load in greylag geese? *Canadian Journal of Zoology*,
755 *83*(3), 389–395. doi:10.1139/z05-022

756 Fitze, P. S., Tschirren, B., Gasparini, J., & Richner, H. (2007). Carotenoid-based plumage
757 colors and immune function: is there a trade-off for rare carotenoids? *American*
758 *Naturalist*, *169*, S137-44. doi:10.1086/510094

759 Foley, J.D., Van Dam, A., 1982. Fundamentals of Interactive Computer Graphics. Addison-
760 Wesley, Reading, M.A.

761 García-de Blas, E., Mateo, R., Viñuela, J., & Alonso-Álvarez, C. (2011). Identification of
762 carotenoid pigments and their fatty acid esters in an avian integument combining
763 HPLC-DAD and LC-MS analyses. *Journal of Chromatography B*, *879*(5–6), 341-348.
764 doi:10.1016/j.jchromb.2010.12.019

García-de Blas, E., Mateo, R., Viñuela, J., Pérez-Rodríguez, L., & Alonso-Alvarez, C. (2013). Free and esterified carotenoids in ornaments of an avian species: the relationship to color expression and sources of variability. *Physiological and Biochemical Zoology*, 86(5), 483–98. doi:10.1086/671812

Gautier, P., Barroca, M., Bertrand, S., Eraud, C., Gaillard, M., Hamman, M., … Faivre, B. (2008). The presence of females modulates the expression of a carotenoid-based sexual signal. *Behavioral Ecology and Sociobiology*, *62*(7), 1159–1166. doi:10.1007/s00265-008-0544-1

George, D. B., Schneider, B. C., McGraw, K. J., & Ardia, D. R. (2017). Carotenoids buffer the acute phase response on fever, sickness behavior and rapid bill color change in zebra finches. *Journal of Experimental Biology*, *220*(16), 2957–2964. doi:10.1242/jeb.155069

Gómez, J., Liñán-Cembrano, G., (2017). SpotEgg: an image-processing tool for automatised analysis of colouration and spottiness. *Journal Avian Biology* 48, 502–512.

Grether, G. F., Kolluru, G. R., & Nersissian, K. (2004). Individual colour patches as multicomponent signals. *Biological Reviews*, *79*(3), 583–610. doi:10.1017/S1464793103006390

Henderson, A. J., Lasselin, J., Lekander, M., Olsson, M. J., Powis, S. J., Axelsson, J., & Perrett, D. I. (2017). Skin colour changes during experimentally-induced sickness. *Brain, Behavior, and Immunity*, *60*, 312–318. doi:10.1016/j.bbi.2016.11.008

Hill, G. E. (2011). Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecology Letters*, *14*(7), 625–634. doi:10.1111/j.1461-0248.2011.01622.x

Hill, G. E., 1990. Female house finches prefer colorful males: Sexual selection for a condition-dependent trait. Anim Behav 40: 563–570.

789 Hill, G. E., Hood, W. R., Ge, Z., Grinter, R., Greening, C., Johnson, J. D., Park, N. R., Taylor,
790 H. A., Andreasen, V. A., Powers, M. J., Justyn, N. M., Parry, H. A., Kavazis, A. N., Zhang,
791 Y. (2019). Plumage redness signals mitochondrial function in the House Finch.
792 *Proceedings of the Royal Society B*, *286*, 20191354.

793 Hill, G. E., Weaver, R. J., & Powers, M. J. (2023). Carotenoid ornaments and the spandrels
794 of physiology: a critique of theory to explain condition dependency. *Biological*
795 *Reviews*, 98(6), 2320–2332. doi:10.1111/brv.13008

796 Ibáñez, A., Polo-Cavia, N., López, P., & Martín, J. (2014). Honest sexual signaling in turtles:
797 experimental evidence of a trade-off between immune response and coloration in
798 red-eared sliders Trachemys scripta elegans. *Die Naturwissenschaften*, *101*(10), 803–
799 811. doi:10.1007/s00114-014-1219-6

800 Jiang, Q., Im, S., Wagner, J. G., Hernandez, M. L., & Peden, D. B. (2022). Gamma-
801 tocopherol, a major form of vitamin E in diets: Insights into antioxidant and anti-
802 inflammatory effects, mechanisms, and roles in disease management. *Free Radical*
803 *Biology and Medicine*, *178*, 347–359. doi:10.1016/j.freeradbiomed.2021.12.012

804 Johnson, J. D., & Hill, G. E. (2013). Is carotenoid ornamentation linked to the inner
805 mitochondria membrane potential? A hypothesis for the maintenance of signal
806 honesty. *Biochimie*, *95*(2), 436–444. doi:10.1016/j.biochi.2012.10.021

807 Kaulmann, A., & Bohn, T. (2014). Carotenoids, inflammation, and oxidative stress-
808 implications of cellular signaling pathways and relation to chronic disease prevention.
809 *Nutrition Research*, *34*(11), 907–929. doi:10.1016/j.nutres.2014.07.010

810 Koch, R. E., & Hill, G. E. (2018). Do carotenoid-based ornaments entail resource trade-offs?
811 An evaluation of theory and data. *Functional Ecology*, *32*(8), 1908–1920.
812 doi:10.1111/1365-2435.13122

Koch, R. E., Hill, G. E., & Koch, R. E. (2016). The importance of carotenoid dose in supplementation studies with songbirds. *Physiological and Biochemical Zoology*, 89(1), 61–71. doi:10.1086/684485

Koch, R. E., Truong, C. N., Reeb, H. R., & Joski, B. H. (2025). Multiple pathways to red carotenoid coloration: House finches (*Haemorhous mexicanus*) do not use CYP2J19 to produce red plumage. Molecular Ecology, doi: 10.1111/mec.17744

Kodric-Brown, A. (1985). Female preferente and sexual selection for male coloration in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, 17, 199–205.

Koutsos, E. A., Calvert, C. C., & Klasing, K. C. (2003). The effect of an acute phase response on tissue carotenoid levels of growing chickens (*Gallus gallus domesticus*). *Comparative Biochemistry and Physiology - A*, *135*(4), 635–646. doi:10.1016/S1095-6433(03)00158-2

Laidre, M. E., & Johnstone, R. A. (2013). Animal signals. *Current Biology*, *23*(18), 829–833. doi:10.1016/j.cub.2013.07.070

Lessells, C. M., & Boag, P. T. (1987). Unrepeatable repetabilities: a common mistake. The Auk, 104, 116–121. doi:10.2307/4087240

Li, N., Ansari, A. R., Sun, Z., Huang, H., Cui, L., Hu, Y., … Liu, H. (2017). Toll like receptor 4 signaling pathway participated in Salmonella lipopolysaccharide-induced spleen injury in young chicks. *Microbial Pathogenesis*, *112*, 288–294. doi:10.1016/j.micpath.2017.10.004

Lieboldt, M. A., Frahm, J., Halle, I., Görs, S., Schrader, L., Weigend, S., … Dänicke, S. (2016). Metabolic and clinical response to Escherichia coli lipopolysaccharide in layer pullets of different genetic backgrounds supplied with graded dietary L-arginine. *Poultry Science*, *95*(3), 595–611. doi:10.3382/ps/pev359

Lopes, R. J., Johnson, J. D., Toomey, M. B., Ferreira, M. S., Araújo, P. M., Melo-Ferreira, J., … Carneiro, M. (2016). Genetic basis for red coloration in birds report genetic basis for red coloration in birds. *Current Biology*, 26(11), 1427–1434. doi:10.1016/j.cub.2016.03.076

López, P., Gabirot, M., & Martín, J. (2009). Immune challenge affects sexual coloration of male Iberian wall lizards. *Journal of Experimental Zoology Part A*, *311*(2), 96–104. doi:10.1002/jez.505

Maynard Smith, J. & Harper, D. (2003). Animal Signals. Oxford University Press.

McGraw, K. J., & Toomey, M. B. (2010). Carotenoid accumulation in the tissues of zebra finches: predictors of integumentary pigmentation and implications for carotenoid allocation strategies. *Physiological and Biochemical Zoology*, *83*(1), 97–109. doi:10.1086/648396

McGraw, K. J., Lee, K., & Lewin, A. (2011). The effect of capture-and-handling stress on carotenoid-based beak coloration in zebra finches. *Journal of Comparative Physiology. A, 197*(6), 683–91. doi:10.1007/s00359-011-0631-z

McGraw, K.J. (2006). Mechanics of carotenoid-based coloration. in G.E. Hill and K.J. McGraw, eds. Bird coloration, VoL. 1: Mechanisms and measurements. Harvard University Press, Harvard.

Meriwether, L. S., Humphrey, B. D., Peterson, D. G., Klasing, K. C., & Koutsos, E. A. (2010). Lutein exposure, in ovo or in the diet, reduces parameters of inflammation in the liver and spleen laying-type chicks (*Gallus gallus domesticus*). *Journal of Animal Physiology and Animal Nutrition*, *94*(5), 115–122. doi:10.1111/j.1439-0396.2010.00990.x

Merrill, L., Naylor, M. F., & Grindstaff, J. L. (2016). Imperfect past and present progressive: Beak color reflects early-life and adult exposure to antigen. *Behavioral Ecology*, *27*(5), 1320–1330. doi:10.1093/beheco/arw029

Metzger, B. J., & Bairlein, F. (2011). Fat stores in a migratory bird: a reservoir of carotenoid pigments for times of need? *Journal of Comparative Physiology. B*, *181*(2), 269–75. doi:10.1007/s00360-010-0511-9

Milani, A., Basirnejad, M., Shahbazi, S., & Bolhassani, A. (2017). Carotenoids: biochemistry, pharmacology and treatment. *British Journal of Pharmacology*, *174*(11), 1290–1324. doi:10.1111/bph.13625

Møller, A. P., & Erritzøe, J. (2002). Coevolution of host immune defence and parasite-induced mortality: Relative spleen size and mortality in altricial birds. *Oikos*, *99*(1), 95–100. doi:10.1034/j.1600-0706.2002.990110.x

Møller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N. & Surai, P. F. (2000). Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian and Poultry Biology Reviews*, 11, 137–159.

Mounien, L., & Tourniaire, F. (2019). Nutrients adipose tissue and adipose tissue-driven. *Nutrients*, *11*, 1–14.

Mundy, N. I. I., Stapley, J., Bennison, C., Tucker, R., Twyman, H., Kim, K. W., … Slate, J. (2016). Red carotenoid coloration in the zebra finch is controlled by a cytochrome p450 gene cluster. *Current Biology*, 26(11), 1435–1440. doi:10.1016/j.cub.2016.04.047

Negro, J. J., Figuerola, J., Garrido, J., & Green, a. J. (2001). Fat stores in birds: an overlooked sink for carotenoid pigments? *Functional Ecology*, *15*(3), 297–303. doi:10.1046/j.1365-2435.2001.00526.x

885 Palace, V. P., Khaper, N., Qin, Q., & Singal, P. K. (1999). Antioxidant potentials of vitamin A
886 and carotenoids and their relevance to heart disease. *Free Radical Biology and*
887 *Medicine*, *26*(5–6), 746–761. doi:10.1016/S0891-5849(98)00266-4

888 Perez-Rodriguez, L. (2009). Carotenoids in evolutionary ecology: re-evaluating the
889 antioxidant role. *BioEssays*, *31*, 1116–1126. doi:10.1002/bies.200900070

890 Podos, J. (2022) Costs, constraints and sexual trait elaboration. *Animal Behaviour* 184,
891 209-214. doi:10.1016/j.anbehav.2021.05.021

892 Rajput, N., Naeem, M., Ali, S., Zhang, J. F., Zhang, L., & Wang, T. (2013). The effect of
893 dietary supplementation with the natural carotenoids curcumin and lutein on broiler
894 pigmentation and immunity. *Poultry Science*, *92*(5), 1177–1185. doi:10.3382/ps.2012-
895 02853

896 Rathinam, V. A. K., Zhao, Y., & Shao, F. (2019). Innate immunity to intracellular LPS. *Nature*
897 *Immunology*, *20*(5), 527–533. doi:10.1038/s41590-019-0368-3

898 Rice, W. R., & Gaines, S. D. (1994). 'Heads I win, tails you lose': testing directional
899 alternative hypotheses in ecological and evolutionary research. *Science*, *9*, 235–237.

900 Ridout, M., Demetrio, C. G., & Hinde, J. (1998). Models for count data with many zeros. In
901 *Proceedings of the XIXth International Biometric Conference*, pages 179–192.

902 Rodríguez-Estival, J., Martínez-Haro, M., Martín-Hernando, M. a P., & Mateo, R. (2010).
903 Sub-chronic effects of nitrate in drinking water on red-legged partridge (Alectoris
904 rufa): oxidative stress and T-cell mediated immune function. *Environmental Research*,
905 *110*(5), 469–75. doi:10.1016/j.envres.2010.03.008

906 Romano, A., Rubolini, D., Caprioli, M., Boncoraglio, G., Ambrosini, R., & Saino, N. (2011).
907 Sex-Related effects of an immune challenge on growth and begging behavior of barn
908 swallow nestlings. *PLoS ONE*, *6*(7), e22805. doi:10.1371/journal.pone.0022805

Rosenthal, M. F., Murphy, T. G., Darling, N., & Tarvin, K. A. (2012). Ornamental bill color rapidly signals changing condition. *Journal of Avian Biology*, *43*(6), 553–564. doi:10.1111/j.1600-048X.2012.05774.x

Rubin, L. P., Ross, A. C., Stephensen, C. B., Bohn, T., & Tanumihardjo, S. A. (2017). Metabolic effects of inflammation on vitamin A and carotenoids in humans and animal models. *Advances in Nutrition*, *8*(2), 197–212. doi:10.3945/an.116.014167

Ruxton, G. D., & Neuhäuser, M. (2010). When should we use one-tailed hypothesis testing? *Methods in Ecology and Evolution*, *1*(2), 114–117. doi:10.1111/j.2041-210x.2010.00014.x

Scalf, C. S., Chariker, J. H., Rouchka, E. C., & Ashley, N. T. (2019). Transcriptomic analysis of immune response to bacterial lipopolysaccharide in zebra finch (*Taeniopygia guttata*). *BMC Genomics*, *20*(1), 647. doi:10.1186/s12864-019-6016-3

Sköld-Chiriac, S., Nord, A., Tobler, M., Nilsson, J. Å., & Hasselquist, D. (2015). Body temperature changes during simulated bacterial infection in a songbird: Fever at night and hypothermia during the day. *Journal of Experimental Biology*, 218(18), 2961–2969. doi:10.1242/jeb.122150

Siefferman, L., & Hill, G. E. (2005). Evidence for sexual selection on structural plumage coloration in female eastern bluebirds (*Sialia sialis*). *Evolution*, 59(8), 1819–1828. doi:10.1111/j.0014-3820.2005.tb01828.x

Sild, E., Sepp, T., Männiste, M., & Hõrak, P. (2011). Carotenoid intake does not affect immune-stimulated oxidative burst in greenfinches. *Journal of Experimental Biology*, 214(20), 3467–3473. doi:10.1242/jeb.062182

Simons, M. J. P., & Verhulst, S. (2011). Zebra finch females prefer males with redder bills independent of song rate-a meta-analysis. *Behavioral Ecology*, *22*(4), 755–762. doi:10.1093/beheco/arr043

Smith, K. G., & Hunt, J. L. (2004). On the use of spleen mass as a measure of avian immune system strength. *Oecologia*, *138*(1), 28–31. doi:10.1007/s00442-003-1409-y

Stevens, M., Párraga, C. A., Cuthill, I. C., Partridge, J. C., & Troscianko, T. S. (2007). Using digital photography to study animal coloration. *Biological Journal of the Linnean Society*, 90, 211–237.

Svensson, P. A., & Wong, B. B. M. (2011). Carotenoid-based signals in behavioural ecology: A review. *Behaviour*, *148*(2), 131–189. doi:10.1163/000579510X548673

Számadó, S., Samu, F., & Takács, K. (2022). Condition-dependent trade-offs maintain honest signalling. *Royal Society Open Science*, *9*(10). doi:10.1098/rsos.220335

Számadó, S., Zachar, I., & Penn, D. J. (2023b). The trade-offs of honest and dishonest signals. *EcoEvorxiv*, preprint: https://doi.org/10.32942/X2960G

Számadó, S., Zachar, I., Czégel, D., & Penn, D. J. (2023a). Honesty in signalling games is maintained by trade-offs rather than costs. *BMC Biology*, *21*(1), 4. doi:10.1186/s12915-022-01496-9

Tang G & Russell RM (2009). Carotenoids as Provitamin A. In Carotenoids Volume 5: Nutrition and Health. Edited by G. Britton, S. Liaaen-Jensen, H. Pfander. Chapter 8, pp. 149-172. Birkhäuser Verlag, Basel, Germany.

Teemer, S. R., & Hawley, D. M. (2024). Effects of cold temperature and pseudo-infection on sickness behaviour and transmission potential in house finches. *Animal Behaviour*, 209, 179–189. doi:10.1016/j.anbehav.2024.01.003

Tesoriere, L., Bongiorno, a, Pintaudi, a M., D'Anna, R., D'Arpa, D., & Livrea, M. a. (1996). Synergistic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Archives of Biochemistry and Biophysics*, *326*(1), 57–63. doi:10.1006/abbi.1996.0046

Toomey, M. B., Marques, C. I., Araújo, P. M., Huang, D., Zhong, S., Liu, Y., … Corbo, J. C. (2022). A mechanism for red coloration in vertebrates. *Current Biology*, 32(19), 4201-4214. doi:10.1016/j.cub.2022.08.013

Torres, R., & Velando, A. (2007). Male reproductive senescence: The price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby. *Journal of Animal Ecology*, *76*(6), 1161–1168. doi:10.1111/j.1365-2656.2007.01282.x

Weaver, R. J., Koch, R. E., & Hill, G. E. (2017). What maintains signal honesty in animal colour displays used in mate choice? *Philosophical Transactions of the Royal Society B*, *372*(1724). doi:10.1098/rstb.2016.0343

Weaver, R. J., Santos, E. S. A., Tucker, A. M., Wilson, A. E., & Hill, G. E. (2018). Carotenoid metabolism strengthens the link between feather coloration and individual quality. *Nature Communications*, *9*(1), 73. doi:10.1038/s41467-017-02649-z

Xie, H., Rath, N. C., Huff, G. R., Huff, W. E., & Balog, J. M. (2000). Effects of Salmonella typhimurium lipopolysaccharide on broiler chickens. *Poultry Science*, *79*(1), 33–40. doi:10.1093/ps/79.1.33