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1 **Mutual mate preferences and assortative mating in relation to**
2 **a carotenoid-based color trait in blue tits**

3

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6

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14

15

16 Short title: Mate-preferences in blue tits

17

18 **Abstract**

19 Choosing an appropriate sexual partner is a critical decision for many animal species.
20 However, many mechanisms involved in mate choice are still poorly understood. Do
21 both males and females choose their sexual partners, do both sexes use the same
22 criteria for choosing, and do their own phenotype influence the choices they make, are
23 questions that need further investigation. Over two successive experiments conducted
24 in captivity with hand-reared blue tits (*Cyanistes caeruleus*), we manipulated the color
25 of the chest plumage, a secondary sexual trait that reflects an individual's condition, to
26 create two different color morphs (one pale and one colored). We then tested
27 whether both sexes express a preference, whether they are attracted to the same
28 morphs, and if the subjects' own chest color influences the preference they show. Our
29 data reveal that both sexes are choosy, with females tending to be slightly choosier
30 than males. We also show that both sexes preferentially select individuals with a pale
31 chest plumage over colorful individuals, and this was again more pronounced in
32 females. Finally, paler individuals tend to be selected by birds that are themselves
33 pale, even if this phenotype matching was not very robust. Such a preference for paler
34 individuals is intriguing since mates are predicted to associate with individuals
35 displaying higher, not lower, value of quality signals. It could result from adaptive
36 mechanisms related to avoidance of aggressiveness in confined environment,
37 avoidance of conflicting sexual signals within individuals, or from cultural mechanisms
38 leading to preference for individuals that match its own phenotype.

39

40 Keywords: bird, carotenoids, color, mate-choice, sexual selection

41

42 Introduction

43

44 Choosing mates is one of the most important decisions that many sexually reproducing
45 animals have to take. Mate choice indeed impacts an individual's current reproductive
46 success and fitness (Andersson, 1994). Both direct and indirect benefits, such as access
47 to a high-quality territory, parental care, reduced likelihood of getting sexually
48 transmitted disease, or genetic quality for the offspring, can be derived from selecting
49 an appropriate partner (Fisher, 1915; Trivers, 1972; Smith, 1991). In conventional sex-
50 role species, females are typically considered the choosing sex, while males spend
51 their time advertising (Jones and Ratterman, 2009; Davies et al., 2012). As a
52 consequence, mate choice has been mostly studied in terms of male traits and
53 behaviours that affect female preference. However, conspicuous traits are present
54 both in males and females of many species (Amundsen, 2000; Dale et al., 2015; Hare
55 and Simmons, 2019; Doutrelant et al., 2020). While these traits in females have long
56 been considered non-functional byproducts of sexual selection on male traits (Lande,
57 1980; Price, 1996; Kraaijeveld et al., 2007), the possibility that males could also choose
58 their female partners has received comparatively less attention (Amundsen, 2000;
59 Clutton-Brock, 2009; Courtiol et al., 2016; Doutrelant et al., 2020). Like male
60 ornaments, female ornaments could signal reproductive or survival qualities and be
61 involved in male mate choice (Hare and Simmons, 2019). Mutual choosiness is
62 predicted to be particularly important when benefits of choices are evident for both
63 sexes, in particular in monogamous species where reproductive rates are similar for
64 males and females, and in species with biparental care (Johnstone et al., 1996; Kokko
65 and Johnstone, 2002; Kraaijeveld et al., 2007). Male mate preferences have been
66 documented in various taxa, including fishes (Sargent et al., 1986; Amundsen and
67 Forsgren, 2001; Roberts and Mendelson, 2017; Schlupp, 2018), insects (Bonduriansky,
68 2001; Byrne and Rice, 2006), reptiles (Olsson, 1993; LeBas and Marshall, 2000),
69 mammals (Domb and Pagel, 2001; Fitzpatrick et al., 2015), and birds (Amundsen et al.,
70 1997; Griggio et al., 2005; Pryke and Griffith, 2007; Kimmitt et al., 2018). In birds for
71 example, male bluethroats (*Luscinia s. svecica*) have been reported to spend more
72 time close to colorful than drab females in a binary choice experiment (Amundsen et

73 al., 1997). Female coloration positively correlated with tarsus length and body mass,
74 suggesting that coloration can also signal phenotypic quality in females (Amundsen et
75 al., 1997; Roulin et al., 2000; Griggio et al., 2005; Weiss, 2006; Doutrelant et al., 2008;
76 2012). In crested auklets (*Aethia cristatella*), both males and females were attracted
77 by opposite-sex models having experimentally increased crests on the front-head
78 (Jones and Hunter, 1993). On the other hand, evidence for male mate preference has
79 failed to be shown in other studies (Muma and Weatherhead, 1989; Dale and
80 Slagsvold, 1994). Since reproduction is often less costly for males than females
81 (Williams, 2012), and additional paternities can be gained from fertilizing multiple
82 females, males might not always need to be choosy (but see Pizzari et al., 2003). Male
83 choosiness might even be counterselected if choosy males face a competitive
84 disadvantage (Fitzpatrick et al 2015). These contrasted hypotheses highlight the need
85 for additional work on male sexual preferences to understand how often males
86 contribute to sexual selection.

87

88 For a long time, it has also been considered that individuals always try to mate with
89 the highest quality partners. In practice, this has assumed that regardless their own
90 condition or phenotype, females (historically considered the choosing sex, see above)
91 should always choose the best males, and therefore that there is little inter-individual
92 variation in preferences (Cotton et al., 2006). Several studies have however challenged
93 this view and shown that individuals may vary substantially in their mate preferences,
94 with preferences that can sometimes be predicted from the subject's own phenotype.
95 Female black field crickets (*Teleogryllus commodus*) raised on a protein-rich diet were
96 more responsive and choosy to male call quality than females reared on poorer diets
97 (Hunt et al., 2005). Females of the smooth toadlet (*Uperoleia laevigata*), an Australian
98 frog, selected mates that were about 70% of their own body weight (Robertson, 1990).
99 In zebra finches (*Taeniopygia guttata*), females that were reared in enlarged broods,
100 and that were concomitantly considered as being of poorer quality than females raised
101 in small broods, were shown to prefer males that were themselves of lower quality
102 (Holveck and Riebel, 2010; see Griggio and Hoi, 2010 for a similar example in sparrows;
103 but see Wang et al., 2017). Similarly, male and female great tits (*Parus major*) have
104 been shown to prefer opposite-sex individuals that were of similar heterozygosity

105 levels as themselves, even if in the case of relatively homozygous individuals it meant
106 having offspring of low heterozygosity level (Zandberg et al., 2017). In the frog and the
107 great tit examples, the authors also showed that these preferences are adaptive
108 because pairing with the preferred phenotypes led to higher fertilization rates and
109 fitter offspring, respectively (Robertson, 1990; Zandberg et al., 2017). Such kind of self-
110 referent phenotype matching, leading to assortative mating, could be quite
111 widespread. It has however been relatively little studied experimentally so far.

112

113 Here we study experimentally the mate preferences of captive blue tits. Blue tits are
114 socially monogamous, both sexes care for the offspring, and both sexes are
115 ornamented (Andersson et al., 1998), characteristics that, according to what we have
116 reported above, are ideal for testing mutual mate preference (Hunt et al., 1999) and
117 assortative mating (Fargevieille et al., 2017). In blue tits both the UV-blue crest and the
118 yellow chest have been suggested to function as secondary sexual signals (but see
119 Parker, 2013 for a meta-analysis on male UV coloration). Most studies focused on the
120 role that blue crest color variation plays on intra-sexual competition and on inter-
121 sexual selection (Hunt et al., 1998; 1999; e.g. Delhey et al., 2003; Kurvers et al., 2010;
122 Remy et al., 2010; Parker, 2013). The role of the yellow-colored chest has received less
123 attention, but there is correlational and experimental evidence that the intensity of
124 yellow chroma or brightness may reflect an individual's condition and parental ability
125 (Senar et al., 2002; Saks et al., 2003; Peters et al., 2007; Doutrelant et al., 2008; Ferns
126 and Hinsley, 2008; Reudink et al., 2009; 2012; Garcia-Navas et al., 2012; Midamegbe et
127 al., 2013). The yellow color could thus potentially be used as a reliable signal of quality
128 in a mate-choice context. Contrary to the blue color that is structurally based, the
129 yellow color is conferred by carotenoids exclusively acquired through food (Goodwin,
130 1984; Hill and McGraw, 2006; Isaksson et al., 2008). In captivity, where the right
131 balance of carotenoids is difficult to provide, blue and great tits rapidly lose their
132 yellow-based coloration that becomes light-grey (see Fig.S1A), showing how plastic
133 this trait is. Carotenoids are also thought to contribute to prevention of oxidative
134 stress and regulation of immune function (Blount et al., 2003; Biard et al., 2006;
135 Simons et al., 2012; Koch and Hill, 2018).

136

137 Here, we test whether both male and female blue tits choose their partners, and
138 whether the expressed preferences are based on the color of their breast. For this, we
139 manipulated the chest color of captive blue tits and tested the preferences of
140 opposite-sex individuals for variation in this phenotypic trait over two successive
141 experiments. In the first experiment, we manipulated the chest colors of the birds that
142 were presented as stimuli. In the second experiment, we manipulated the chest colors
143 of both the stimulus and the subject birds. We then tested whether both males and
144 females express preferences (experiments 1 and 2), whether they show preferences
145 for the same color phenotypes (experiments 1 and 2), and whether their own
146 phenotype (chest color) influences the preference they express (experiment 2).

147

148 **Methods**

149

150 Ethical note

151 The experiments run in this study were approved by the Animal Experimentation
152 Committee of the Royal Dutch Academy of Sciences (DEC-KNAW; permit number
153 CTE.09-04 and NIOO11.09), and the Animal Care and Use Committee Languedoc-
154 Roussillon (permit number CEEA-LR-1047). The work performed in the field was
155 approved by the prefectural office of Corsica and the Regional Direction of
156 Environment (DIREN) committee (permit numbers 2009-0379, 3467 and 2015615-
157 147).

158

159 Subjects

160 In experiment 1, we used 81 blue tits (*Cyanistes caeruleus*; 44 males and 37 females)
161 that were taken as nestlings in the long-term studied populations in Corsica (Muro and
162 Pirio, see Reparaz et al., 2014). Whole broods of chicks (N=17 broods) were collected
163 from their nests when they were 7 to 10-day-old, and were transferred to the laboratory
164 for standardized hand rearing (see Drent et al., 2003; Titulaer et al., 2012). Briefly, birds
165 were transported to the Netherlands Institute of Ecology (NIOO-KNAW) by car. During
166 the travel, and later on at the institute, chicks were fed every half-hour, for 14 hours per
167 day (7:00 am - 9:00 pm), with a diet consisting of a mixture of curd cheese, ground beef

168 heart, baby cereal, multivitamin solution and calcium carbonate, supplemented with
169 wax moth larvae and bee larvae, until independence. After reaching independence
170 (about 35 days after hatching) and being molecularly sexed, the birds were temporarily
171 transferred to an individual home cage of 0.9 x 0.4 m x 0.5 m, before being released in
172 single-sex groups in outdoor aviaries, with acoustic but no visual contacts between
173 aviaries. Adult diet consisted of a mixture of egg, cow heart, vitamins, and minerals,
174 supplemented with dry food containing insects (Orlux Insect Patee, Versele-Laga,
175 Deinze, Belgium) and peanuts. Birds also had access to mealworms, grit, and sunflower
176 seeds. Food and water were provided *ad libitum*.

177

178 In experiment 2, we used 72 blue tits (40 males and 32 females) that were also taken as
179 nestlings, aged 8 to 12-day-old, in the long-term studied population of La Rouvière, near
180 Montpellier. Hand-rearing procedures were similar to those of experiment 1, except
181 that it was conducted at the Center for Functional and Evolutionary Ecology (CEFE-
182 CNRS) at Montpellier, France. Diet consisted here in a solution of hand-rearing powder
183 (Nutribird A21 and A19, Versele-Laga, Deinze, Belgium), supplemented with wax moth
184 larvae, bee larvae, and mealworms. After they reached independence, birds were fed
185 with mealworms, and a cake made of eggs, sunflower margarine, sugar, flour and
186 protein-rich pellets (Country's Best Show1-2 Crumble, Versele-Laga, Deinze, Belgium).
187 Both mealworms and cake were supplemented with commercial powders containing
188 mostly vitamins and minerals (Nutribird A21, Versele-Laga; and Nekton-S, Nekton
189 GmbH, Pforzheim, Germany). Food and water were provided *ad libitum*. Birds were also
190 housed in single-sex groups in outdoor aviaries. Here however, visual and physical
191 contact were possible through the mesh of adjacent aviaries. Aviaries housing groups of
192 females were away from aviaries housing groups of males, with no visual and physical
193 contacts possible between males and females. For logistic reasons inherent to food
194 distribution, birds from a given aviary belonged to the same color treatment (provided
195 in the food, see below), and treatments alternated between aviaries, so that each bird
196 could see and interact with same-sex birds from both treatments.

197

198 Color treatments

199 In experiment 1, we manipulated the chest color of male and female blue tits using color
200 markers (a procedure used in other studies, Delhey et al., 2007; Remy et al., 2010; Ligon
201 and McGraw, 2016). Only birds used as stimuli in the mate-preference apparatus (32
202 birds: 16 males and 16 females, see below) were artificially colored, not the ones that
203 were used as subjects (49 birds: 28 males and 21 females). Half of the stimulus birds (8
204 males and 8 females) had their chest feathers colored in yellow (Yellow group, Fig. 1A),
205 with a marker that best mimic the natural color in free-living blue tits (Prismacolor Art
206 Marker, canary yellow PM19, Newell Rubbermaid, Atlanta, USA), while the other half of
207 the birds (8 males and 8 females) had their chest feathers colored in grey (Prismacolor
208 Art Marker, warm grey PM-99, Newell Rubbermaid, Atlanta, USA), the color that blue
209 and great tits commonly acquire when held in captivity for long periods of time (Fig. 1A).
210 Resulting colors were measured with a spectrometer (Avaspec, Avantes, The
211 Netherlands) and with a DH-2000 Deuterium Tungsten Halogen Light Source and a 200
212 μm optic-fiber probe. Spectrograms obtained from the two kinds of artificially colored
213 birds were close to spectrograms obtained in non-manipulated birds (wild yellow birds
214 and captive grey birds, respectively; Fig. 1A and C, table S1).

215

216 In experiment 2, we manipulated the chest color of male and female blue tits through
217 the addition of carotenoids in their diet. In order to test for self-reference phenotype
218 matching mechanisms, the chest plumage of both stimulus and subject birds were
219 manipulated early in life, when birds were starting to feed independently (i.e. about 35
220 days old, in early June). Thirty five birds had their diet (cake and mealworms, see above)
221 supplemented with a powder containing 10% of canthaxanthin at a concentration of
222 5000 ppm (Carophyll-Red 10%, produced by DSM Nutritional Products Ltd, Basel,
223 Switzerland), while 37 birds had their diet supplemented with a placebo made of the
224 exact same powder as in the other group, but with no pigment in it (placebo specifically
225 made for the experiment by DSM Nutritional Products Ltd, Basel, Switzerland).
226 Treatments thus only differed in the presence/absence of canthaxanthin, which colors
227 the chest plumage in dark yellow/orange. Spectrograms of placebo and canthaxanthin
228 tits are shown at fig. 1B. We opted for canthaxanthin rather than other carotenoids
229 present in the diet of wild tits like lutein, or zeaxanthin because those other carotenoids
230 have not always been efficient in enhancing the chest color of blue tits (e.g. Biard et al.,

231 2006), and because we had experience with canthaxanthin and knew it efficiently colors
232 the chest plumage of blue tits (P. Perret, unpubl. data). Furthermore, using
233 canthaxanthin allowed us to oppose a pale yellow/grey to a dark yellow/orange morph,
234 two experimental groups that consisted in color phenotypes rarely encountered in the
235 wild, thus avoiding to oppose a natural phenotype (natural yellow) with an artificial one
236 (pale yellow/grey). Treatments were stopped two weeks before the start of the
237 behavioral tests. After the first series of binary preference tests, we started the diet
238 supplementation again, but we reversed the experimental groups, i.e. birds that were
239 first supplemented with canthaxanthin received the placebo, and vice versa for the
240 other group, which reversed the plumage colors of the two groups. That way each bird
241 was tested in both groups.

242

243 Mate preference testing

244 About two weeks before the start of the tests, birds were settled in individual cages in
245 rooms where opposite-sex birds could not see each other. Tested and stimulus birds
246 were housed in separate rooms. During that time, birds were exposed to a long
247 photoperiod (≥ 15 hours of light per day). Long photoperiods indeed trigger a cascade
248 of neuroendocrine reactions that lead to production and secretion of sexual steroids by
249 the gonads, particularly 17β -estradiol (E2) in females, and promote the expression of
250 reproductive behaviors (Dawson et al., 2001). In songbirds, increasing plasma E2 in
251 females, either directly through injection or implants of E2, or indirectly through
252 exposure to long photoperiods, is often used to infer behavioral sexual preferences to
253 a relevant stimulus like male song or male presence (Searcy, 1992; Byers and Kroodsma,
254 2009; Caro et al., 2010; Reparaz et al., 2014). By exposing blue tits to long days prior to
255 testing, we increased the chances that the observed choices are actual sexual
256 preferences and not social preferences, even though it is hard to firmly distinguish the
257 two.

258

259 Experiment 1: Among the 81 birds used, 32 (16 males and 16 females) were used as
260 stimulus birds. These 32 birds were randomly distributed across the two color
261 treatments (yellow and grey markers, see above). The remaining 49 birds were not

262 artificially colored and used as subjects. Each subject was tested only once and exposed
263 to one pair of unrelated birds of the opposite sex. Sixteen pairs (8 pairs of each sex) of
264 stimulus birds were formed and used in two to four mate-preference trials. Tests were
265 performed in 2013 (April 23 to May 15), with birds that were born in 2010 and 2011.

266

267 Experiment 2: Among the 72 birds used, 24 (12 males and 12 females) were used as
268 stimulus birds. Contrary to experiment 1, both stimulus and tested birds were subjected
269 to the color treatments (canthaxanthin and placebo supplements in the diet, see above).
270 In addition, the members of the 12 pairs of stimulus birds were not selected at random
271 here, but pairs were made of brothers or sisters. Choosing genetically similar birds for a
272 given pair reduces any genetically-related and early-environment-related variances
273 between the members of a pair and hence enhances the importance of the artificially
274 manipulated color of the chest as a choice criterion during the behavioural tests. Each
275 pair of stimulus birds was used in three to five mate-preference trials. Tests were
276 performed in 2015 (December 16 to December 23), with birds that were born in spring.
277 At that age, birds were sexually mature (Silver et al., 1992; Dawson et al., 2001), and
278 were set into a reproductive-like status by the long photoperiod (see above). After the
279 end of the first series of tests, continuous exposure to a long photoperiod led to a state
280 of photorefractoriness that coincides with the onset of a new molt of all feathers
281 (Dawson et al., 2001). Once the first birds had started molting, all birds were moved to
282 a shorter photoperiod for six weeks, which restored photosensitivity and allowed for a
283 new cycle of stimulation of the reproductive system by the long days of April (Silverin,
284 1994; Dawson et al., 2001). At the end of the first series of tests the color treatments
285 were reversed for all birds (see above), so that birds from each group could grow breast
286 feathers of the color that previously characterized the other group. The second series
287 of tests took place in Spring 2016 (April 28 to May 3, 2016), keeping the same pairs of
288 stimulus birds as in the first trials.

289

290 The test apparatus used for assessing potential partner preferences was identical in
291 both experiments and similar to the one described in Reparaz et al. (2014). Briefly, two
292 identical test chambers were used simultaneously. Each test chamber was made of a
293 large neutral area (approximately 4m²) and two semi-enclosed areas (approximately

294 1m² each) in front of the cages in which the stimulus birds were placed (Fig. S1). Artificial
295 wooden "trees" were disposed in the neutral zone and in each stimulus zone. Each
296 stimulus cage was constructed of wood and wire mesh, and affixed to a rolling base. A
297 'natural sun' light bulb (Arcadia Compact Bird Lamp 20W, Arcadia Products, Redhill,
298 United Kingdom) was installed in the top of each stimulus bird cage to allow for UV-
299 coloration visibility, and each cage was equipped with four wooden perches. A curtain
300 was installed in front of each two cages to conceal the stimulus birds from the subject
301 bird as needed throughout the testing. Three wide-angle cameras were affixed in each
302 test chamber, with one in the neutral area, and one in each of the two stimulus zones,
303 to capture both subject and stimulus bird activities throughout the trials (Fig. S1).

304

305 Tested and stimulus birds were introduced in their respective compartments, with the
306 curtains closed to prevent visual contacts between them. They were given 10 minutes
307 to acclimate to the chamber/cages. After the acclimation period, the curtains were
308 raised, and the first half of the mate preference trial took place for 20 minutes. After
309 that time, the curtains were closed, and the stimulus cages were switched, in order to
310 account for any side-bias of the subject birds. The second half of the trial took place over
311 20 minutes. After testing, subjects were returned to their home cages. Each bird was
312 tested only once in experiment 1, twice in experiment 2, i.e. once as canthaxanthin bird,
313 and once as placebo bird, with four months in between the tests to allow birds to molt
314 and change their chest plumage color.

315

316 Video analyses and data processing

317 Video footages from the mate preference tests were analyzed using the computer
318 programs Observer XT (version 10.5, Noldus, Wageningen, The Netherlands) for
319 experiment 1, and Solomon Coder (version beta 15.11.19, <https://solomoncoder.com>)
320 for experiment 2. Footages were analyzed in terms of the time that subject birds spent
321 in each of the three possible zones (neutral, right stimulus, left stimulus). Data from the
322 two 20-min half trials of each test were pooled together for statistical analyses. Mate
323 preference was inferred from the amount of time (in seconds) a tested bird spent near
324 a particular stimulus bird. We also defined two other response variables that we called

325 "interest" and "preference strength" (see Reparaz et al., 2014). *Interest* was calculated
326 to estimate the overall motivation of the subject birds during the experiment. This
327 variable indicates the proportion of time that the subjects spent close to stimulus birds,
328 and was calculated as follows:

329

$$330 \quad \frac{\textit{Time spent with stimulus 1} + \textit{Time spent with stimulus 2}}{\textit{Total duration of the test}}$$

331

332 The "total duration of the test" represents the cumulative time spent in the three
333 possible zones of the test chamber. *Preference strength* indicated the strength of a
334 subject's preference for any of the two stimulus birds it encountered in the test.
335 Which stimulus bird is chosen is not considered here, preference strength only
336 illustrates the magnitude of the bias for one of the two stimuli, whichever it is. In the
337 present case, it is thus a simple way to assess how affirmative a bird is in its choice,
338 and it has been shown to be context-dependent (Reparaz et al., 2014). Preference
339 strength is defined as the relative amount of time spent with the chosen individual
340 (the bird with which the subject spent >50% of the stimulus-zone time) compared to
341 the time spent with both stimuli, and calculated as follows:

342

$$343 \quad \frac{\textit{Time spent with chosen stimulus}}{\textit{Time spent with stimulus 1} + \textit{Time spent with stimulus 2}}$$

344

345 *Statistical analyses*

346 We used a step approach to analyze the data from simple, starting from easily
347 understandable tests on counts, to more elaborated tests on the exact time birds
348 spent in the different zones of the apparatus.

349

350 *Interest*

351 For both experiment 1 and 2, we started with a simple binomial test that compared
352 the number of subject birds that spent the majority of their time in the zone of the
353 stimulus birds with the number of birds that spent more time in the neutral zone. We
354 next tested the effect of several explanatory variables on the level of interest. As

355 interest is a proportion, it was arcsine transformed to achieve normality. In
356 experiment 1, we tested whether the level of interest differed between males and
357 females, and varied depending on the time of the day, by including the sex of the
358 subject birds (*female* or *male*) and time of day (continuous and centered to produce a
359 variable with a mean of zero) as explanatory variables in a linear model. Preliminary
360 analyses of the datasets showed that the behaviors of the birds were sometimes
361 influenced by the time at which the birds were tested. To account for this bias, time of
362 day will be included as a nuisance variable in all analyses that include covariates. For
363 experiment 2, we also added an effect of the treatment of the subject birds
364 (*canthaxanthin* or *placebo*), since in this experiment we not only compared the
365 preferences of males and females, but also considered their color phenotypes, we
366 used a linear mixed-model with individual ID as a random intercept to account for the
367 fact that each bird was tested on two occasions.

368

369 *Preference strength*

370 As preference strength is a proportion, it was also arcsine transformed to achieve
371 normality. In experiment 1, we tested whether preference strength differed between
372 males and females, and varied depending on the time of the day, by including the sex
373 of the subject birds (*female* or *male*) and time of day (continuous and centered to
374 produce a variable with a mean of zero) as explanatory variables in a linear model. In
375 the model, preference strength was weighted by interest. In other words, subjects that
376 spent more time with the stimulus birds in general (i.e. higher interest) were given more
377 statistical weight in the analyses than subjects that were less interested (see Reparaz et
378 al., 2014). For experiment 2, we also added an effect of the treatment of the subject
379 birds (*canthaxanthin* or *placebo*), since in this experiment we not only compared the
380 preferences of males and females, but also considered their color phenotypes, and we
381 used a linear mixed-model with individual ID as a random intercept to account for the
382 fact that each bird was tested on two occasions.

383

384 *Which birds are chosen?*

385 For both experiments 1 and 2, we started with a simple binomial test that compared
386 the number of subject birds that spent more time close to yellow (exp 1) or

387 canthaxanthin (exp 2) birds than to control (exp 1) or placebo (exp 2) birds. We then
388 tested the effect of several explanatory variables on these counts, using generalized
389 linear (mixed) models with binomial distributions. In experiment 1, we tested whether
390 the number of birds that spent more time close to yellow or control birds differed
391 between males and females, and varied depending on the time of the day, by
392 including the sex of the subject birds and time of day as explanatory variables on a
393 binary response variable (*control* or *yellow*) in a generalized linear model. For
394 experiment 2, we also added an effect of the treatment of the subject birds, and its
395 interaction with sex (to test for a possible self-reference phenotype matching
396 mechanism) on the binary response variable (*canthaxanthin* or *placebo* bird chosen) in
397 a generalized linear mixed model with individual ID as a random intercept.

398

399 Finally, instead of only considering counts of birds (which gives the same value to all
400 choices, independently of whether the subject spent 51 or 100% of its time with the
401 chosen stimulus), we analyzed the exact time (in seconds) subject birds spent close to
402 each stimulus using linear mixed models. In experiment 1, we included the sex of the
403 subject bird (*female* or *male*), the treatment of each stimulus bird (*control* or *yellow*),
404 and their interactions as fixed effect. Since in the dataset we considered two time
405 values for each subject bird (one time value for each stimulus), we included subject
406 identity as a random intercept. Like for the analysis of preference strength, time was
407 weighted by interest. We performed the same analysis for experiment 2, adding the
408 effect of the treatment of the subject bird (*canthaxanthin* or *placebo*), its 2- and 3-way
409 interactions with sex of subject birds (*female* or *male*) and treatment of stimulus birds
410 (*canthaxanthin* or *placebo*), and the effect of time of testing (continuous, centered). In
411 experiment 2, subject identity was also introduced as a random intercept to account
412 for the fact that each subject has been tested twice, and that there are two time
413 values per subject for each test.

414

415 Analyses were performed in *R* (version 3.2.1)(R-Core-Team, 2015), using the functions
416 *binom.test*, *lm*, *glm*, and the functions *lmer* and *glmer* of the package *lme4* (Bates et
417 al., 2015). Models were simplified using backward elimination of the non-significant
418 terms, starting with the higher order interactions (Crawley, 2007). P-values were

419 obtained either by model comparisons between a model that includes and another
420 that excluded the term of interest, or using the *anova* function of the package
421 *lmerTest* (Kuznetsova et al., 2016) in the cases of *lmer* analyses. Post-hoc tests
422 following (nearly) significant interactions were performed using the package *lsmeans*
423 (Lenth, 2016). Confidence intervals of model estimates plotted on the figures were
424 calculated using the package *bootpredictlme4* (Duursma, 2017).

425

426 **Results**

427 Experiment 1

428 *Interest for stimulus birds*

429 On average, subject birds spent 74% of their time close to the stimulus birds, with
430 birds spending more time in the stimulus-bird zone than in the larger neutral zone in
431 39 of the 49 tests performed (binomial test, $p < 0.001$). The level of interest for the
432 stimulus birds did not differ between the sexes of the subject birds, and was not
433 influenced by the time of day the birds were tested (table 1).

434

435 *Preference strength*

436 On average female tended to express clearer preferences than males (females:
437 0.67 ± 0.10 , males: 0.62 ± 0.09 ; mean \pm sd). This sex effect on preference strength was
438 however not fully significant ($p = 0.063$, see table 1). There was no effect of the time of
439 day the birds were tested (table 1).

440

441 *Which stimulus bird do they choose?*

442 If we count the number of subjects that spent more time with yellow or control birds,
443 significantly more subjects selected control birds than yellow birds, with controls
444 selected in 33 out of 49 tests (binomial test, $p = 0.02$). This preference for Control birds
445 occurred both in males and females as revealed by the absence of a sex effect on
446 which stimulus is chosen (general linear model, $z = 0.30$, $p = 0.76$), and was not
447 influenced by the time birds were tested ($z = 1.23$, $p = 0.22$).

448 Analyzing the exact time the subject birds spent close to each of the two stimulus
449 birds, instead of simply accounting for which stimulus bird was preferred overall, led

450 to similar conclusions, i.e. subjects spent more time close to control than to yellow
451 birds ($p=0.008$, table 1). In this analysis, there was also a trend for an interaction
452 between the sex of the subject and the treatment of the stimulus birds ($p=0.096$, see
453 table 1), suggesting that the preference for control birds might be more pronounced in
454 females than in males (Fig. 2A).

455

456 Experiment 2

457 *Interest for stimulus birds*

458 On average, subject birds spent 61% of their time close to the stimulus birds, with
459 birds spending more time in the stimulus-bird zone than in the, larger, neutral zone in
460 58 of the 90 tests performed (binomial test, $p=0.008$). The level of interest for the
461 stimulus birds did not differ between the treatments or sexes of the subject birds
462 (table 2). There was however an effect of the time of the day at which the birds were
463 tested, with birds slightly increasing their interest level as the day progresses (table 2).

464

465 *Preference strength*

466 Males did not differ from females in their preference strength (females: 0.66 ± 0.13 ;
467 males: 0.72 ± 0.14 , mean \pm sd) (table 2). Time of day and the treatment in which the
468 subjects were did not influence the strength of preference either (table 2).

469

470 *Which stimulus bird do they choose?*

471 The result of the binomial test that compared the number of occasions subject birds
472 spent more time close to canthaxanthin than to placebo birds, reveals that in the 86
473 tests where the birds made a choice (i.e. did not stay all the time in the neutral zone,
474 which only occurred four times), Canthaxanthin and Placebo stimulus birds were
475 selected in 39 and 47 cases, respectively. Thus based on counts there is no overall
476 preference for a stimulus category ($p=0.45$). This analysis does not account for the sex
477 of the subject birds, neither for their own treatment. Incorporating those factors, their
478 interaction and the effect of time of day in a generalized linear mixed-model with a
479 binary response variable (Canthaxanthin or Placebo) did not lead to any significant
480 result (table S2).

481 By contrast, the result of the linear mixed-model using the exact time spent close to
482 each stimulus bird (instead of the count) reveals a significant interaction between the
483 sex of the subjects and the treatment of stimulus birds ($p=0.002$, Table 2, Fig. 2B).
484 Post-hoc analyses reveal that females spent more time close to placebo than to
485 canthaxanthin birds ($p=0.001$), while males seem to spend similar amounts of times
486 with each phenotype ($p=0.33$). There was also a trend for assortative choice, indicated
487 by the (not fully significant) interaction between the treatment of the subject and the
488 stimulus birds ($p=0.058$; Table 2 and fig. 3). If we run a post-hoc test on this interaction
489 we find that Placebo subjects (males and females confounded) preferentially select
490 Placebo stimuli ($p=0.01$), and canthaxanthin subjects tend to spend more time with
491 canthaxanthin stimuli ($p=0.08$). This should however be interpreted cautiously since
492 the interaction is not fully significant.
493
494

495 **Discussion**

496

497 Our experiments show that male and female blue tits do not differ in their overall
498 interest for prospective mates. Similarly, males expressed comparable levels of
499 preference than females, suggesting that mutual mate choice exists in this species. The
500 only sexual difference found was in the second experiment, where the preference for
501 paler congeners was more pronounced in females than in males. Finally, we did not
502 find strong evidence for an assortative mate preference in blue tits, even though we
503 found that pale birds tended to select birds that are themselves pale.

504

505 The results described here quite clearly show that male and female blue tits are
506 mutually attracted by the opposite sex. They add to the growing body of literature that
507 suggests that male mate choice is common in birds and other organisms (reviewed in
508 Hare and Simmons, 2019; Doutrelant et al., 2020). We did not measure mate choice
509 *per se* in our apparatus since birds were not allowed to pair and reproduce together,
510 but rather a sexual, or eventually social (see below), mate preference. Actual mate
511 choice in the wild depends on a number of other parameters than mate preferences,
512 including environmental conditions, availability of preferred phenotypes, time and
513 effort allocated to prospecting mates, time of season and intra-sexual competition
514 (Jennions and Petrie, 1997; Cotton et al., 2006; Botero and Rubenstein, 2012; Kuijper
515 et al., 2012; Auld et al., 2017; Zandberg et al., 2017). Nevertheless, mate choice is the
516 manifestation of a mate preference (Cotton et al., 2006). In any case, similar mutual
517 mate preferences in this species were also described in earlier works by Hunt and
518 colleagues (1999), who specifically tested the preferences for the UV-blue coloration
519 of the crest, and in a recent meta analysis conducted on four populations over 10
520 years, which suggested a low but positive assortative mating for both the blue and the
521 yellow ornaments (Fargevieille et al., 2017).

522

523 Carotenoid-based colors have been found to signal capacity to raise an immune
524 response (Faivre et al., 2003; Saks et al., 2003; reviewed in Simons et al., 2012) and so
525 could potentially signal high-quality over low- quality individuals (Andersson, 1994;

526 Jones and Ratterman, 2009; Doutrelant et al., 2012; Wells et al., 2015). A pale plumage
527 contains less carotenoids, it is therefore surprising that in both experiments, our blue
528 tits did not prefer the most colored morph, corresponding to birds displaying more
529 carotenoid in their plumage and, by extension, potentially higher quality mate. In
530 experiment 1, pale birds were selected more often than colorful birds. This result was
531 robust as indicated by the fact that it was already significant in the initial binomial test
532 based on counts (number of birds that spent more time near to controls). It also
533 suggested the existence of an assorted mating (see below). This preference for pale
534 birds was apparent in both sexes (table 1), although it tended to be more pronounced
535 in females (Fig. 2A). Experiment 2 confirmed these results. Even if the same binomial
536 test did not reveal any striking preference this time, analyzing the exact time spent
537 with the different stimulus birds, indeed showed again that female blue tits
538 preferentially selected the least colorful males (Fig. 2B).

539 Preference for partners presenting lower values of signals has already been reported
540 in the literature, both from studies conducted in the field and in captivity. Female pied
541 flycatchers (*Ficedula hypoleuca*) and house sparrows of some wild populations were
542 described to prefer less-ornamented partners (Saetre et al., 1997; Griffith et al., 1999).
543 If the reason for this preference was quite clearly established in the first case, with
544 females that would show this preference to more surely avoid hybridization with the
545 closely related collared flycatcher (*Ficedula albicollis*) (Saetre et al., 1997), the sparrow
546 case was less clear. In fishes, captive male sticklebacks (*Gasterosteus aculeatus*) were
547 shown to prefer drab over red females. The author argued that avoidance of redder
548 females might in fact be a side-effect of a mechanism of avoidance of aggressive
549 interactions in male-male competition, and not a preference for drab females *per se*
550 (Nordeide, 2002). As we can see, in the few cases where it has been reported,
551 preference for less-ornamented partners generally arises from specific, context-
552 dependent, situations (Griffith et al., 1999), and not from a general preference for dull
553 colors. In the case of blue tits, we can only speculate on why this preference for pale-
554 chested birds occurred, and we think that four main hypotheses can be drawn from
555 these results, hypotheses that will need to be tested in the future.

556

557 First, we know that mate preference can be influenced by genetic and cultural factors
558 (i. e. Dugatkin, 1996). A genetically inherited preference for paler individuals seems
559 highly improbable, but a mating preference mechanism based on sexual imprinting
560 early in life, or on the most common social phenotype encountered thereafter, could
561 explain the results found, at least in experiment 1. In this experiment we only colored
562 the stimulus birds, not the subject birds, and only soon before the start of the
563 experiment. All subjects and their aviary mates have had a pale chest since their first
564 pre-basic molt, and were therefore used to encounter and interact with other pale
565 congeners. However, while pale birds was the most common phenotype they met for
566 most of the time, birds had a natural, yellow-colored chest during their first months of
567 life in captivity (pers. observations), thanks to the carotenoids present in the food
568 brought by their parents at the nest (chicks were brought to the lab when they were
569 around 10-days old, but were raised in natural conditions by their parents before that,
570 see methods). Birds only acquired a pale chest after their first pre-basic molt, which
571 occurred a few months after birth (July for birds born in May). In addition, the parents
572 that feed them at the nest for the first 10 days of life were of the natural, yellow
573 phenotype. An early sexual imprinting on pale-chest birds could therefore not explain
574 the observed preference for pale birds. It remains however possible that social
575 associations later in life could have played a role, and this was taken into account in
576 the design of experiment 2. In this experiment, bird food was indeed supplemented
577 with canthaxanthin or a placebo *before* the onset of their first pre-basic moult,
578 meaning that contrary to what happened in experiment 1, birds in experiment 2 went
579 directly from their initial yellow plumage to either one of the two experimental colors,
580 only half of which were pale. Furthermore, birds were housed in aviaries in a way that
581 they could always see both color morphs (see methods). The preference for pale-
582 chested birds in this second experiment could thus hardly be explained by early sexual
583 imprinting, or by social habituation later in life.

584

585

586 Second, more colored individuals might be more aggressive individuals (Senar, 2006;
587 Tobias et al., 2012) (but see McGraw and Hill, 2000). If pairing with an aggressive
588 partner might confer direct and indirect benefits in terms of territory defense and

589 genes conferring more vigor to offspring, it may also come at the cost of being more
590 aggressed. In captivity, where space is often limited, pairing with an aggressive partner
591 can lead to reproductive failure (Caro et al., 2007). Therefore, in confined environment
592 like the one in which our birds are raised, mate preference rules might differ from
593 what has been theorized (Wang et al., 2017), and selecting a paler, potentially less
594 aggressive, partner might be advantageous, especially for females that are slightly
595 smaller than males (Blondel et al., 1999). We know in our populations that males and
596 females with higher value of yellow chroma are more aggressive toward human
597 intruders (Mercier-Gauthier, Doutrelant, Dubuc Messier, Charmantier, Réale, unpubl.
598 data), and that duller females are less aggressive toward other blue tit females
599 (Midamegbe et al., 2011). Alternatively, even if we believe that the results reported
600 here depict a sexual preference because birds were primed into a reproductive state
601 (see methods), we cannot exclude that there might have been a social dimension to
602 the preferences expressed, with males and females choosing a partner with which to
603 spend time rather than to breed. In this case, selecting a less aggressive social partner
604 would seem more appropriate. A different experimental setup with more than two
605 possible choices, and which would include one or several individuals of the same sex
606 as the focal bird, could have shed some light on this social pairing possibility (e.g.
607 Griggio et al., 2011). In any case, like in the stickelback example (Nordeide, 2002),
608 avoiding aggressiveness might be a possible mechanism explaining the preferences for
609 paler individuals observed in our experiments.

610

611 Third, since potential competitors and mates are most probably assessed based on a
612 variety of signals (Bro-Jorgensen, 2010; Ligon and McGraw, 2016), it might be the
613 overall homogeneity of signals that matters, more than one signal taken
614 independently from the others. In blue tits, individuals with a yellower plumage also
615 tend to have more UV in the blue part of the plumage (Mercier Gauthier et al., unpub.
616 data). Accordingly, Kurvers et al. (2010) found that female blue tits expressed a
617 preference for males with UV-enhanced crowns, but only if those males also had a
618 yellower chest plumage, otherwise females selected the UV-reduced males. This
619 suggests that choosing birds might pay attention to an ensemble of traits and prefer
620 those individuals that are more homogenous in their different color signals. In our

621 experiments, like in most other experiments manipulating color phenotypes, we only
622 manipulated one single trait (i.e. the color of the chest). Since the diet that our birds
623 receive in captivity is obviously less diversified than what free-roaming birds eat in the
624 wild, it is possible that long-term housing in captivity induces a general decrease in
625 plumage quality. Although we only manipulated the chest color of our birds, we also
626 collected some blue crown feathers from the birds used in our second experiment.
627 Comparing the brightness, hue and UV chroma of those UV-blue feathers between the
628 two groups shows that the diet supplemented with canthaxanthin did not affect any of
629 those parameters (Brightness: $p=0.7$; hue: $p=0.2$; chroma: $p=0.9$; data not shown). This
630 suggests that only enhancing the chest color might have increased the contrast
631 between the different parts of the plumage, which in turn could have been
632 counterselected. This could explain why less-colored birds were preferred in both
633 experiments. Future studies of mate-preference, adopting a multi-modal approach
634 manipulating several plumage traits simultaneously, will shed light on whether birds
635 assess potential mates based on a variety of signals.

636

637 Finally, assortative mating could also explain the results described in this study. In
638 experiment 1, all subjects had a pale chest, and they preferentially selected birds that
639 were themselves pale, leading to a clear preference for individuals that were assorted
640 to the subjects. This result in fact led to experiment 2, where one of the goals was to
641 further test this hypothesis of assortative mating, presumably based on a mechanism
642 of self-referent phenotype matching. In this second experiment, where the colors of
643 both the stimulus and the subject birds were manipulated at an early stage, there was
644 a trend for an interaction between the treatments of the subject and of the stimulus
645 birds, and in the predicted direction: birds tended to spend more time with congeners
646 of the same chest phenotype as their own. Self-reference phenotype matching was
647 certainly not robust in our second experiment (not visible in the simple tests based on
648 counts of birds, and interaction not fully significant when analyzing the exact time
649 spent), but sufficiently to suggest that it might exist in blue tits, and sufficiently to call
650 for additional studies on this specific question. A recent study testing for assortative
651 mating for coloration in wild blue tits analyzed the matching of more than 1500 pairs
652 across multiple populations and over 10 years. If authors found some evidence for

653 positive assortative mating across populations, there were large spatio-temporal
654 variations (Fargevieille et al., 2017), confirming that mate preference can be very labile
655 in the wild (Chaine and Lyon, 2008).

656

657 In conclusion, this study suggests that mutual mate preference exists in blue tits,
658 which makes sense in a species where both sexes invest a lot in parental care
659 (Johnstone et al., 1996; Kokko and Johnstone, 2002). Results also show that the color
660 of the breast does influence mate preference. We additionally asked whether the
661 phenotype of the subjects influences the preference they express, but did not fully
662 resolve this question yet. Using a more integrative measure of quality like the one
663 proposed by Wang et al. (2017), which takes more than one phenotypic trait into
664 account, seems a promising way to further our understanding of the importance of
665 phenotype matching in mate preferences. Asking this question both in captivity and in
666 the wild also seems important to give a broader picture of the mate preference
667 dynamics in this, and other species.

668

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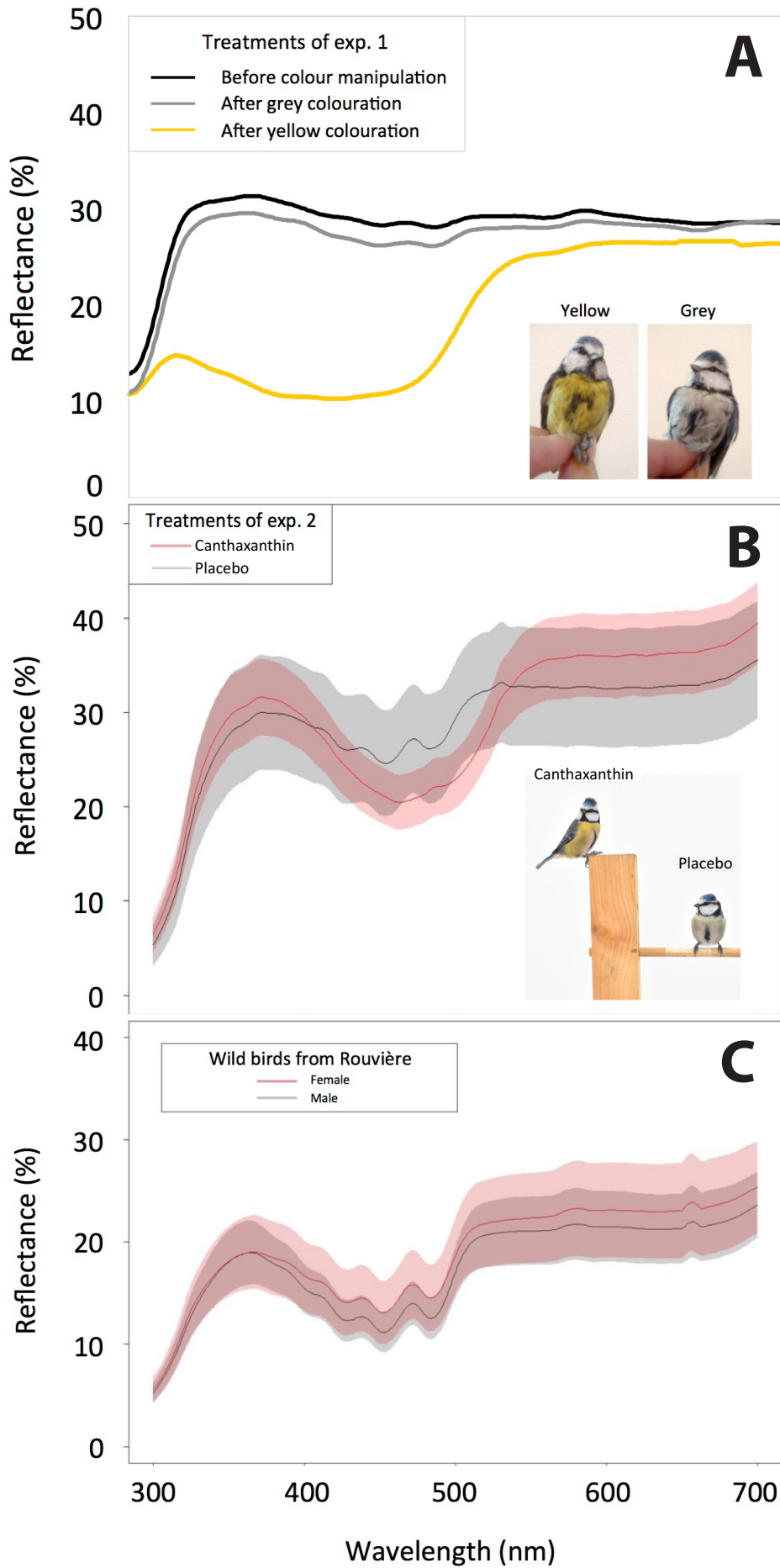
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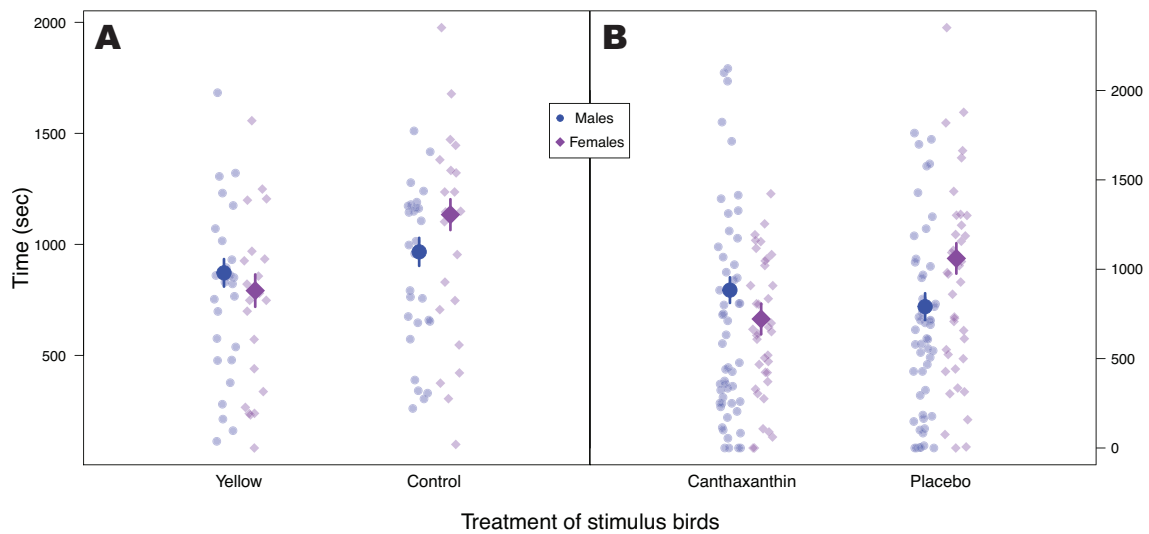
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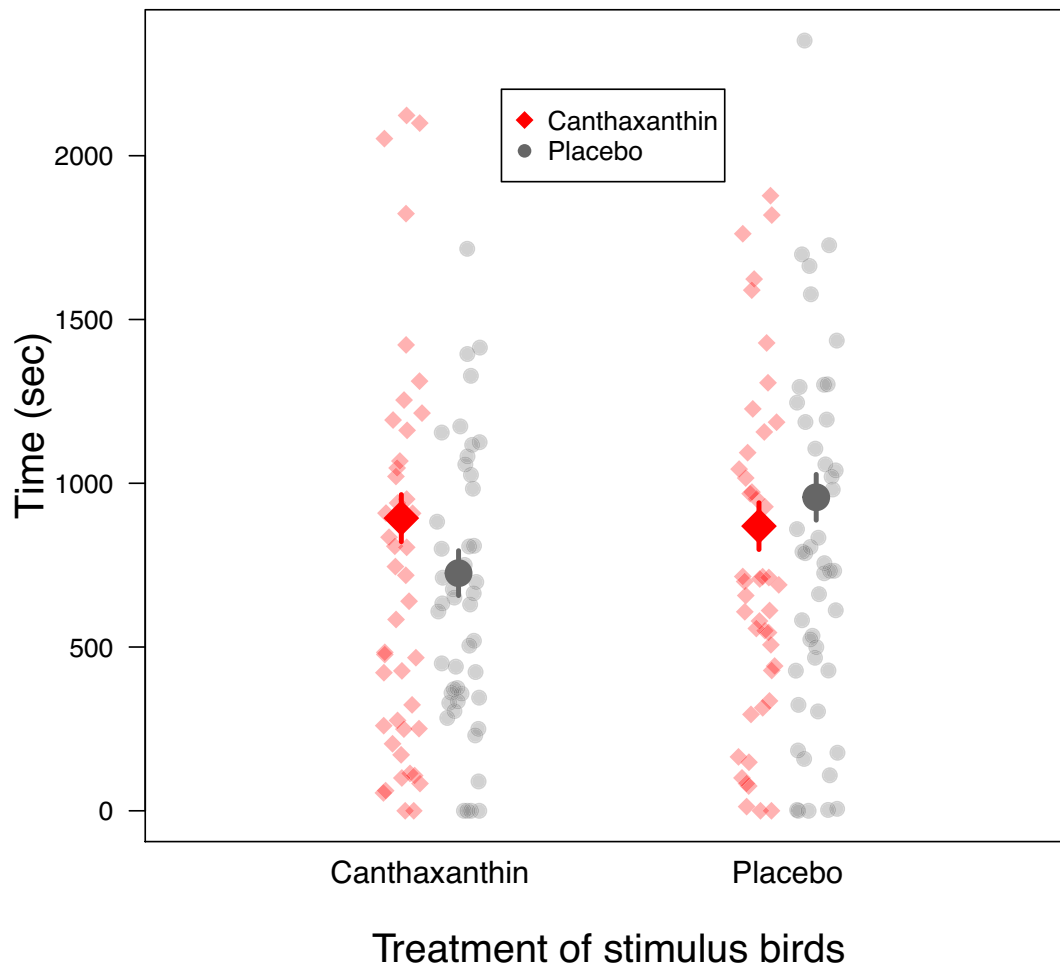


931 **Figure 1: Reflectance spectra of blue tit breast feathers.** (A) Spectra of birds used in
932 experiment 1. Colours obtained by painting the feathers with colour markers. (B)
933 Spectra of birds used in experiment 2. Colours obtained by manipulation of the diet of
934 the birds. (C) Spectra from wild blue tits at La Rouvière, France (measurements
935 performed on breast feathers : see Fargevieille et al., 2017).
936



937

938 **Figure 2: Sexual differences in the color morphs selected by captive blue tits.** (A) In
939 experiment 1, control birds were preferred over yellow birds ($p=0.008$), and this
940 preference for pale individuals tended to be slightly more pronounced in females than
941 in males (interaction between sex of the chooser and treatment of the stimulus bird:
942 $p=0.096$). (B) In experiment 2, this interaction was significant ($p=0.002$) and reveals
943 that female blue tits spent significantly more time close to Placebo than Canthaxanthin
944 stimuli (post-hoc test: $p=0.001$), while males spent similar amounts of time with each
945 phenotype (post-hoc test: $p=0.331$). Small symbols represent individual data points,
946 bigger symbols and error bars represent the statistical model estimates and S.E.
947



948

949 *Figure 3: Assortative mating in captive blue tits?* The nearly significant
 950 interaction between treatments of the subjects and of the stimulus birds
 951 ($P=0.058$) suggests that blue tits tend to prefer birds that are of the same color
 952 phenotype as theirs. This is particularly visible in placebo subjects (post-hoc test:
 953 $p=0.01$), less in canthaxanthin birds ($p=0.08$). Small symbols represent individual
 954 data points, bigger symbols and error bars represent the statistical model
 955 estimates and S.E. For the sake of simplicity, the estimates presented here come
 956 from a model including the treatments of stimulus and chooser birds, their
 957 interaction, and time of day. Sex and its interactions (table 2) were not included.
 958

959 **Table 1: Analysis of the variables that potentially influence the interest of the subject**
 960 **birds, their preference strength, and the time spent close to the stimulus birds in**
 961 **experiment 1 (n=49).** Variables in bold represent the minimal adequate models,
 962 eliminated variable (in grey) are presented in the reverse order in which they were
 963 removed from the model. Intercept includes Sex of subject = female, Treatment of
 964 stimulus = control.
 965

Trait analysed	Variable	Estimate	S.E.	F	p-value
<i>Interest</i>	(Intercept)	1.06	0.04		
	Time of day	-3.5e-04	2.5e-04	2.01	0.163
	Sex of subject	0.05	0.08	0.12	0.733
<i>Preference strength</i>	(Intercept)	0.96	0.02		
	Sex of subject	-0.06	0.03	3.62	0.063
	Time of day	1.2e-04	8.6e-05	1.83	0.183
<i>Time</i>	(Intercept)	1039.49	52.57		
	Treatment of stimulus	-202.62	74.34	7.43	0.008**
	Sex of subject	-43.77	74.82	0.34	0.560
	Time of day	-0.12	0.21	0.33	0.568
	Treatment stimulus x Sex subject	247.76	147.28	2.83	0.096

966

967 **Table 2: Analysis of the variables that potentially influence the interest of the**
 968 **subject birds, their preference strength, and the time spent close to the stimulus**
 969 **birds in experiment 2 (n=47).** Variables in bold represent the minimal adequate
 970 models, eliminated variable (in grey) are presented in the reverse order in which
 971 they were removed from the model. Intercept includes Sex of subject = female,
 972 Treatment of subject and stimulus = Canthaxanthin.
 973

Trait analysed	Variable	Estimate	S.E.	F	p-value
<i>Interest</i>	(Intercept)	0.90	0.04		
	Time of day	3.5e-04	1.5e-04	5.71	0.019*
	Sex of subject	-0.09	0.08	1.26	0.268
	Treatment of subject	-0.04	0.06	0.50	0.482
<i>Preference strength</i>	(Intercept)	0.97	0.03		
	Sex of subject	0.05	0.04	1.78	0.190
	Time of day	1.1e-04	7.9e-05	2.12	0.150
	Treatment of subject	-0.04	0.04	1.29	0.262
<i>Time</i>	(Intercept)	721.74	91.52		
	Sex of subject	161.50	123.59	0.28	0.609
	Treatment of stimulus	338.82	103.54	3.08	0.081
	Time of day	0.35	0.17	4.27	0.044*
	Treatment stimulus x Sex subject	-431.27	140.39	9.44	0.002**
	Treatment of subject	-39.26	72.94	0.29	0.591
	Treatment subject x Treatment stimulus	264.13	138.37	3.64	0.058
	Treatment subject x Treatment stimulus (<i>placebo</i>) x Sex subject	76.13	203.51	0.07	0.932

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