

# Dietary carotenoid supplementation facilitates egg laying in a wild passerine

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1 During egg laying, female birds face a trade-off between self-maintenance and investment into  
2 current reproduction. Providing eggs with resources is energetically demanding, since in most  
3 species females lay one egg per day. However, the costs of egg laying not only relate to  
4 energetic requirements, but also depend on the availability of specific resources that are vital  
5 for egg production and embryonic development. One of these compounds are carotenoids,  
6 pigments with antioxidant properties and immuno-stimulatory functions, which are crucial  
7 during embryonic development. In this study, we explore how carotenoid availability alleviates  
8 this trade-off and facilitates egg laying in the blue tit. Blue tit females lay one egg per day and  
9 have the largest clutch size of all European passerines. We performed a lutein  
10 supplementation experiment, and measured potential consequences for egg laying capacity  
11 and egg quality. We found that lutein-supplemented females had less laying interruptions and  
12 thus completed their clutch faster than control females. No effects of treatment were found  
13 on the onset of egg laying or clutch size. Experimentally enhanced carotenoid availability did  
14 not elevate yolk carotenoid levels or egg mass, but negatively affected eggshell thickness. Our  
15 results provide hence evidence on the limiting role of carotenoids during egg laying, However,  
16 the benefits of laying faster following lutein supplementation were counterbalanced by a  
17 lower accumulation of calcium in the eggshell. Thus, even though single components may  
18 constrain egg laying, it is the combined availability of a range of different resources which  
19 ultimately determines egg quality and thus embryonic development.

20

21 Key words: Carotenoid allocation, self-maintenance, egg production, trade-off, maternal  
22 effects.

## 23 Introduction

24 Life-history theory predicts that increased investment into current reproduction  
25 provides immediate fitness benefits via enhanced reproductive success, while it impinges at  
26 the same time on the amount of resources that can be maintained for self-maintenance and

27 thus for future reproduction (Stearns 1992). In birds, females face this trade-off between  
28 current and future reproduction among others when allocating resources to their eggs, as this  
29 increases offspring viability, but the costs of egg production compromise their rearing capacity  
30 and their prospects for future reproduction as well as survival (Monaghan et al. 1998; Visser  
31 and Lessells 2001). The high costs of egg production and the difficulties to maintain egg quality  
32 throughout laying are also reflected in the changes in egg composition along the egg sequence  
33 (Nager et al. 2000; Williams and Miller 2003).

34 Variation in egg composition along the laying sequence relates on the one hand to the  
35 energetic requirements for egg production that involve the acquisition of nutrients to be  
36 allocated to the eggs (Carey 1996; Monaghan and Nager 1997). Such energy or nutrient related  
37 effects on egg production have been studied in food supplementation experiments (Harrison  
38 et al. 2010). Indeed, providing females with more nutrients advanced the timing of  
39 reproduction (Vafidis et al. 2016), and had positive effects on clutch size (Korpimäki and  
40 Hakkarainen 1991) or egg size (Ardia et al. 2006). Laying capacity depends on the other hand  
41 on the availability of specific resources that are essential for embryonic development. One of  
42 these essential dietary micronutrients are carotenoids. These pigments are involved in a wide  
43 range of physiological processes, playing among others an immuno-stimulatory (Pérez-  
44 Rodríguez et al. 2008) and antioxidant role (Cohen and McGraw 2009; Watson et al. 2018).  
45 Carotenoids are crucial at early stages of development, since they reduce embryonic ROS  
46 damage (Surai and Speake 1998) and enhance offspring immune system before (Surai et al.  
47 2001) and after hatchling (McWhinney et al. 1989; Haq et al. 1996). Furthermore, they have an  
48 effect on offspring growth (Biard et al. 2007) and influence the development of traits like  
49 plumage or beak colouration that play an important role in parent-offspring communication  
50 (Tschirren et al. 2003; Morales and Velando 2013).

51 However, carotenoids cannot be endogenously synthesized by vertebrates and must  
52 be acquired from the diet. This implies that carotenoids could become limiting during highly

53 demanding periods, and that their use could be constrained by both their availability or by an  
54 individual's ability to find them. Thus, allocating carotenoids to eggs is expected to impose a  
55 physiological cost for females (Surai et al. 2001; McGraw et al. 2005; Karadas et al. 2005).  
56 Carotenoid demand for self-maintenance processes is greater during the breeding season and,  
57 particularly, during egg laying, a period framed by a situation of high oxidative stress and  
58 immune-depression (Sheldon and Verhulst 1996; Hansell et al. 2005). This challenging period  
59 may –depending on clutch size- be extensive, since carotenoid acquisition and transfer to the  
60 eggs starts already days prior to egg laying (e.g., in birds, 5 days prior to laying; Surai 2001).  
61 Egg quality is hence likely reduced under low carotenoid availability (Bortolotti et al. 2003).  
62 Indeed, previous studies have found that eggs laid by carotenoid supplemented females  
63 contained higher yolk carotenoid concentrations (see Blount et al. 2002; Biard et al. 2007).

64         There is also some evidence that egg production per se could be limited by low  
65 carotenoid availability (Blount et al. 2000, 2004), potentially with negative effects on clutch  
66 size in conditions of low carotenoid availability (Eeva and Lehikoinen 2010). Furthermore,  
67 when carotenoids are limited females may also extend the laying period by lowering their egg  
68 laying rate. In birds, females normally lay one egg per day until the clutch is completed, but  
69 laying interruptions of one or several days can occur (reviewed in Astheimer 1985; see also  
70 Nilsson and Svensson 1993). These interruptions have been reported to be more frequent  
71 under harsh conditions such as cold weather (Lessells et al. 2002), high pollution (Eeva and  
72 Lehikoinen 2010) and poor calcium availability (Graveland 1996; Bureš and Weidinger 2003;  
73 Eeva and Lehikoinen 2010). Yet, such delays in the reproductive schedule may negatively affect  
74 fitness because they increase the time in which females and their clutches are vulnerable to  
75 predators (Milonoff 1989), may decouple the timing for chick rearing with the peak of food  
76 availability (Durant et al. 2005), or may increase hatching asynchrony, if females start  
77 incubating before clutch completion (Magrath 1990). However, no study has explored the  
78 effects of carotenoid availability on egg laying interruptions.

79           In this study, we explored the previous question in a small passerine, the blue tit  
80 (*Cyanistes caeruleus*). In this species, females make a substantial investment into their clutch,  
81 which can weigh up to 150% of their own body mass (Perrins and Birkhead 1983; Stenning  
82 2018). As income breeders, blue tit females must acquire all the resources allocated to the  
83 clutch from their diet, for a period of up to 2 weeks. Dietary carotenoid availability is likely of  
84 central importance as it is known to have significant implications for blue tit embryonic and  
85 post-hatching development (e.g. Surai and Speake 1998; Biard et al. 2007; Valcu et al. 2019).  
86 Moreover, blue tit females supplemented with carotenoids at laying have been found to  
87 allocate more carotenoids to the egg yolk and to raise chicks with enhanced carotenoid-based  
88 coloration (Biard et al. 2006). We tested whether experimentally enhanced carotenoid  
89 availability prior and during laying affected the occurrence of laying interruptions, as well as  
90 clutch size and laying date. We predicted that carotenoid supplemented females would have  
91 less laying interruptions, may advance egg laying and lay larger clutches. We also explored  
92 whether carotenoid supplementation influenced various aspects of egg quality like the amount  
93 of carotenoids in the yolk, as well as egg mass and shell thickness.

94

## 95           **Material and methods**

### 96           **General methods**

97           The study was carried out in Miraflores de la Sierra, Community of Madrid, central  
98 Spain (40° 48'N, 03° 47'W) during the spring of 2017. We studied a nest-box breeding blue tit  
99 population in a deciduous forest that is dominated by Pyrenean oak (*Quercus pyrenaica*). The  
100 blue tit is a territorial-monogamous passerine that has one of the largest clutch size and the  
101 largest variation in clutch size among all European passerines (Stenning 2018). Eggs weigh on  
102 average 1.17 g (n= 1001; Stenning 2018), ranging from 0.97-1.41 g (Nilsson and Svensson  
103 1993). As many other passerines (Perrins 1970), blue tit females lay one egg per day, but laying

104 interruptions are frequent and are more abundant under environmental constraints and food  
105 unavailability (Nilsson and Svensson 1993; Matthysen et al. 2010; Stenning 2018).

106 From the beginning of April onwards, nest-boxes were visited every two days to  
107 determine the start of nest building. Blue tit nests are mainly made of moss, which is formed  
108 and lined with soft material such as hair and feathers. Nest building is mainly done by the  
109 female. As soon as the nest cup was defined (a hole in the moss not coated with soft  
110 material), which is typically 6.2 days prior to egg laying (range 1-14 days), we started lutein  
111 supplementation (see the following section).

112 Once egg laying started, nests were visited every second day and eggs were marked on  
113 the day they were found. Blue tit females lay one egg per day, although laying interruptions  
114 are frequent, so visiting nests every second day is sufficient to notice any laying interruptions.  
115 The fifth egg was collected immediately and substituted by a fake egg to prevent females from  
116 replacing it. Lutein supplementation (see below) continued throughout egg laying and stopped  
117 with the onset of incubation. Lutein supplementation lasted on average 15.7 days prior to  
118 clutch completion (range: 9-23 days), as blue tit females tend to start incubating before the  
119 clutch is complete (Salvador 2012; Stenning 2018). A total of 92 nests were included in this  
120 experiment, and the average clutch size was 9.36 (n=92, range 6-14).

121 We trapped adults during parental feeding visits (8-12 days after hatching; hatching  
122 date = day 0) by means of nest-box traps. Adults were weighed with a Pesola spring balance to  
123 the nearest 0.01 g.

124 In this study, we did not include hatching date effects due to a second experimental  
125 design in which we cross-fostered clutches two days before the expected hatching date. Thus,  
126 we avoided including hatching date in the analyses of the present study due to possible late  
127 prenatal effects related to incubation of foster females.

128

129 **Manipulation of lutein availability**

130           Once the nest cup was defined but not lined, we put a transparent plastic feeder into  
131 the nest-box (2.5x4.5x4.5 cm), pinned to the inner back nest-box wall. We chose this stage of  
132 nest building to ensure that the nest owners would continue breeding, since most nest  
133 usurpations occur at earlier stages. Nests were sequentially assigned to either a control group  
134 or to a lutein-supplemented group. Initially, we created 60 control nests and 32 lutein-  
135 supplemented nests. This unbalance between treatments was designed in the context of the  
136 second experiment mentioned above, in which we needed twice the number of control nests  
137 than lutein-supplemented nests. In the present study, we nevertheless used all data available.  
138 One nest was deserted during food supplementation as result of a nest usurpation by another  
139 blue tit pair.

140           Lutein was provided every second day, and each dosage consisted of 50 mg of Versele  
141 Laga Yel-lux Oropharma (lutein 8000 mg/Kg), which corresponds to 0.4 mg of lutein. According  
142 to Partali et al. (1987), one lepidopteran larvae in the natural diet of blue tits contains on  
143 average 5.3 µg of lutein and thus the dosage used would correspond, approximately, to 75  
144 prey items. At least, blue tits chicks consume on average 100 lepidopteran larvae per day (Gibb  
145 and Betts 1963), and thus our experimental manipulation every two days lies within the  
146 natural range. Each lutein dose was mixed with 5 g of commercial fat with nuts (GRANA Oryx).  
147 Control nests received the same amount of fat but without lutein. Each lutein dose was  
148 weighted in advance with a digital analytical balance (accuracy 0.001 mg) and stored in  
149 Eppendorf tubes at 4°C in the dark to prevent oxidation. Lutein doses were mixed with the  
150 corresponding amount of fat just before supplementation at the nest.

151           We supplemented nests until the female started incubation. At each visit, we weighed  
152 the amount of food that remained at the feeder using a Pesola spring balance (to the nearest  
153 0.01 g), cleaned the feeder and refilled it with 5 g with the corresponding treatment. We then  
154 calculated the total amount of food that was consumed over the two days. We also noted the  
155 first day when at least 0.5 g of food was consumed, in order to estimate the number of days it

156 took a given female to start consuming food (hereafter also termed “food neophobia”). In a  
157 subsample of nests (n=15), we confirmed by means of video recordings that males rarely  
158 visited the nest-box and, thus, we assume that any food that disappeared was consumed  
159 mainly by the female.

160

### 161 **Egg measurements**

162 We collected the 5<sup>th</sup> egg on the day of laying (n=80 clutches; 51 from control nests and  
163 29 from lutein-supplemented nests) and weighed it in the field to the nearest 0.01 g. We  
164 registered egg mass in 79 eggs because one egg of the lutein-supplemented group was broken  
165 during the egg collection. Eggs were kept cool and within the same day they were stored at -  
166 80°C until the analyses. For the analysis, we defrosted all eggs on the same day and separated  
167 the yolk, the albumen and the eggshell, and weighed the yolk using a digital analytical balance  
168 (accuracy 0.001 g). We added to the yolk twice the volume of water and vortexed this mixture  
169 at the highest speed for 1 min. Yolks were again stored at -80°C until the following day, when  
170 they were defrosted again for carotenoid analyses. We could use 76 samples for yolk mass and  
171 carotenoid analysis (48 from control nests and 28 from lutein-supplemented nests) because  
172 yolk and albumen was mixed in a few samples after defrosting.

173 From that yolk-water mixture, 100 µl were transferred to a new Eppendorf and 400 µl  
174 of pure ethanol were added. The mixture was then centrifuged at 1500 G during 5 min at room  
175 temperature, and the aqueous phase was transferred to an Eppendorf tube. Optical density  
176 was obtained at 450 nm using a Synergy™ HT Multi-model Microplate Reader (BioTek®  
177 Instruments, Inc.). Carotenoid concentrations were obtained from a lutein analytical standard  
178 (Sigma-Aldrich®). Plate number was registered for each sample and controlled for in statistical  
179 analyses. In a subsample of eggs (n=30), we also analysed lutein concentration by high-  
180 performance liquid chromatography (HPLC) with ethanol extraction following Alonso-Álvarez



181 et al. (2004), and both measurements were positively correlated ( $r_{30}=0.41$ ,  $P=0.02$ ). We  
182 calculated yolk carotenoid content by multiplying carotenoid concentration and yolk mass.  
183 Eggshell thickness of all the 80 collected eggs was measured using a digital tube  
184 micrometer (Mitutoyo Ip-65) with ball-point ends and precision of 0.001 mm, following  
185 Morales et al. (2013). We took 9 measures per egg (if possible, 3 in each of the following  
186 eggshell locations: blunt end, sharp end and equator). When it was not possible to identify the  
187 specific location of the eggshell, it was categorized as “indeterminate”. Measures at these  
188 different locations showed high repeatability (blunt end:  $r=0.8$ ,  $F=10.7$ ,  $P < 0.001$ ; sharp end:  
189  $r=0.8$ ,  $F=12.7$ ,  $P < 0.001$ ; equator:  $r=0.8$ ,  $F=17.3$ ,  $P < 0.001$ ; indeterminate:  $r=0.8$ ,  $F=4.4$ ,  $P <$   
190  $0.001$ ; all measures pooled:  $r=0.7$ ,  $F_{1,79}=21.4$ ,  $P < 0.001$ ). Thus, we calculated the mean of each  
191 eggshell location. We clearly identified equator location for 48 shells, 33 shells for sharp end  
192 location and 24 for shells from blunt end location.

193

#### 194 **Statistical Analyses**

195 First, we tested whether the total amount of food consumed and food neophobia  
196 differed between the control and the lutein-supplemented group. Food neophobia was  
197 analysed using a Generalized Linear Model (GLZ) with Poisson distribution and a log link  
198 function, including treatment, the total amount of food consumed, female body mass and all  
199 double interactions with treatment as predictor variables. The total amount of food consumed  
200 was analysed with General Linear Models (GLM), including treatment, female body mass and  
201 the interactions between both as predictor variables.

202 Second, we tested the effect of treatment on laying capacity variables (laying date,  
203 number of laying interruptions and clutch size) and egg quality (egg mass and yolk carotenoid  
204 content) by using GLMs, except for the number of laying interruptions, which was analysed  
205 using a GLZ with Poisson distribution. In these models, we included treatment, the total  
206 amount of food consumed, female body mass and all double interactions between treatment

207 and the rest of the parameters as predictor variables. We also included clutch size and its  
208 interaction with treatment as predictor variables in the analysis of laying interruptions. Plate  
209 number was included as predictor in yolk carotenoid analysis.

210 Finally, shell thickness was analysed using a mixed model including nest id as random  
211 factor in order to account for repeated measures at different shell locations, and treatment,  
212 the total amount of food consumed, female body mass, eggshell location (blunt end, sharp end  
213 or equator) and all double interactions with treatment as fixed predictor variables.

214 We used SAS 9.4 (SAS Inst., Cary, NC, USA) for all statistical analyses. Backward  
215 elimination of non-significant interactions ( $\alpha=0.05$ ) was used to acquire minimal models. The  
216 models were checked for residual normality with a Shapiro normality test. In the text, we  
217 report minimal models after backward elimination, while full models are shown in tables.

218

## 219 **Results**

### 220 **Total amount of food and food neophobia**

221 Treatment did not significantly affect the total amount of food consumed (coef. = -3.86  
222  $\pm 1.85$ ,  $F_{1,69} = 3.77$ ,  $P = 0.052$ ), although lutein-supplemented females tended to eat more food  
223 than control females. Lutein-supplemented females showed less food neophobia than control  
224 females (coef. =  $0.39 \pm 0.18$ ,  $\chi^2_1 = 4.87$ ,  $P = 0.027$ ). Food neophobia did not depend on female's  
225 body mass or the total amount of food consumed (both  $P > 0.1$ ). None of the interactions with  
226 treatment was significant (Table 1).

227

### 228 **Egg laying capacity**

229 There was no treatment effect on the onset of egg laying (coef. =  $-1.20 \pm 0.80$ ,  $F_{1,68} =$   
230  $2.28$ ,  $P = 0.14$ ) or on clutch size (coef. =  $0.03 \pm 0.08$ ,  $\chi^2_1 = 0.1$ ,  $P = 0.75$ ). Lutein-supplemented  
231 females had less laying interruptions than control females (coef. =  $1.17 \pm 0.63$ ,  $\chi^2_1 = 4.54$ ,  $P =$   
232  $0.033$ ). Females that consumed more food had less laying interruptions (coef. =  $-0.14 \pm 0.05$ ,

233  $\chi^2_1 = 11.30, P < 0.001$ ), while no effects of female body mass were found (coef. =  $-0.35 \pm 0.49$ ,  
234  $\chi^2_1 = 0.54, P = 0.46$ ). All interactions with treatment were not significant (see Table 1).

235

### 236 **Egg quality**

237 No effects of treatment (coef. =  $0.02 \pm 0.02, F_{1,59} = 0.75, P = 0.39$ ) or of the total  
238 amount of food consumed (coef. =  $-0.001 \pm 0.001, F_{1,59} = 1.32, P = 0.25$ ) were found on egg  
239 mass. Heavier females laid heavier eggs (coef. =  $0.06 \pm 0.02, F_{1,59} = 11.92, P = 0.001$ ). The  
240 interactions with treatment were not significant (Table 2).

241 The yolk carotenoid content of the fifth egg was not affected by treatment (coef. = -  
242  $0.005 \pm 0.25, F_{1,55} = 0.02, P = 0.98$ ) or by the total amount of food consumed (coef. =  $0.005 \pm$   
243  $0.01, F_{1,55} = 0.37, P = 0.72$ ). Females with higher body mass laid eggs with higher yolk  
244 carotenoid content (coef. =  $0.56 \pm 0.23, F_{1,55} = 12.15, P = 0.019$ ). All other variables and the  
245 interactions with treatment had no significant effect (Table 2).

246 Lutein-supplemented females laid eggs with thinner egg shells than control females  
247 (coef. =  $0.003 \pm 0.001, F_{1,119} = 11.68, P < 0.001$ ). Eggshell thickness did not vary with female  
248 body mass or with the total amount of food consumed (both  $P > 0.2$ ). Eggshell thickness  
249 depended on the eggshell location (coef. =  $0.002 \pm 0.001, F_{1,119} = 3.27, P = 0.0084$ ; see also  
250 Table 2), sharp end locations being thicker than blunt end locations (least square difference:  
251 coef. =  $0.002 \pm 0.001, t_{127} = 2.19, P = 0.030$ ), but not to equator locations (coef. =  $0.001 \pm$   
252  $0.001, t_{127} = 1.58, P = 0.1163$ ).

253

### 254 **Discussion**

255 We hypothesized that dietary carotenoid availability during egg laying is likely of  
256 central importance for female blue tits. Indeed, experimentally enhanced carotenoid  
257 availability allowed lutein-supplemented females to have less laying interruptions. However,  
258 this came at a potential cost as lutein-supplemented females laid thinner eggs than control

259 females. Intriguingly, other aspects of a female's laying capacity or the allocation of  
260 carotenoids to the yolk were not affected. The potential causes of these findings are discussed  
261 below.

262

### 263 **Egg laying capacity**

264 Lutein-supplemented females started eating from the feeders before control females,  
265 which was unexpected. This lower food neophobia was independent of female body mass,  
266 indicating that it is not driven by female quality differences between groups. One possible  
267 explanation could be that blue tits are able to forage selectively on carotenoid-rich foods, via  
268 the perception of differences in food colour or smell. Thus, carotenoid treatment could have  
269 been more attractive for females, which is supported by a study in great tits (*Parus major*), a  
270 species closely related to the blue tit, which chooses to forage on carotenoid-enriched food  
271 when exposed to choice tests (Senar et al. 2010). However, even though lutein-supplemented  
272 females tended to consume more extra food, this was not reflected in their egg mass (see  
273 below).

274 As expected, we did find an effect of treatment on the number of laying interruptions.  
275 Lutein-supplemented females completed their clutch in fewer days than control females, while  
276 clutch size was similar. This is the first evidence of the effects of lutein availability on laying  
277 interruptions. This effect remained after controlling for female body mass. Previous studies in  
278 the blue tit showed that laying interruptions occur frequently in non-food-supplemented  
279 females or as a consequence of environmental constraints (Yom-Tov and Wright 1993). Our  
280 study focuses on one micronutrient and the results suggest that carotenoids are an essential  
281 resource that modulates laying intervals. If blue tit females maintain their clutch size (Nilsson  
282 and Svensson 1993; García-Navas and Sanz 2011), but need more than one day for a certain  
283 egg, this will ultimately cause a delay in the timing of hatching. Longer laying times can have  
284 serious consequences for parental rearing capacity (Perrins 1970), egg viability (Milonoff

285 1989), and nestling survival (Martin and Hannon 1987; Nilsson 1990; Hochachka 1990). The  
286 latter may arise via increased hatching asynchrony, if incubation starts before the clutch is  
287 completed, which disadvantages later hatching chicks (Magrath 1990).

288 Lutein treatment had no effects on laying date, while several studies have shown a  
289 positive effect of food supplementation on the timing of reproduction (Martin 1987; Meijer  
290 and Drent 1998; Robb 2008). Yet, these effects may relate to caloric restrictions and not to the  
291 availability of specific nutrients. However, due to our experimental design we did not expect  
292 large effects on laying date. In the current study, feeding both control and lutein-  
293 supplemented females with bird fat allowed us to avoid metabolic or energetic effects not  
294 related with female carotenoid requirements, and to focus on the role of carotenoids.  
295 Furthermore, the onset of feeding may have been too short to affect the onset of laying.

296 There was no effect on clutch size either (Harrison et al. 2010), despite the fact that  
297 previous studies showed that carotenoids may be limiting for egg production (Biard et al.  
298 2005). In the study year, the average clutch size in control and experimental females was  $9.36$   
299  $\pm 0.15$  (range: 6-14,  $n=92$ ), which does not differ from the following two years in which females  
300 were not supplemented ( $9.56 \pm 0.14$ , range 4-15,  $n=206$ ). Thus, it seems that the plasticity in  
301 clutch size is limited at least in the study population, and does not depend greatly in food  
302 availability or in other specific substances (see also Moreno et al. 1989 in the pied flycatcher  
303 *Ficedula hypoleuca*).

304

### 305 **Egg quality**

306 Unexpectedly, we did not find differences in yolk carotenoid content between our treatments.  
307 This is in contrast to previous studies showing effects of carotenoid-supplementation on yolk  
308 carotenoid content in wild blue tits (Blount et al. 2002; Biard et al. 2005) and in other captive  
309 bird species (Surai and Speake 1998; Surai and Sparks 2001; Bortolotti et al. 2003). However,  
310 these studies substantially manipulated carotenoids potentially various magnitude orders

311 above the natural consumption (more than 100 times the daily consumption) (daily amount of  
312 lutein supplemented: 500 mg in Biard et al. 2005; 1.75 in Remeš et al. 2007). Here, we supplied  
313 females with carotenoids within the biological range (0.4 mg in this study; see Partali et al.  
314 1987). Our results suggest that perhaps females used the extra carotenoids for other  
315 physiological functions related to self-maintenance and to enhance the laying capacity  
316 (Hargitai et al. 2006; Navara et al. 2006; Isaksson et al. 2018). Thus, in the context of a trade-  
317 off between allocation to eggs and to self-maintenance (Morales et al. 2008; Giordano et al.  
318 2014), self-maintenance is prioritized until carotenoid supplementation goes beyond the levels  
319 required by the female, when it may indeed be reflected in higher yolk carotenoid contents.  
320 However, it has to be considered that we assume that carotenoid transfer to the follicle occurs  
321 each 24 hours (Salvante and Williams 2002), and, thus, if females had allocated more  
322 carotenoids to the yolk, we should have detected differences between treatments in the fifth  
323 collected egg. Thus, the lack of an effect on yolk carotenoid content in our study is more likely  
324 explained by females using carotenoids for self-maintenance functions than being an artefact  
325 of our methodology.

326         We did not find effects of treatment on egg mass, but both control and lutein-  
327 supplemented females received additional resources that could have been used for yolk  
328 formation. Yet, egg mass was still positively related to female body mass, which may not  
329 necessarily reflect condition differences, but could be due to morphological constraints with  
330 larger females being able to lay larger eggs.

331         We found a treatment effect on eggshell thickness, with lutein-supplemented females  
332 laying thinner eggs than control females. One explanation is that laying interruptions allow  
333 females to accumulate calcium resources, which is reflected in increased shell thickness. Thus,  
334 carotenoid supplementation facilitated egg laying, but less number of laying interruptions  
335 during egg sequence prevented the deposition of calcium in the eggshell, either because  
336 lutein-supplemented females had less time for foraging on calcium-rich resources between

337 eggs (note that even short foraging bouts result in increased quantities of calcium; Flint et al.,  
338 1998) or because their eggs stayed on average less time in the oviduct compared to control  
339 eggs. Eggshell is the physical barrier between the embryo and the environment and its  
340 thickness has profound consequences on fitness related to incubation efficiency by heat  
341 transference (Soliman et al. 1994), microbial infection (D'Alba et al. 2014), water loss (Drent  
342 and Woldendorp 1989) and egg viability (Mellanby 1992). Therefore, our results suggest that a  
343 consequence effect of laying faster could be a lower accumulation of calcium in the eggshell,  
344 which indicates that the combined availability of different resource determines egg quality.

345 To conclude, this study provides the first evidence that experimentally enhanced  
346 carotenoid availability allowed blue tit females to complete their clutch faster. This suggests  
347 that carotenoids are a limiting resource in the blue tit, a species that lays large clutches in a  
348 very short time interval. Yet, lutein supplementation did not lead to higher yolk carotenoid  
349 content, which suggests that females used the extra carotenoids for self-maintenance or to  
350 enhance their laying capacities. The supplementation of a single compound, here lutein, also  
351 revealed a trade-off between laying in short sequence and calcium deposition in the eggshell,  
352 since lutein-supplemented females laid eggs with thinner shells. To summarize, our results  
353 emphasize the limiting role that carotenoids play for blue tit females during the egg  
354 production.

355

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358

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364

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556 Table 1. Full models before backward deletion of non-significant interactions showing the effect of carotenoid supplementation on laying capacity and food  
557 consumption. General lineal models were performed for laying date and food neophobia analyses. Laying interruptions and clutch size models were  
558 performed using Generalized lineal models. Coefficients are shown for control nests. Significant differences are marked in bold.

	<i>Laying interruptions (days)</i>	<i>Laying date</i>	<i>Clutch Size</i>	<i>Total food consumed (g)</i>	<i>Food neophobia (days)</i>
<i>Intercept</i>	<i>coef</i> = -1.13 ± 20.77	<i>coef</i> = 16.09 ± 13.49	<i>coef</i> = 2.49 ± 1.42	<i>coef</i> = -14.85 ± 34.20	<i>coef</i> = -0.23 ± 3.15
<i>Treatment (control)</i>	<i>coef</i> = 7.65 ± 21.49 $\chi^2_1 = 0.13$ <i>P</i> = 0.71	<i>coef</i> = 18.03 ± 16.31 $F_{1,66} = 1.22$ <i>P</i> = 0.27	<i>coef</i> = -0.42 ± 1.72 $\chi^2_1 = 0.06$ <i>P</i> = 0.80	<i>coef</i> = 5.94 ± 41.38 $F_{1,68} = 0.02$ <i>P</i> = 0.89	<i>coef</i> = -1.29 ± 3.60 $\chi^2_1 = 0.13$ <i>P</i> = 0.72
<i>Total food consumed (g)</i>	<i>coef</i> = -0.39 ± 0.26 $\chi^2_1 = 2.15$ <b><i>P</i> = 0.001</b>	<i>coef</i> = -0.14 ± 0.08 $F_{1,66} = 3.59$ <i>P</i> = 0.063	<i>coef</i> = -0.01 ± 0.01 $\chi^2_1 = 1.00$ <i>P</i> = 0.38		<i>coef</i> = -0.01 ± 0.02 $\chi^2_1 = 0.64$ <i>P</i> = 0.16
<i>Female body mass (g)</i>	<i>coef</i> = -0.01 ± 2.24 $\chi^2_1 = 0.00$ <i>P</i> = 0.87	<i>coef</i> = -0.11 ± 1.35 $F_{1,66} = 1.87$ <i>P</i> = 0.18	<i>coef</i> = -0.04 ± 0.14 $\chi^2_1 = 0.06$ <i>P</i> = 0.91	<i>coef</i> = 2.59 ± 3.41 $F_{1,68} = 1.04$ <i>P</i> = 0.31	<i>coef</i> = 0.07 ± 0.32 $\chi^2_1 = 0.05$ <i>P</i> = 0.41
<i>Clutch Size</i>	<i>coef</i> = 0.09 ± 0.46 $\chi^2_1 = 0.04$ <i>P</i> = 0.70				
<i>Total food consumed* Treatment</i>	<i>coef</i> = 0.26 ± 0.27 $\chi^2_1 = 0.93$ <i>P</i> = 0.23	<i>coef</i> = -0.10 ± 0.10 $F_{1,66} = 0.97$ <i>P</i> = 0.33	<i>coef</i> = -0.01 ± 0.01 $\chi^2_1 = 0.48$ <i>P</i> = 0.49	<i>coef</i> = -0.98 ± 4.12 $F_{1,68} = 0.06$ <i>P</i> = 0.81	<i>coef</i> = -0.001 ± 0.02 $\chi^2_1 = 0.00$ <i>P</i> = 0.96
<i>Female body mass* Treatment</i>	<i>coef</i> = -0.42 ± 2.30 $\chi^2_1 = 0.03$ <i>P</i> = 0.85	<i>coef</i> = -2.01 ± 1.63 $F_{1,66} = 1.51$ <i>P</i> = 0.22	<i>coef</i> = -0.35 ± 0.49 $\chi^2_1 = 0.09$ <i>P</i> = 0.77		<i>coef</i> = 0.17 ± 0.36 $\chi^2_1 = 0.21$ <i>P</i> = 0.65
<i>Clutch Size* Treatment</i>	<i>coef</i> = -0.38 ± 0.49 $\chi^2_1 = 0.58$ <i>P</i> = 0.44				

559 Table 2. Full models before backward deletion of non-significant interactions showing the effects of treatment on egg quality. General lineal models were  
 560 performed for egg mass and yolk carotenoid content. A mixed model was performed for eggshell thickness. Coefficients are shown for control nests and  
 561 sharp end location. Significant differences are marked in bold.

	<i>Egg mass (g)</i>	<i>Yolk carotenoids (μg)</i>	<i>Eggshell thickness (mm)</i>	
<i>Intercept</i>	<i>coef</i> = 0.57 ± 0.29	<i>coef</i> = 0.51 ± 4.00	<i>coef</i> = 0.05 ± 0.02	562
				563
<i>Treatment (control)</i>	<i>coef</i> = -0.01 ± 0.36 <i>F</i> <sub>1,57</sub> = 0.00 <i>P</i> = 0.98	<i>coef</i> = -5.57 ± 4.94 <i>F</i> <sub>1,53</sub> = 1.27 <i>P</i> = 0.26	<i>coef</i> = 0.001 ± 0.02 <i>F</i> <sub>1,118</sub> = 0.05 <i>P</i> = 0.96	564
				565
<i>Total amount of food consumed (g)</i>	<i>coef</i> = -0.002 ± 0.002 <i>F</i> <sub>1,57</sub> = 1.68 <i>P</i> = 0.20	<i>coef</i> = 0.01 ± 0.03 <i>F</i> <sub>1,53</sub> = 0.17 <i>P</i> = 0.69	<i>coef</i> = -0.0004 ± 0.0001 <i>F</i> <sub>1,118</sub> = 0.02 <i>P</i> = 0.90	566
<i>Female body mass (g)</i>	<i>coef</i> = 0.06 ± 0.03 <i>F</i> <sub>1,57</sub> = 10.19 <b><i>P</i> = 0.0023</b>	<i>coef</i> = 0.18 ± 0.40 <i>F</i> <sub>1,53</sub> = 3.60 <i>P</i> = 0.063	<i>coef</i> = 0.001 ± 0.002 <i>F</i> <sub>1,118</sub> = 1.19 <i>P</i> = 0.28	567
<i>Lab plate</i>		<i>coef</i> = 0.58 ± 0.16 <i>F</i> <sub>1,53</sub> = 12.97 <b><i>P</i> &lt; 0.001</b>		568
<i>Eggshell location (sharp end)</i>			<i>coef</i> = 0.002 ± 0.001 <i>F</i> <sub>5,118</sub> = 3.28 <b><i>P</i> = 0.0083</b>	570
				571
<i>Total amount of food consumed*</i>	<i>coef</i> = 0.002 ± 0.002 <i>F</i> <sub>1,57</sub> = 0.47 <i>P</i> = 0.49	<i>coef</i> = 0.08 ± 0.08 <i>F</i> <sub>1,53</sub> = 0.10 <i>P</i> = 0.76	<i>coef</i> = -0.0001 ± 0.0001 <i>F</i> <sub>1,118</sub> = 0.28 <i>P</i> = 0.60	572
<i>Treatment</i>				573
<i>Female body mass*</i>	<i>coef</i> = 0.001 ± 0.04 <i>F</i> <sub>1,57</sub> = 0.00 <i>P</i> = 0.98	<i>coef</i> = 1.03 ± 1.03 <i>F</i> <sub>1,53</sub> = 1.31 <i>P</i> = 0.26	<i>coef</i> = -0.0002 ± 0.0002 <i>F</i> <sub>1,118</sub> = 0.01 <i>P</i> = 0.93	574
<i>Treatment</i>				

575 Legends to figures:

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577 Figure 1. Laying capacity of control and lutein-supplemented blue tit females (*Cyanistes caeruleus*): a) Number of laying interruptions during egg laying  
578 (days; GLZ,  $P = 0.033$ ); b) Laying date according to the Julian calendar (GLM,  $P = 0.14$ ); c) Clutch size (GLZ,  $P = 0.75$ ). Error bars denote standard errors (mean  
579  $\pm$  SE;  $n=92$ ). Sample sizes for each treatment are shown.

580

581 Figure 2. Egg quality measures (mean  $\pm$  SE) of control and lutein-supplemented blue tit females (*Cyanistes caeruleus*): a) Egg mass of the 5<sup>th</sup> collected egg (g;  
582 GLM,  $n = 79$ ,  $P = 0.39$ ); b) Yolk carotenoid content of the 5<sup>th</sup> collected ( $\mu\text{g}$ ; (GLM,  $n = 76$ ,  $P = 0.98$ ); c) Eggshell thickness of the 5<sup>th</sup> collected (mm; GLMM,  $n =$   
583  $80$ ,  $P < 0.001$ ); values of shell thickness are the mean measured in all locations pooled (blunt end, sharp end and equator). Sample sizes for each treatment  
584 are shown.

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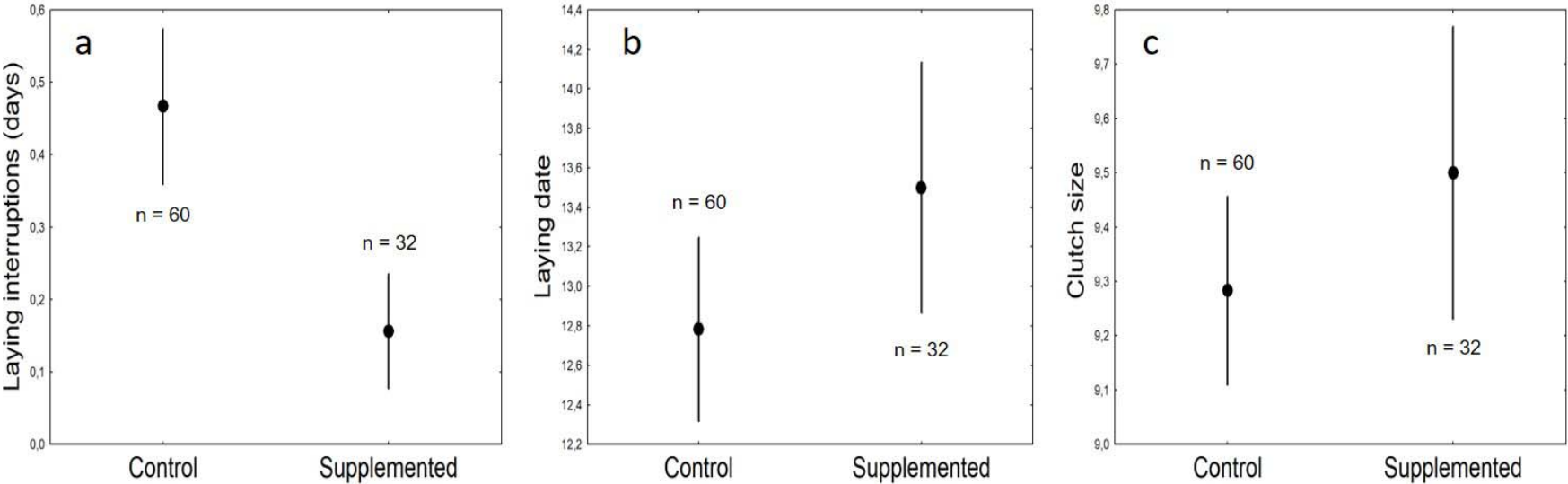
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595 **Fig. 1**

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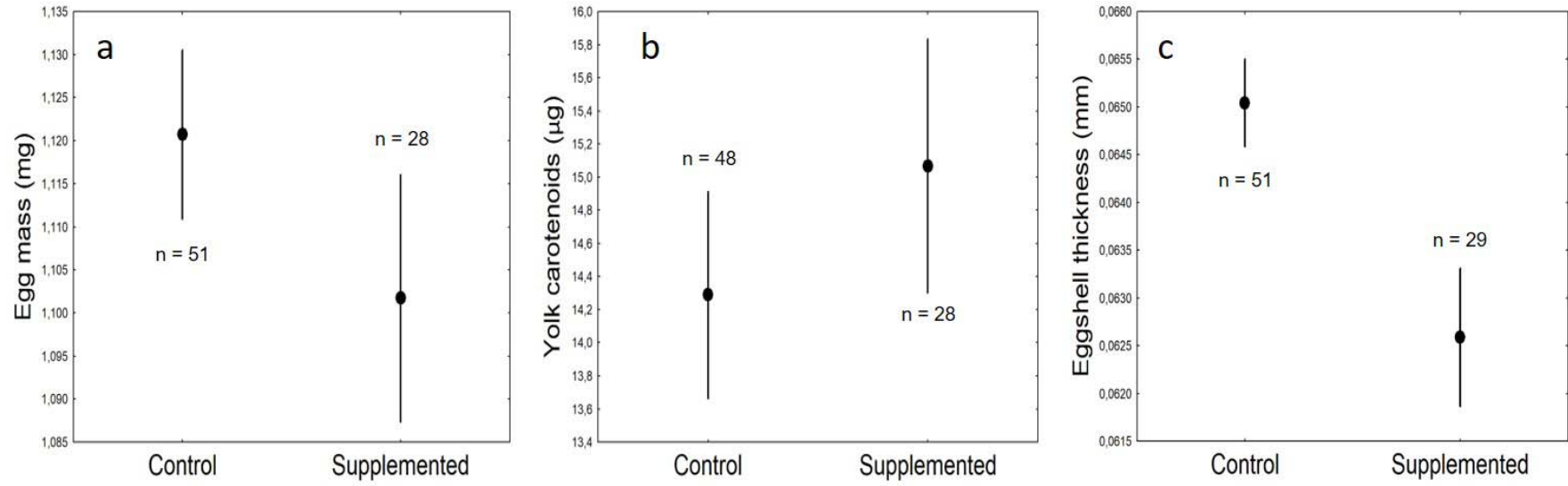
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604 **Fig. 2**

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