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## Feeding partridges with organic or conventional grain triggers cascading effects in life-history traits

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18 **Abstract**

19 Farmland birds are declining across Europe and North America and the research of  
20 factors behind is the subject of extensive researches. Agricultural intensification is now  
21 recognized as a major factor governing the loss of biodiversity with strong evidence that  
22 pesticides induced direct bird mortality at a high dose. However, less attention has been given  
23 to the long-term effects of chronic exposure to low dose of pesticides. Here, we used an  
24 experimental procedure in which grey partridges were fed with untreated grains obtained from  
25 either organic (no pesticide) or conventional agriculture (with pesticide) for 26 weeks, thus  
26 strictly mimicking wild birds foraging on fields. We then examined a suite of life-history traits  
27 (ecophysiological and behavioural) that may ultimately, influence population dynamics. We  
28 show for the first time that ingesting low pesticide doses over a long period has long-term  
29 consequences on several major physiological pathways without inducing differential mortality.  
30 Compared to control partridges, birds exposed to chronic doses i) had less developed  
31 carotenoid-based ornaments due to lower concentrations of plasmatic carotenoids, ii) had  
32 higher activated immune system, iii) showed signs of physiological stress inducing a higher  
33 intestinal parasitic load, iv) had higher behavioural activity and body condition and v) showed  
34 lower breeding investment. Our results are consistent with a hormetic effect, in which exposure  
35 to a low dose of a chemical agent may induce a positive response, but our results also indicate  
36 that breeding adults may show impaired fitness traits bearing population consequences through  
37 reduced breeding investment or productivity. Given the current scale of use of pesticides in  
38 agrosystems, we suggest that such shifts in life-history traits may have a negative long-term  
39 impact on wild bird populations across agrosystems. We stress that long-term effects should no  
40 longer be ignored in pesticide risk assessment, where currently, only short-term effects are  
41 taken into account.

42 **Keywords:** carotenoid, immune system, long-term effect, parasite, pesticides residuals,  
43 realistic exposure

44

## 45 **1. Introduction**

46 In just five decades, farmland bird abundance has declined by 56% in the UK and similar  
47 patterns were observed in other Western European countries (Chamberlain et al., 2000;  
48 Robinson & Sutherland, 2002; Inger et al., 2015) and North America (Rosenberg et al., 2019).  
49 Although air pollution (specifically ozone) has been recently shown to be responsible for part  
50 of bird decline in United-States (Liang et al. 2020), there is overwhelming support that  
51 agricultural intensification, in particular habitat transformation and insect food resource loss,  
52 have been causing such trends (Robinson & Sutherland, 2002; Newton, 2017). Until recently,  
53 however, there was very little evidence that pesticides could also have contributed to such  
54 declines, but continental and country scale analyses have suggested it might be so (Goulson,  
55 2014; Hallmann et al., 2014). Environmental stressors such as pollutants and pesticides may  
56 increase allergies and immunopathologies (Rowley & Monestier, 2005; Carey et al., 2006)  
57 leading to immune system disorders and reduce immunity and body condition (Carey et al.,  
58 2006; Franco et al., 2009; Lopez-Antia et al. 2018; Zhao et al. 2020). Birds' immune systems  
59 rely on compounds only obtained through their diet, such as carotenoids that not only scavenge  
60 free radicals but also enhance immune system responses (Møller et al., 2000). Immune defences  
61 are thus costly in terms of resources (McGraw & Ardia, 2003; Viney et al., 2005), especially  
62 since carotenoids are also involved in bird colouration as sexual secondary traits, leading to a  
63 trade-off between ornamentation and detoxification (Blount et al., 2003; McGraw & Ardia,  
64 2003; Baeta et al., 2008). Recently, plasma carotenoid concentrations and carotenoid-based  
65 pigments have been shown to be affected by exposure to heavy metals and Persistent Organic  
66 Pollutant (García-de Blas et al., 2014; Vallverdú-Coll et al., 2015; 2016). Pesticides may also

67 affect breeding capacity and increase in the long-term the probability of death through  
68 deteriorated body condition (Mitra et al., 2011; Lopez-Antia et al., 2015b). Immune function,  
69 carotenoid-based ornament and body condition may, therefore, be affected by pesticides and  
70 such effect on individual fitness may cascade into long-term population decline (Hansen et al.,  
71 2004).

72         However, despite pathways through which pesticides may affect birds' life-history traits  
73 are now well documented (e.g., Mitra et al. 2011; Gibbons et al. 2015), there is still little direct  
74 evidence from field studies. One main reason is the practical difficulty of elaborating a robust  
75 design in the field. For instance, there is virtually no area in most farmland landscapes which  
76 are pesticide-free since most of the soils and even agricultural products were reported to be  
77 contaminated by pesticides (Hoai et al. 2011; Camenzuli et al. 2015; Qu et al. 2016). Hence,  
78 there is no control situation. A possible alternative is to consider landscapes in which pesticides  
79 are almost absent at large scale, or diets (and food) coming from such landscapes and then given  
80 to hand-reared birds. Organic farming is an alternative to conventional agriculture, offering  
81 higher sustainability in food production, biodiversity and human health (Sandhu et al., 2010a;  
82 Gonthier et al., 2014). Organic farmers are not allowed to use synthetic inputs, and indeed  
83 represent such a proxy of control situation, although organic farming and conventional farming  
84 do not only differ in respect to pesticide use (see discussion below).

85         Few studies have so far considered pesticide effects in farmland birds (though see  
86 Lopez-Antia et al., 2013; 2015a; 2015b) and those used usually high toxic dose to induce  
87 detectable effects on survival rates (e.g., Lopez-Antia et al., 2014), thus neglecting sublethal  
88 effects. The mechanisms through which pesticides may directly cause declines in bird  
89 populations may be direct mortality at high doses (Mineau & Tucker, 2002; Mitra et al., 2011)  
90 or sub-lethal effects at medium doses (Lopez-Antia et al., 2013; 2014; 2015a,b,c). Far less  
91 attention has been given, however, to the delayed sub-lethal effects of pesticides at very low,

92 chronic, doses, such as the ones that might be expected to occur in conventional farmland  
93 landscapes, because such effects can be very weak and may only occur after several years of  
94 exposure (Mitra et al., 2011). Current levels of pesticide residues in farmland bird food are not  
95 well known, though supposed to be low. Since many farmland birds are winter seedeaters and  
96 spring insect foragers (Perkins et al., 2007), they may be highly exposed to such pesticides  
97 (Bartuszevige et al., 2002 for an example). Even small quantities of these residues present in  
98 the seeds may lead to sublethal effects if this food is taken over a long period of time (Parween  
99 et al., 2016). Moreover, the pesticides may act through various pathways such as  
100 neurotransmitters, hormones, reproduction, immune response, resistance to pathogens and  
101 avoidance of predators (Coors & De Meester, 2008; Mitra et al., 2011). In the context of chronic  
102 exposures to different pesticide residues, and given their different modes of action, we may  
103 hypothesise that life-history traits of birds exposed to these chronic doses would be affected  
104 compared to control.

105         How this may happen however remains an open question, since the effects may not  
106 necessarily be negative. For instance, there is increasing evidence that environmental stressors  
107 like chemical contaminants may have a positive effect on life-history traits at low dose (Majer  
108 et al., 2019). Indeed, the hormetic dose-response to environmental stressors predicts a biphasic  
109 dose response with positive effects at low dose becoming negative at a higher dose (Costantini  
110 et al., 2010; Mattson & Calabrese, 2010; Hashmi et al., 2014). This effect has been described  
111 in a wide range of organisms in response to different environmental stressors, and hormesis in  
112 birds has gained recent attention (Hashmi et al., 2014). Interestingly, hormetic responses may  
113 represent an evolutionary adaptation of metabolic systems that promotes survival for an  
114 organism living in a stressful environment, allowing bird populations higher flexibility to cope  
115 and survive with environmental perturbations (Costantini, 2009; Costantini & Borremans,  
116 2019). Deciphering whether the effects of low doses of pesticide residues can be positive or

117 negative is critical to determine the role of chronic exposition in the fate of farmland bird  
118 populations.

119 We report here on an experiment that aimed at filling this gap by quantifying the  
120 sublethal effects resulting from pesticide residues exposition on a range of life-history traits  
121 under realistic field conditions in the grey partridge, *Perdix perdix*, a charismatic farmland bird.  
122 This species has declined by about 90% over the last 50 years in Europe (Kuijper & Oosterveld,  
123 2009; Aebischer & Ewald, 2010). Partridges are exposed to a wide range of pesticides through  
124 the food they forage in farm fields and sub-lethal effects may contribute to the current decline  
125 of their populations though neither direct nor acute effects were described so far (Bro et al.,  
126 2015; Millot et al., 2015). In the present study, we tested the sublethal effects of being fed with  
127 food from organic or conventional farming, differing therefore in pesticide content, and  
128 controlling for its quality. We thus developed a novel experimental procedure, in which our 6-  
129 month-old hand-reared grey partridges were provided with natural grains being grown either  
130 from organic or conventional agriculture, i.e., seeds were not treated on their own but resulted  
131 from different cropping practices, thus food varied in pesticide content. Over 26 weeks for two  
132 consecutive years, we surveyed partridges' behaviour, breeding performance and bodily and  
133 physiological conditions, allowing to measure lagged and sublethal effects of naturally ingested  
134 low doses of pesticides in grains from conventional farms.

135

## 136 **2. Materials and Methods**

137 **2.1 Experimental design.** For this experiment, we used 40 (2017) and 38 (2018) 6-month-old  
138 hand-reared grey partridges obtained from a farming centre. One male and one female were  
139 randomly housed in pairs (i.e., 20 and 19 pairs) in outdoor cages (580 x 109 x 170 cm). Pairs  
140 were acclimatized to the new boxes for three weeks before starting the experiments, with *ad*  
141 *libitum* tap water and food. At this stage, all were fed with organic wheat seeds (week -3 to

142 week 0; see Fig. S1 for details and below for details about food). Pairs were then randomly  
143 assigned to either the “organic” group or the “conventional” group (10 pairs each in 2017 and  
144 9 pairs in 2018). All life-history traits assessed in this study (see below) showed no difference  
145 between the two groups in 2017 and 2018 at start of the season, (week 0; see electronic  
146 supplementary material, Table S1).

147         The organic group was fed *ad libitum* with grains obtained from the harvest of certified  
148 organic crops (and purchased from organic producers). Diet was composed of two-thirds wheat  
149 and one-third maize in 2017 and wheat only in 2018. We changed the composition of food to  
150 get a replicate of our experiments (organic *versus* conventional). The ratio of two-thirds of  
151 wheat and one-third of maize in 2017 had been recommended by partridge breeders. As wheat  
152 and maize were provided *ad libitum*, birds had the opportunity to choose their foods. In 2017  
153 and 2018, organic wheat was from variety LG ABSALON and ATTLASS respectively (Table  
154 1a). The conventional group was also fed with two-thirds wheat and one-third maize in 2017  
155 and wheat only in 2018, but seeds were purchased from producers that grew the crops using  
156 conventional agriculture with various pesticide applications during the cropping season (Table  
157 2). Wheat varieties involved in conventional treatment; NEMO was the same for both years  
158 (Table 1a). Unfortunately, we could not get this information for maize from producers.  
159 Moreover, birds of both groups in both years received organic food pellets, 2 days a week in  
160 2017 (always Monday and Thursday) and 3 days a week in 2018 (always Friday to Monday),  
161 also *ad libitum*. The precise composition of these granules is given in electronic supplementary  
162 material, Table S2. The addition of granules was mandatory to maintain enough proteins to the  
163 diets since a strict grain diet would not allow birds to lay eggs.

164         Given grains differed between the two treatments regarding presumed pesticide loads,  
165 but also possibly regarding quality (since varieties differed), we quantified pesticide residues  
166 (Table 2), energy content (Table 1b) and searched for quality content of the different varieties



167 (Table 1a). Pesticide residues were quantified by GIRPA (Beaucouzé, Loire Atlantique, France)  
168 using a multi-residual method with a gas chromatograph coupled to a mass spectrometer (GC-  
169 MS, ISO 17025, the limit of detection was 0.003 mg/kg). Pesticide residues were found at  
170 various concentrations in the conventionally grown grains (up to 0.048 mg/kg in wheat in 2018  
171 and 2.90 mg/kg in maize in 2017) whereas no residues were detected in the organic grains  
172 (Table 2). These values are consistent with those found in other studies quantifying pesticide  
173 residues in cereal grains (Lozowicka et al., 2014; Mebdoua & Ounane, 2019). The energy  
174 content for both food types was also quantified as the heat released by combustion using a  
175 calorimeter (Table 1b). This was carried out twice at the Centre Européen de Recherches et  
176 d'Analyses (Dijon, France). There was no significant difference between the food ingested by  
177 the organic group and the conventional group (Table 1b). Protein class, specific weight, Zeleny  
178 index and grains hardness of the different wheat indicated that organic and conventional  
179 varieties had highly similar quality traits (Table 1a).

180 Water and food were replaced each week. During the 26-week season, from the end of  
181 January (week 0) to the end of July (week 26), birds were kept on a natural light cycle. After 3  
182 weeks of acclimatization (week -3 to week 0) when both groups were fed organic grains, the  
183 birds were assessed at least three different times (Fig. S1) to assess: (1) immunocompetence  
184 using a PHA test and physiological stress through measurement of different indices, (2) the  
185 intensity of carotenoid-based colouration and plasma carotenoid concentration, (3) the  
186 behaviour and body condition and (4) breeding traits. In 2017 only, we used the natural  
187 infection by intestinal parasites (coccidia) to assess the effect of food on intestinal parasite load.  
188 No anticoccidial drugs were provided during the experiment.

189

190 **2.2 Intestinal parasites (coccidia).** To avoid unnecessary stress of repeated captures to collect  
191 individual faeces, we chose to analyse the intestinal parasitic load on pairs of birds. The day

192 before the collection of faeces, the cages were cleaned carefully to remove all faeces. Each pair  
193 was left without disturbance for exactly 24 hours, then fresh faeces were collected from the  
194 floor and the faecal samples were weighed to the nearest  $\pm 0.01$  g with an electronic balance  
195 (ScoutPro) and placed in a 50 mL Falcon tube. Samples were then gently homogenized with 14  
196 mL of Sheather's solution (45% sugar). Parasites were counted in a McMaster chamber with  
197 600  $\mu$ L of the sample solution which had been left for 10 min to allow the oocysts and eggs to  
198 float (Filipiak et al., 2009). There were two counts for each faecal sample and the mean number  
199 of oocysts was used. Coccidia concentrations were expressed as the mean number of parasites  
200 per gram of faecal sample. Counts were performed at weeks 1, 4, 7, 10 and 13.

201

202 **2.3 Haematocrit, white blood cell count and heterophil/lymphocyte ratio.** A blood sample  
203 was taken from the brachial vein using a sterile needle and heparinised micro-capillary tubes.  
204 These tubes were then centrifuged at 5000 r.p.m. for 5 min immediately afterwards to determine  
205 haematocrit (the proportion of the tube filled with red blood cells divided by the total sample  
206 volume in the capillary tube, as an index of anaemia) (Biard et al., 2015). Lengths were  
207 measured with an electronic calliper (accuracy of 0.1mm). A thin blood smear was made from  
208 a droplet of the blood sample from each bird. The slides were air-dried and immediately fixed  
209 in absolute methanol for 5 min and stained with Giemsa solution for 40 minutes. The blood  
210 smears were examined under 1000x magnification with an oil immersion microscope to  
211 estimate the total numbers and proportions of different types of leucocytes. The total proportion  
212 of white blood cells (WBC) was estimated by counting the number of leucocytes found for  
213 10000 erythrocytes. The ratio of the relative numbers of heterophils to lymphocytes (H/L ratio)  
214 for 100 leucocytes was used as an index of physiological stress (Ots et al., 1998). Blood  
215 sampling and all related counts were performed in weeks 0, 5 and 10.

216

217 **2.4 Plasma carotenoid concentration.** We centrifuged blood from the same samples  
218 immediately after collection (4,000 r.p.m. at 4°C for 5 min) to recover the plasma which was  
219 stored in Eppendorf tubes at -80°C until analysis. The plasma carotenoid concentration was  
220 assessed using a standardised colorimetric technique (Biard et al., 2010). Twenty microliters of  
221 plasma were diluted in 180µL of absolute ethanol; gently vortexed and flocculent proteins were  
222 precipitated by centrifugation at 1500g for 10 min. The optical density of the supernatant was  
223 determined with a spectrophotometer at 450 nm. This was performed in weeks 0, 5 and 10.

224

225 **2.5 PHA assay.** The ability to trigger a cell-mediated immune response was measured using a  
226 simplified phytohaemagglutinin (PHA) skin test protocol (Biard et al., 2009). Grey partridges  
227 were injected with 100µL of 10 mg/mL of PHA (PHA-P, Sigma-Aldrich, Lyon, France)  
228 dissolved in phosphate-buffered saline solution (PBS) in the centre of the right-wing web  
229 (patagium). All individuals were injected in the morning between 9:00 and 12:00 am. The  
230 patagium thickness at the injection site was measured just before and exactly 24h after the  
231 injection using a pressure-sensitive thickness gauge with an accuracy of 0.01 mm (Teclock SM-  
232 112, Alpa SpA, Milano, Italy). Each individual was measured twice. The PHA test was  
233 performed only once per bird, at week 5.

234

235 **2.6 Eye ring colouration.** In the grey partridge, the red patch just behind the eye acts as a  
236 secondary carotenoid sexual trait, involved in mate choice (Svobodová et al., 2013). High-  
237 resolution digital images were taken without flash or zoom twice on each side of the head using  
238 the same digital camera (Olympus, Stylus TG-2) under standardized conditions (same light,  
239 same spatial position between camera and birds) to measure the intensity of the red colour of  
240 the eye ring in weeks 0, 5 and 10 (Fig. S1). A standard grey strip was placed close to the head  
241 to control for any subtle variation in illumination between images. For each image, we

242 calculated the red (R), green (G), and blue (B) components of the eye ring using Adobe  
243 Photoshop (v 7.0) by selecting a square of 1 cm<sup>2</sup> of only the pigmented area (see the procedure  
244 in Lopez Antia et al., 2013). The same components were calculated for the grey reference to  
245 compensate for possible differences in colour between images. The average of the RGB  
246 components from the four pictures was then calculated. The intensity of red colouration  
247 (redness) was calculated as R divided by the average of R, G, and B. This was performed in  
248 weeks 0, 5 and 10.

249

250 **2.7 General behaviour.** The behaviour of individual birds in the aviaries was scored to derive  
251 a time budget, using cameras (Reconyx, UltraFireTM). After acclimatization for 30 minutes,  
252 10 second-videos were recorded every minute for two hours. The videos were then watched  
253 individually to score “active behaviour” (eating, moving, vigilant, grooming) and "passive  
254 behaviour" (sleeping, immobile) for each 10-second video. Only the first behaviour observed  
255 in video was recorded when several activities occurred within the 10 seconds. Video recordings  
256 were made in weeks 0, 5 and 10 at the same hour.

257

258 **2.8 Body Mass Index.** To monitor the body condition of grey partridges during the course of  
259 the experiment, right and left tarsus lengths were measured twice with a digital calliper with an  
260 accuracy of 0.1mm at the beginning of the experiment (week 0). Body mass was recorded to  
261 the nearest 0.1 g using an electronic balance (Scout Pro) for weeks 0, 3, 6, 7, 8, 9 and 10 (Fig.  
262 S1). Using body mass and mean tarsus length (right and left), we evaluated the body condition  
263 using the scaled mass index (see Peig and Green, 2009).

264

265 **2.9 Clutch and egg sizes.** The first eggs were laid in week 16 in 2017 and week 17 in 2018.  
266 Once the first egg was laid, all boxes were checked three times a week (on Monday, Wednesday

267 and Friday) to collect eggs over the whole season (8 weeks). All eggs were measured (maximum  
268 length and diameter) using the same digital calliper and weighed to the nearest 0.1 g using an  
269 electronic balance (Scout Pro). In 2017 only, the eggs laid 4<sup>th</sup> and 8<sup>th</sup> in the laying sequence  
270 (laying ranks 4 and 8) for each female, were sacrificed to assess the thickness of eggshell (see  
271 Lopez-Antia et al., 2013 for the details of the method).

272

273 **2.10 Statistical analyses.** Prior to analyses, the normality and homoscedasticity of the variable  
274 distributions were checked and transformed appropriately if required (see below). The  
275 difference in reaction to PHA between the organic and conventional groups, sex and year was  
276 tested with a linear regression model (LM) with *F*-ratio statistics. The haematocrit, H/L ratio  
277 (log-transformed), carotenoid concentration (log-transformed), colour (log-transformed), body  
278 condition, inactivity (square root transformed) and egg volume were compared using Linear  
279 Mixed-Effects Models (LMM) including the treatment (organic *versus* conventional), sex, and  
280 year as fixed effects, week of measure as a repeated effect to account for repeated measurements  
281 (time effect in the statistical model). Bird identity was treated as a random effect except for egg  
282 volume and intestinal parasite analyses, in which the random effect was the pair identity, and  
283 the models did not include the sex. Eggshell thickness was analysed similarly but the model did  
284 not include the year as data were available only for 2017. Generalized Linear Mixed-Effects  
285 Models with negative binomial error (NB-GLMM) were used instead of LMMs to analyse the  
286 variation in the white blood cell counts and intestinal parasites, but the statistical procedure was  
287 identical to that for the LMMs. Egg-laying chronology was analysed using Cox mixed-effects  
288 model, using the pair identity as a random effect. The LMMs, NB-LMMs and Cox model were  
289 associated with an analysis of deviance based on  $\chi^2$ -statistics for unbalanced design. For all  
290 tests, *P* values are given at 5% significance level threshold. All statistical tests were performed  
291 using R software (v. 3.5.0, R Core Team 2018) implemented with *nlme* for LMM, *glmmADMB*

292 for NB-GLMM, *car* for deviance analyses, *smatr* for the scale mass index computation and  
293 *coxme* for Cox mixed-effects model.

294

### 295 **3. Results**

296 There were no significant differences between years and treatments in the survivorship  
297 of adult partridges. In the conventional group, there were five deaths (two in 2017 and three in  
298 2018) while in the organic group there were three deaths (two in 2017 and one in 2018) (Fisher  
299 exact test:  $P = 0.67$ ). On the other hand, all behavioural, morphological and physiological traits  
300 either differed significantly or had significantly different trends between the two groups  
301 throughout the season.

302 Firstly, for the physiological traits, we found that the intestinal parasite load in the  
303 conventional group increased significantly during the course of the experiment while that of  
304 organic pairs remained stable (treatment x time interaction:  $P = 0.01$ )(Fig. 1a, Table S3).  
305 Moreover, the haematocrit of the conventional group (both sexes, but particularly males)  
306 decreased significantly through the season, the trend being particularly pronounced at the end  
307 of the season, while it remained stable in the organic group (time x treatment x sex interaction:  
308  $P < 0.01$ ) for both years (Fig. 2a, Table S3). For both sexes, the WBC count (treatment x time  
309 interaction:  $P < 0.0001$ ) and H/L ratio (treatment x time interaction:  $P = 0.02$ ) significantly  
310 increased in the conventional group while there was a decrease in the organic group, for both  
311 years (Fig. 2b, 2c, Table S3). Finally, the inflammatory response to PHA was significantly  
312 higher in the conventional group with a more marked effect in 2018 than 2017 (Fig. 2d, Linear  
313 regression model, treatment:  $F_{1,69} = 73.60$ ,  $P < 0.0001$ ; year:  $F_{1,69} = 123.69$ ,  $P < 0.0001$ ) for  
314 both sexes (sex:  $F_{1,69} = 1.52$ ,  $P = 0.22$ ; treatment x sex interaction:  $F_{1,69} = 0.41$ ,  $P = 0.52$ ).

315 Secondly, for the behavioural traits, we found that eye-ring redness (carotenoid-based  
316 colouration) increased slightly for both males and females over the season in both years, but it

317 increased and reached higher values in the organic males compared to the conventional males,  
318 especially in 2017 (time x treatment x year interaction:  $P = 0.03$ ; Fig. 3a, Table S3). There was  
319 an even more marked difference for the concentration of plasma carotenoids for both years,  
320 which was similar for the two groups at the beginning of the season, but then decreased steadily  
321 in both sexes of the conventional group relative while it increased sharply in the organic group  
322 (time x treatment interaction:  $P < 0.0001$ ; Fig. 3b, Table S3). In both sexes and years, the  
323 inactivity time of the organic group increased from 15% to about 40% (at week 10), while in  
324 the conventional group, it remained stable at about 15%; the difference was more marked for  
325 males than for females (time x treatment x sex interaction:  $P < 0.01$ , Fig. 3c, Table S3).  
326 Similarly, pesticide exposition (conventional group) affected body condition (Table S3). In  
327 males, the body condition of both groups declined slightly with time. For females, in the  
328 conventional group, the body condition improved (especially after week 6), while for females  
329 in the organic group, the body condition remained stable (interaction time x treatment x sex:  $P$   
330  $< 0.01$ ) (Fig. 3d). This effect on body condition was not the result of different daily food  
331 consumption rates as these were similar between groups (Table 1c).

332         Thirdly, for breeding, treatment did not affect the onset of laying (week 16 in 2017 and  
333 week 17 in 2018 for both groups) nor did it affect the clutch size in laying females (mean  
334 number of eggs  $\pm$  s.e.m.:  $12.17 \pm 3.30$  for the organic group *versus*  $12.11 \pm 1.67$  for the  
335 conventional group in 2017 and  $12.43 \pm 2.26$  *versus*  $12.25 \pm 2.86$  in 2018, N=8 females for  
336 both years; Wilcoxon rank sum test: for 2017,  $W = 26$ ,  $P = 0.95$ , for 2018:  $W = 28.5$ ,  $P = 1.00$ ).  
337 This absence of an effect of pesticide exposition on laying was confirmed by a Cox mixed  
338 effects regression model for the egg laying dynamics (treatment:  $\chi^2 = 1.17$ ,  $df = 1$ ,  $P = 0.28$ ;  
339 year:  $\chi^2 = 0.0004$ ,  $df = 1$ ,  $P = 0.98$ ; treatment x year:  $\chi^2 = 2.06$ ,  $df = 1$ ,  $P = 0.15$ ). However,  
340 pesticide exposure affected the egg volume which was lower in the conventional group than in  
341 the organic group, although this was only significant in 2017 (treatment x year interaction:  $P <$

342 0.01; Fig. 1b, Table S3). On the other hand, eggshell thickness for the conventional group (mean  
343  $\pm$  s.e.m. in mm:  $1.12 \pm 0.02$  and  $1.15 \pm 0.02$  for the eggs with laying ranks 4 and 8 respectively)  
344 was greater than for the organic group (mean  $\pm$  s.e.m. in mm:  $0.99 \pm 0.03$  and  $1.02 \pm 0.04$ , Table  
345 S3).

346

347

#### 348 **4. Discussion**

349 Although our experimental approach consisted in providing untreated grains differing  
350 only in the source (grains from organic farms and grains from conventional farms), we were  
351 able to detect a suite of cascading consequences on life-history traits after only 10 weeks, which  
352 led to significant divergences between the two groups for every trait we monitored in both years  
353 despite modest sample sizes. However, we acknowledge that organic and conventional systems  
354 differ in a set of parameters, which may affect bird in various pathways. Organic farming may  
355 provision more continuous and more abundant resources to birds across the landscape and  
356 throughout the season. Indeed, the ban of synthetic herbicides and mineral fertilizers increases  
357 floral, but also insect diversity or density (Bengtsson et al., 2005; Gabriel & Tschardtke, 2007;  
358 Ponce et al., 2011). Moreover, organic farmland is often sown with a greater variety of crops  
359 than conventionally farmed land (Hole et al., 2005; Barbieri et al., 2017) and comprises larger  
360 areas of semi-natural elements (Gibson et al., 2007). These two processes however cannot affect  
361 our results since birds are hand-reared. Only grains origins differed in the two treatment groups  
362 since food pellets were identical, and the proportions and quantity of grains were also identical.  
363 Despite varieties differed between organic and the conventional diets, we detected no statistical  
364 difference in quality between the organic and the conventional diets, either for energetical  
365 content (Table 1b) or from producer's information regarding protein contents (Table 1a).  
366 Finally, feeding rates were similar between the two groups of partridges (Table 1c). Therefore,



367 the only remaining difference in the grain's food provided to the two groups was the pesticide  
368 content: no detectable pesticide was found in organic food, but 1 to 4 pesticides were detected  
369 in the conventional food depending on the year. We are therefore confident that the differences  
370 detected in the behaviour and physiology between our two groups of partridges are the result  
371 of pesticide load, though we cannot exclude that another factor, unmeasured in this study, may  
372 further drive additional differences. The rapid development of different analytical techniques  
373 to assess simultaneously the detection and the quantification of different molecules of pesticides  
374 in avian blood samples (Rivera-Rodriguez et al., 2007; Taliensky-Chamudis et al. 2017;  
375 Byholm et al. 2018) will make it possible to quantify the prevalence of different molecules in  
376 wild grey-partridge in the near future.

377         Since the pesticide exposure did not increase mortality and the divergence appeared  
378 gradually with the time between groups, this indicates sub-lethal effects. The main effects of  
379 pesticide exposure included: (i) less-developed carotenoid-based traits and lower concentration  
380 of plasma carotenoids, (ii) more active immune systems (stronger response to PHA, increased  
381 concentration of white blood cells), (iii) physiological stress (lower haematocrit, higher H/L  
382 ratio), (iv) higher intestinal parasitic load, (v) higher behavioural activity (males) and higher  
383 body condition (females) and (vi) lower breeding investment (smaller eggs, though with thicker  
384 eggshell). Despite a change in the source of the conventionally grown grains between the two  
385 years, hence, *a priori*, different pesticide loads, the differences between groups were consistent  
386 between the years. The effects were stronger in 2017 than in 2018, as the pesticide load was  
387 also higher in 2017 than in 2018 (Table 2). Twenty-three active substances were used during  
388 the cropping season in 2017 with 4 detected as residues *versus* 11 active substances in 2018,  
389 with one residue (Table 2). The sexes also reacted differently: pesticide exposure in males  
390 resulted in more active birds than in the control group, with no apparent cost for body condition,  
391 while females traded body condition and breeding investment since pesticide-exposed females

392 improved their body condition at the expense of egg size. This sex difference could be due to  
393 pesticides since they can act as endocrine disruptors through different pathways associated with  
394 the hypothalamic–pituitary–gonadal axis (Grue et al., 1997). This includes endocrine processes,  
395 hence the secretion of steroid hormones, potentially affecting the production of sexual  
396 ornaments and/or the motivation to breed. For example, some pesticides may mimic the action  
397 of oestrogens (steroid hormones), resulting in an increase in the oestradiol concentration  
398 (associated with a decrease in testosterone) and leading to a decrease in adult fertility (Khalil  
399 et al., 2017). In some cases, pesticides may modulate the enzyme Cytochrome P450 aromatase  
400 responsible for the conversion of androgens to oestrogens (Saxena et al., 2015) and  
401 consequently the regulation of male sexual behaviour (Ubuka & Tsutsui, 2014). Prolactin and  
402 oestradiol are also impacted and increased to different extents depending on the molecules  
403 involved (Mohanty et al., 2017; Pandey et al., 2017). The effects of pesticides on hormones  
404 have already been explored in the red-legged partridge, showing contrasting results (Lopez-  
405 Antia et al., 2013; Lopez-Antia et al., 2015a). Such effects on sex-specific hormones in the grey  
406 partridge should be better assessed in future studies.

407 Partridges exposed to pesticides usually show physiological stress such as lower  
408 haematocrit, being indicative of anaemia, similarly to birds ingesting neonicotinoids (Mitra et  
409 al., 2011; Lopez-Antia et al., 2013). However, stress increased the activity of the immune  
410 system with a stronger response to PHA as well as an increase in the white blood cell count, an  
411 effect rarely reported so far (e.g., Lopez-Antia et al., 2013). Such an effect is well known for  
412 the human immune system structure and functions (Mokarizadeh et al., 2015) and an increase  
413 of the expression of the immune system was found with other contaminants such as mercury  
414 (Carey et al., 2006). More indices of quality of immune system such as the capacity of  
415 partridges to produce immunoglobulin should be designed in future studies to evaluate the  
416 delayed sublethal effects of pesticides on bird immunocompetence completely. Whatever the

417 mechanisms involved, both a high level of expression of the immune system and the  
418 physiological stress may impact individual fitness like more damages by parasites and diseases  
419 (Van der Sluijs et al., 2015), a consequence that seems to be confirmed here by the increased  
420 intestinal parasitic load in the treated group compared to controls.

421 Plasma carotenoid concentrations and carotenoid-based colouration of partridges was  
422 also lower in the conventional group and given that red-eye colouration intensity as also found  
423 in red-legged partridges (Lopez-Antia et al., 2013; 2014; 2015), a likely result of functional  
424 trade-offs. Pesticides ingestion may trigger a detoxification process, the latter producing  
425 reactive oxygen species that must be neutralized by the constitutive antioxidant system  
426 including carotenoid (Møller et al., 2000). This neutralization thus depletes the carotenoids  
427 stock for eye-ring pigmentation, especially in the conventional group (Faivre et al., 2003; Baeta  
428 et al., 2008). Since red-eye colouration intensity is involved in partridge mate choice  
429 (Svobodova et al., 2013; 2016), the increased behavioural activity of males in the conventional  
430 group may perhaps be interpreted as a response to decreased attractiveness to females. If this  
431 mechanism operates in the wild, it may have potential deleterious consequences in regard to  
432 mating success and/or predator detection since predation is a major cause of mortality in grey  
433 partridges in France (Bro & Millot, 2013).

434 Pesticides are known in birds to perturb key reproductive hormones (Lopez-Antia et al.,  
435 2015a; Lv et al. 2016; Pandey et al., 2017), which may decrease egg production, delay egg-  
436 laying, reduce egg size and eggshell thickness (Hernández et al., 2008; Lopez-Antia et al., 2013;  
437 2018, Liu et al., 2019). Here, we found mixed results: while females from the conventional  
438 group laid smaller eggs, which may affect chick survival (Blomqvist et al., 1997), their clutch  
439 size was similar to females from the organic group (see Lopez-Antia et al., 2015a for a similar  
440 result on red-legged partridges), and most surprisingly, they had actually thicker eggs.  
441 Pesticides such as organochlorides inhibit calcium pumps in the membrane of eggs and thus,

442 reduce the transport of calcium carbonate from blood to the shell (Mitra et al., 2011), leading  
443 to the thinning of eggshells (Lopez-Antia et al., 2013; Gibbons et al., 2015). This result may  
444 suggest a trade-off between egg size and eggshell thickness: a reduced egg size may allow a  
445 higher investment in eggshell. We also found that pesticide-exposed birds had a higher body  
446 condition, an unexpected result since the reverse has generally been found (Subapriya et al.,  
447 2007; Lopez-Antia et al., 2013). We suggest that the consumption of pesticides may have  
448 induced dysfunctions of metabolic pathways associated with fat storage, as shown in rats  
449 exposed to persistent organic pollutants (Ruzzin et al., 2010). There are potential costs  
450 associated with bird overweight, such as reducing their ability to escape predators (Binazzi et  
451 al., 2011).

452         Finally, some of our results are consistent with a hormetic dose-effect, since we detected  
453 increased performance of exposed individuals to chronic doses of pesticide residues, at least in  
454 some traits, such as higher activated immune system and body condition. Immunological  
455 hormetic response (Calabrese, 2005; Costantini et al., 2010) and stimulatory effect of the  
456 chemical on the growth of birds have already been reported in chickens (Costantini et al., 2010;  
457 Hasmi et al., 2014 and references therein) although the several physiological and molecular  
458 mechanisms involved have not yet been fully elucidated (Costantini et al., 2010). However,  
459 whether these hormetic effects have positive effects for exposed birds is still questioned, as  
460 revealed by exponentially growing numbers of papers showing positive effects (even  
461 transgenerationally) on one hand, but some showing negative effects on the other hand (see  
462 Agathokleous & Calabrese, 2019; Calabrese & Agathokleous, 2020 for recent examples).  
463 Possible hormesis dose-response in partridges should be therefore further investigated as well  
464 as exploring a possible preconditioning effect (i.e., bird being better able to survive with  
465 exposure to higher levels of pesticide residue if encountered on subsequent occasions) as has  
466 been suggested in some birds (Costantini et al., 2010; Costantini, 2019).

467

## 468 **5. Conclusion**

469 Our innovative experimental design highlights that realistic pesticide exposure levels  
470 may have lagged sub-lethal consequences resulting in major life-history traits shifts. Although  
471 this has yet to be proven, these changes in life-history traits may impair individuals' fitness in  
472 the wild. These shifts include, for instance, increased predation risk due to increased activity,  
473 lower mating success due to reduced secondary sexual traits, or lower survival due to higher  
474 intestinal parasite load, immune system disorders and fat storage. Our results thus shed new  
475 lights on farmland bird decline and their causes, by showing for the first time that ingesting low  
476 doses of pesticides may have lagged sub-lethal consequences on several major physiological  
477 pathways. There is, therefore, an urgent need to assess the long-term sub-lethal effects of  
478 pesticides on farmland birds, for pesticide risk assessment where only short-term effects are  
479 currently considered on model species under laboratory conditions, which may not reliably  
480 predict consequences of pesticide exposure on wild birds (EFSA, 2009).

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498

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500

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508

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## 790 **Figures Legend**

791

792 **Fig. 1.** (a) Number of intestinal parasites in both sexes for the organic and conventional groups  
793 during the course of the experiment in 2017 and (b) Egg volume laid by females for the control  
794 and pesticide groups according to the laying rank (1 to 8) in 2017 and 2018.

795

796 **Fig. 2.** Blood parameters and response to phytohaemagglutinin (PHA) for conventional and  
797 organic groups according to sex (or both sexes pooled). Average values are shown and given  
798 through the season (weeks 0, 5 and 10; see text). (a) Haematocrit (percentage of red blood cells  
799 in blood sample), (b) white blood cell count, (c) Heterophil to lymphocyte (H/L) ratio for 100  
800 leucocytes as an index of physiological stress and (d) Cellular immune response (wing web  
801 swelling after PHA injection): the horizontal lines are the medians; the boxes enclose the middle  
802 two quartiles; the vertical lines are the 1.5 x interquartile ranges; the points are the outliers.

803

804 **Fig. 3.** (a) Intensity of red coloration around the eye, (b) Plasma carotenoid concentration, (c)  
805 Percentage of inactivity (time sampling every minute for two hours) and (d) Body condition.  
806 Graphs for each group and sex (or both sexes pooled) through the season (weeks 0, 5 and 10  
807 for a, b, c and weeks 0, 3, 6, 7, 8, 9, 10 for d; see text).

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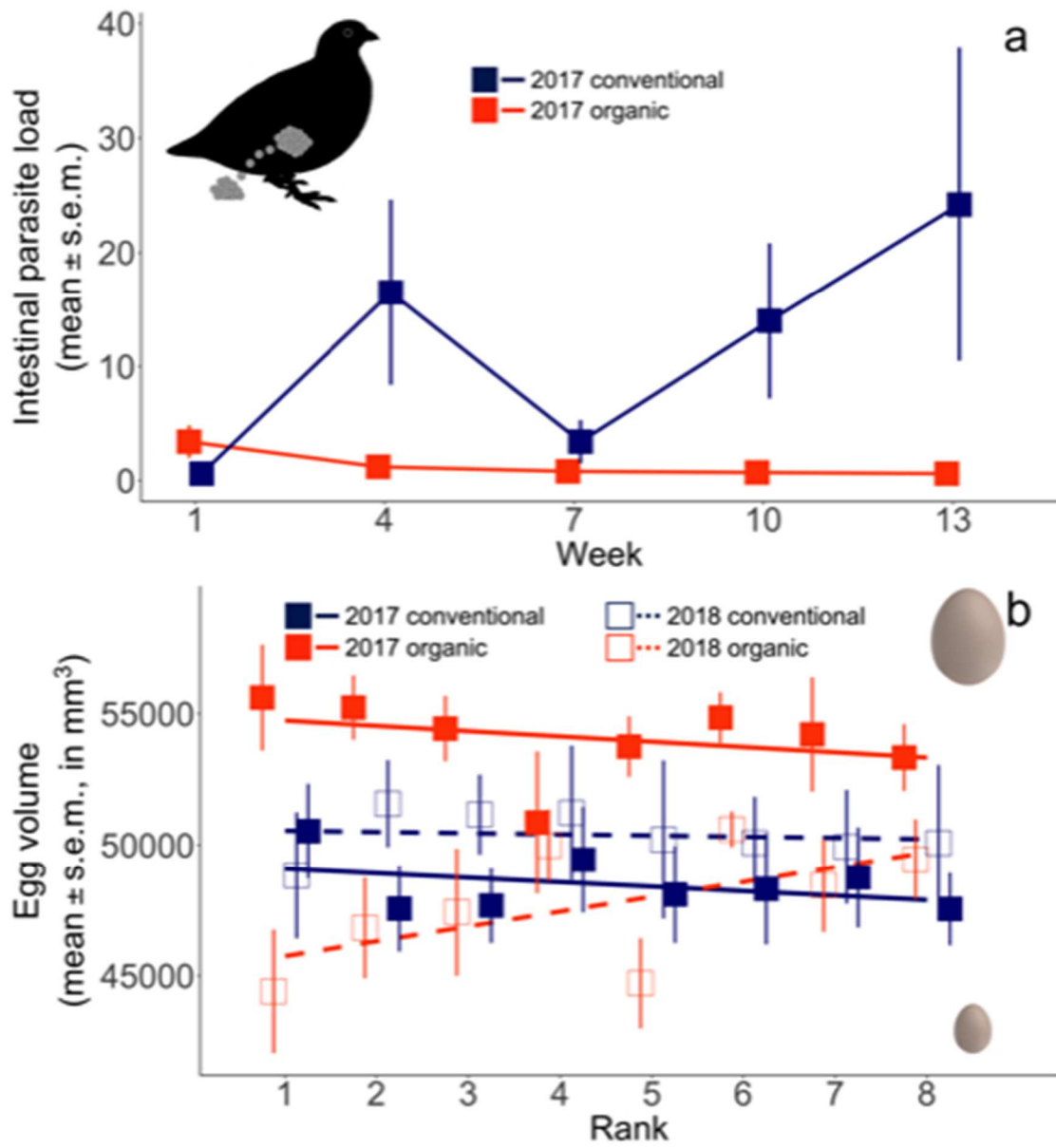
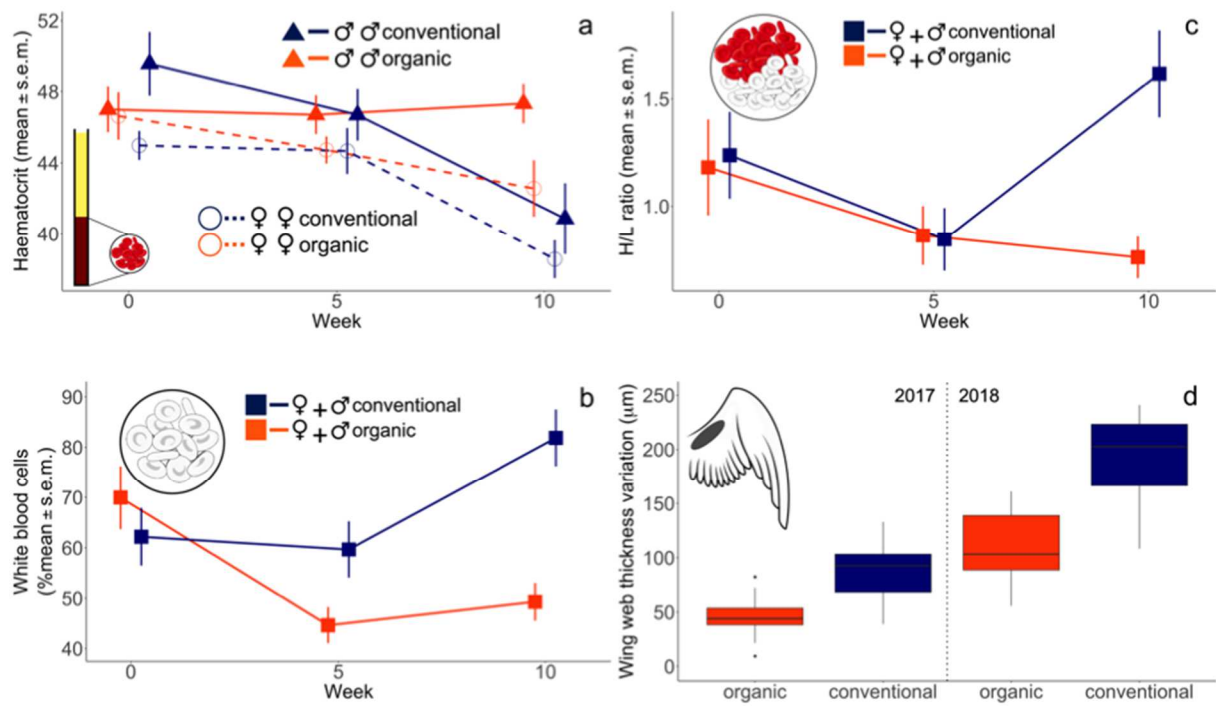


Figure 1  
Moreau et al.

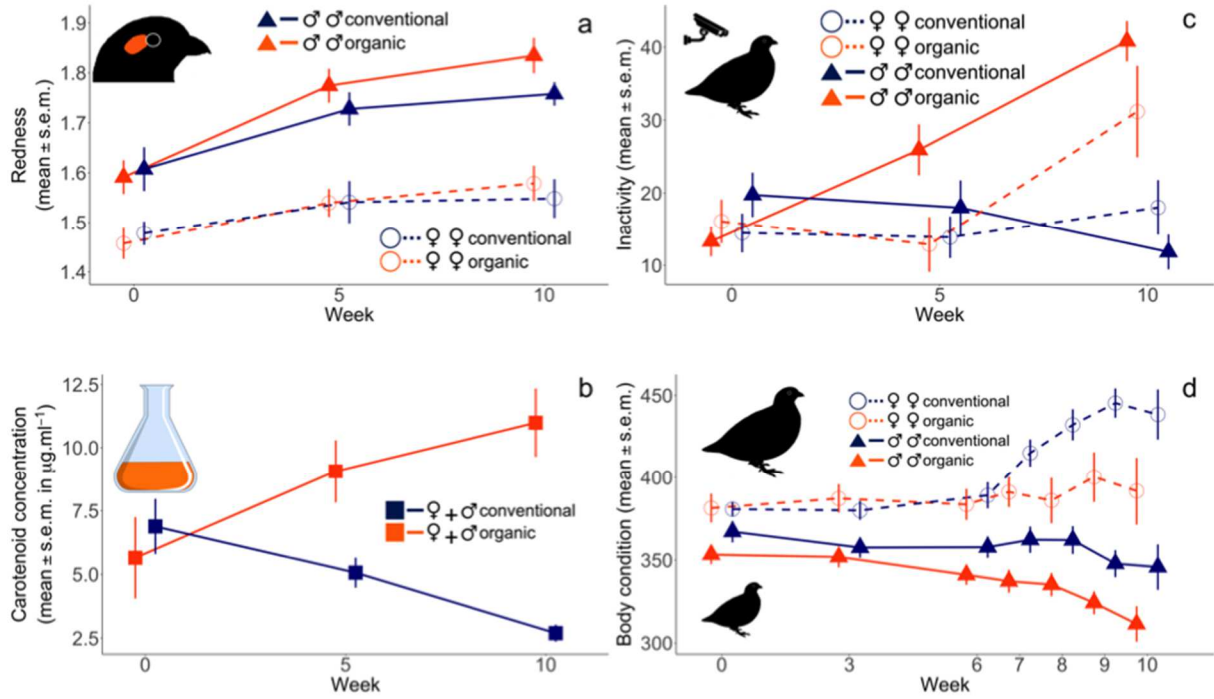
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Figure 2  
Moreau et al.



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Figure 3  
Moreau et al.

870 **Table 1.** (a) Information regarding different wheat varieties, purchased from organic and conventional farmers in 2017 and in 2018 (this information  
871 was not available for maize varieties). Data were obtained from ARVALIS company, the French arable crops Research and Development Institute  
872 (<https://www.arvalis-infos.fr/index.html>). (b) Energy content (in J per gram) for sample of 200 g wheat or maize crushed in 2017 and wheat only  
873 in 2018, analysed twice by calorimeter (Centre Européen de Recherches et d'Analyses, Dijon, France). The results of this assay showed no  
874 differences between the conventional and organic food in either year (the 95% confidence intervals overlap). In 2017, both groups were fed with  
875 two thirds wheat and one third maize, and we used this ratio to calculate the 95% confidence intervals. (c) Consumption rate (mean  $\pm$  s.e.m. in  
876 grams of food per day consumed) of the two groups in 2017 (for 9 pairs) and 2018 (for 8 pairs) assessed during two different weeks (6 and 15).  
877 Consumption rate was assessed by offering *ad libitum* food at the beginning of the day (07:00 a.m.) and weighing the remaining food 24 hours  
878 later. Pesticide exposure did not have any effect in either year (LMM: treatment:  $\chi^2 = 0.18$ , df = 1, P = 0.67; year:  $\chi^2 = 9.91$ , df = 1, P < 0.01;  
879 treatment x year:  $\chi^2 = 0.06$ , df = 1, P = 0.81).

880

		2017				2018	
		Organic		Conventional		Organic	Conventional
		Wheat	Maize	Wheat	Maize	Wheat	Wheat
(a) Characte- ristics	Variety	LG ABSALON	-	NEMO	-	ATTLASS	NEMO
	Protein content class <sup>1</sup>	6	-	6	-	6	6
	Specific weight <sup>2</sup>	7	-	7	-	6	7
	Zeleny Indice <sup>3</sup>	35-40	-	25-40	-	25-40	25-40
	Grains Hardness	Medium-Hard	-	Medium-Hard	-	Hard	Medium-Hard
(b) Energy (J g <sup>-1</sup> )	Energy1	16000	16300	16500	16200	15300	16100
	Energy2	16000	16400	16600	15800	15800	15800
	mean $\pm$ s.e.m	16000 $\pm$ 30	16400 $\pm$ 33	16600 $\pm$ 45	16000 $\pm$ 187	15700 $\pm$ 390	15800 $\pm$ 63
	95% IC	[16100-16200]		[16200-16500]		[14900-16500]	[15600-15900]
(c) Consumption	Week 6	38.6 $\pm$ 9.4		35.8 $\pm$ 11.1		36.2 $\pm$ 4.7	45.4 $\pm$ 6.1
	Week 15	24.7 $\pm$ 4.0		29.5 $\pm$ 10.4		62.6 $\pm$ 4.5	61.1 $\pm$ 6.4

881

882 <sup>1</sup>The protein content is expressed in class on a scale from 1 (very low) to 9 (very high).

883 <sup>2</sup>Specific weight represents the density of a batch of wheat and is expressed in class from 1 (very low) to 9 (very high).

884 <sup>3</sup>Zeleny indice (or dry gluten content) represents an indicator of the quantity and quality of proteins through their ability to swell in an acid medium.

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888 **Table 2.** List of active substances used during the cropping season for both wheat and maize in  
 889 2017 and for wheat in 2018 (information provided by the farmers), and active substances and  
 890 their concentrations found in conventionally grown grains by chemical analysis. No pesticide  
 891 residues were detected in the organic grains. In total, 500 pesticides were searched for.

892 2017

Chemical Substance	Pesticide type	Partridge food	Detected in grains	Concentration
Chlorothalonil	Fungicide	Wheat		
Clodinafop-propargyl	Herbicide	Wheat		
Cloquintocet-mexyl	Herbicide	Wheat		
Cyproconazole	Fungicide	Wheat		
Cyprodinil	Herbicide	Wheat		
Deltamethrine	Insecticide	Maize	X	0.25 mg/kg
Desmediphan	Herbicide	Maize		
Diflufenicanil	Herbicide	Wheat		
Esfenvalerate	Herbicide	Wheat		
Ethofumesate	Insecticide	Wheat		
Florasulam	Herbicide	Maize		
Flufenacet	Herbicide	Wheat		
Fluxapyroxad	Fungicide	Wheat		
Isoproturon	Herbicide	Wheat	X	0.70 mg/kg
Metamitron	Herbicide	Maize		
Metconazole	Fungicide	Wheat		
Metsulfuron-methyl	Herbicide	Wheat		
Phenmédiphan	Herbicide	Wheat		
Pinoxaden	Herbicide	Wheat		
Piperonyl butoxide	Synergiser	Maize	X	2.90 mg/kg
Pirimiphos methyl	Insecticide	Maize	X	0.12 mg/kg
Propiconazole	Fungicide	Wheat		
Prothioconazole	Fungicide	Wheat		

893

894 2018

Chemical Substance	Pesticide type	Partridge food	Detected in grains	Concentration
4-chlorophenoxyacetic acid	Herbicide	Wheat		
Benzovindiflupyr	Fungicide	Wheat		
Chlorpyrifos-methyl	Insecticide	Wheat	x	0.048 mg/kg
Dichlorprop-P	Herbicide	Wheat		
Diflufénicanin	Herbicide	Wheat		
Imadocloprid	Insecticide	Wheat		
Iodosulfuron-methyl	Herbicide	Wheat		
Mefenpyr-diethyl	Herbicide	Wheat		
Mésosulfuron-méthyl	Herbicide	Wheat		
Metconazole	Fungicide	Wheat		
Picolinafen	Herbicide	Wheat		

895

896 Pesticide residues were quantified by GIRPA (Beaucouzé, Loire Atlantique, France) using a  
 897 multi-residual method with a gas chromatograph coupled to a mass spectrometer (GC-MS)  
 898 (ISO 17025) with a quantification threshold of 0.010 mg/kg and detection threshold of 0.003  
 899 mg/kg.





physiology  
behaviour  
life history traits

29 weeks

