



Feeding partridges with organic or conventional grain triggers cascading effects in life-history traits[☆]

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ABSTRACT

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

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1. Introduction

In just five decades, farmland bird abundance has declined by 56% in the UK and similar patterns were observed in other Western European countries (Chamberlain et al., 2000; Robinson and Sutherland, 2002; Inger et al., 2015) and North America (Rosenberg et al., 2019). Although air pollution (specifically ozone) has been recently shown to be responsible for part of bird decline in

United-States (Liang et al., 2020), there is overwhelming support that agricultural intensification, in particular habitat transformation and insect food resource loss, have been causing such trends (Robinson and Sutherland, 2002; Newton, 2017). Until recently, however, there was very little evidence that pesticides could also have contributed to such declines, but continental and country scale analyses have suggested it might be so (Goulson, 2014; Hallmann et al., 2014). Environmental stressors such as pollutants and pesticides may increase allergies and immunopathologies (Rowley and Monestier, 2005; Carey et al., 2006) leading to immune system disorders and reduce immunity and body condition (Carey et al., 2006; Franco et al., 2009; Lopez-Antia et al., 2018; Zhao et al., 2020). Birds' immune systems rely on compounds only obtained through their diet, such as carotenoids that not only

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scavenge free radicals but also enhance immune system responses (Moller et al., 2000). Immune defences are thus costly in terms of resources (McGraw and Ardia, 2003; Viney et al., 2005), especially since carotenoids are also involved in bird colouration as sexual secondary traits, leading to a trade-off between ornamentation and detoxification (Blount et al., 2003; McGraw and Ardia, 2003; Baeta et al., 2008). Recently, plasma carotenoid concentrations and carotenoid-based pigments have been shown to be affected by exposure to heavy metals and Persistent Organic Pollutant (García-de Blas et al., 2014; Vallverdú-Coll et al., 2015; 2016). Pesticides may also affect breeding capacity and increase in the long-term the probability of death through deteriorated body condition (Mitra et al., 2011; Lopez-Antia et al., 2015b). Immune function, carotenoid-based ornament and body condition may, therefore, be affected by pesticides and such effect on individual fitness may cascade into long-term population decline (Hansen et al., 2004).

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

Few studies have so far considered pesticide effects in farmland birds (though see Lopez-Antia et al., 2013; 2015a; 2015b) and those used usually high toxic dose to induce detectable effects on survival rates (e.g., Lopez-Antia et al., 2014), thus neglecting sublethal effects. The mechanisms through which pesticides may directly cause declines in bird populations may be direct mortality at high doses (Mineau & Tucker, 2002; Mitra et al., 2011) or sub-lethal effects at medium doses (Lopez-Antia et al., 2013; 2014; 2015a,b,c). Far less attention has been given, however, to the delayed sub-lethal effects of pesticides at very low, chronic, doses, such as the ones that might be expected to occur in conventional farmland landscapes, because such effects can be very weak and may only occur after several years of exposure (Mitra et al., 2011). Current levels of pesticide residues in farmland bird food are not well known, though supposed to be low. Since many farmland birds are winter seedeaters and spring insect foragers (Perkins et al., 2007), they may be highly exposed to such pesticides (Bartuszevige et al., 2002 for an example). Even small quantities of these residues present in the seeds may lead to sublethal effects if this food is taken over a long period of time (Parween et al., 2016). Moreover, the pesticides may act through various pathways such as neurotransmitters, hormones, reproduction, immune response, resistance to pathogens and avoidance of predators (Coors and De Meester, 2008; Mitra et al., 2011). In the context of chronic exposures to different pesticide residues, and given their different modes of action, we may hypothesise that life-history traits of birds exposed to these chronic doses would be affected compared to control.

How this may happen however remains an open question, since the effects may not necessarily be negative. For instance, there is increasing evidence that environmental stressors like chemical

contaminants may have a positive effect on life-history traits at low dose (Majer et al., 2019). Indeed, the hormetic dose-response to environmental stressors predicts a biphasic dose response with positive effects at low dose becoming negative at a higher dose (Costantini et al., 2010; Mattson and Calabrese, 2010; Hashmi et al., 2014). This effect has been described in a wide range of organisms in response to different environmental stressors, and hormesis in birds has gained recent attention (Hashmi et al., 2014). Interestingly, hormetic responses may represent an evolutionary adaptation of metabolic systems that promotes survival for an organism living in a stressful environment, allowing bird populations higher flexibility to cope and survive with environmental perturbations (Costantini, 2009; Costantini and Borremans, 2019). Deciphering whether the effects of low doses of pesticide residues can be positive or negative is critical to determine the role of chronic exposition in the fate of farmland bird populations.

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

2. Materials and methods

2.1. Experimental design

For this experiment, we used 40 (2017) and 38 (2018) 6-month-old hand-reared grey partridges obtained from a farming centre. One male and one female were randomly housed in pairs (i.e., 20 and 19 pairs) in outdoor cages (580 x 109 x 170 cm). Pairs were acclimatized to the new boxes for three weeks before starting the experiments, with *ad libitum* tap water and food. At this stage, all were fed with organic wheat seeds (week -3 to week 0; see Fig. S1 for details and below for details about food). Pairs were then randomly assigned to either the "organic" group or the "conventional" group (10 pairs each in 2017 and 9 pairs in 2018). All life-history traits assessed in this study (see below) showed no difference between the two groups in 2017 and 2018 at start of the season, (week 0; see electronic supplementary material, Table S1).

The organic group was fed *ad libitum* with grains obtained from the harvest of certified organic crops (and purchased from organic producers). Diet was composed of two-thirds wheat and one-third maize in 2017 and wheat only in 2018. We changed the composition of food to get a replicate of our experiments (organic *versus* conventional). The ratio of two-thirds of wheat and one-third of maize in 2017 had been recommended by partridge breeders. As wheat and maize were provided *ad libitum*, birds had the

opportunity to choose their foods. In 2017 and 2018, organic wheat was from variety LG ABSALON and ATTLASS respectively (Table 1a). The conventional group was also fed with two-thirds wheat and one-third maize in 2017 and wheat only in 2018, but seeds were purchased from producers that grew the crops using conventional agriculture with various pesticide applications during the cropping season (Table 2). Wheat varieties involved in conventional treatment; NEMO was the same for both years (Table 1a). Unfortunately, we could not get this information for maize from producers. Moreover, birds of both groups in both years received organic food pellets, 2 days a week in 2017 (always Monday and Thursday) and 3 days a week in 2018 (always Friday to Monday), also *ad libitum*. The precise composition of these granules is given in electronic supplementary material, Table S2. The addition of granules was mandatory to maintain enough proteins to the diets since a strict grain diet would not allow birds to lay eggs.

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

Water and food were replaced each week. During the 26-week

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

2.2. Intestinal parasites (coccidia)

To avoid unnecessary stress of repeated captures to collect individual faeces, we chose to analyse the intestinal parasitic load on pairs of birds. The day before the collection of faeces, the cages were cleaned carefully to remove all faeces. Each pair was left without disturbance for exactly 24 h, then fresh faeces were collected from the floor and the faecal samples were weighed to the nearest ± 0.01 g with an electronic balance (ScoutPro) and placed in a 50 mL Falcon tube. Samples were then gently homogenized with 14 mL of Sheather's solution (45% sugar). Parasites were counted in a McMaster chamber with 600 μ L of the sample solution which had been left for 10 min to allow the oocysts and eggs to float (Filipiak et al., 2009). There were two counts for each faecal sample and the mean number of oocysts was used. Coccidia concentrations were expressed as the mean number of parasites per gram of faecal sample. Counts were performed at weeks 1, 4, 7, 10 and 13.

2.3. Haematocrit, white blood cell count and heterophil/lymphocyte ratio

A blood sample was taken from the brachial vein using a sterile needle and heparinised micro-capillary tubes. These tubes were then centrifuged at 5000 r.p.m. for 5 min immediately afterwards to determine haematocrit (the proportion of the tube filled with red blood cells divided by the total sample volume in the capillary tube,

Table 1

(a) Information regarding different wheat varieties, purchased from organic and conventional farmers in 2017 and in 2018 (this information was not available for maize varieties). Data were obtained from ARVALIS company, the French arable crops Research and Development Institute (<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 in 2018, analysed twice by calorimeter (Centre Européen de Recherches et d'Analyses, Dijon, France). The results of this assay showed no differences between the conventional and organic food in either year (the 95% confidence intervals overlap). In 2017, both groups were fed with 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 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). Consumption rate was assessed by offering *ad libitum* food at the beginning of the day (07:00 am) and weighing the remaining food 24 h 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, $P < 0.01$; treatment x year: $\chi^2 = 0.06$, df = 1, $P = 0.81$).

		2017				2018	
		Organic		Conventional		Organic	Conventional
		Wheat	Maize	Wheat	Maize	Wheat	Wheat
(a) Characteristics	Variety	LG ABSALON	—	NEMO	—	ATTLASS	NEMO
	Protein content class ^a	6	—	6	—	6	6
	Specific weight ^b	7	—	7	—	6	7
	Zeleny Indice ^c	35–40	—	25–40	—	25–40	25–40
	Grains Hardness	Medium-Hard	—	Medium-Hard	—	Hard	Medium-Hard
(b) Energy (J g ⁻¹)	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

^a The protein content is expressed in class on a scale from 1 (very low) to 9 (very high).

^b Specific weight represents the density of a batch of wheat and is expressed in class from 1 (very low) to 9 (very high).

^c 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.

Table 2

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

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		
Phenmediphan	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		
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		
Diflufenicanin	Herbicide	Wheat		
Imadocloprid	Insecticide	Wheat		
Iodosulfuron-methyl	Herbicide	Wheat		
Mefenpyr-diethyl	Herbicide	Wheat		
Mesosulfuron-méthyl	Herbicide	Wheat		
Metconazole	Fungicide	Wheat		
Picolinafen	Herbicide	Wheat		

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

as an index of anaemia) (Biard et al., 2015). Lengths were measured with an electronic calliper (accuracy of 0.1 mm). A thin blood smear was made from a droplet of the blood sample from each bird. The slides were air-dried and immediately fixed in absolute methanol for 5 min and stained with Giemsa solution for 40 min. The blood smears were examined under 1000× magnification with an oil immersion microscope to estimate the total numbers and proportions of different types of leucocytes. The total proportion of white blood cells (WBC) was estimated by counting the number of leucocytes found for 10000 erythrocytes. The ratio of the relative numbers of heterophils to lymphocytes (H/L ratio) for 100 leucocytes was used as an index of physiological stress (Ots et al., 1998). Blood sampling and all related counts were performed in weeks 0, 5 and 10.

2.4. Plasma carotenoid concentration

We centrifuged blood from the same samples immediately after collection (4000 r.p.m. at 4 °C for 5 min) to recover the plasma which was stored in Eppendorf tubes at −80 °C until analysis. The plasma carotenoid concentration was assessed using a standardised colorimetric technique (Biard et al., 2010). Twenty microliters of plasma were diluted in 180 µL of absolute ethanol; gently vortexed and flocculent proteins were precipitated by centrifugation at

1500g for 10 min. The optical density of the supernatant was determined with a spectrophotometer at 450 nm. This was performed in weeks 0, 5 and 10.

2.5. PHA assay

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

2.6. Eye ring colouration

In the grey partridge, the red patch just behind the eye acts as a secondary carotenoid sexual trait, involved in mate choice (Svobodová et al., 2013). High-resolution digital images were taken

without flash or zoom twice on each side of the head using the same digital camera (Olympus, Stylus TG-2) under standardized conditions (same light, same spatial position between camera and birds) to measure the intensity of the red colour of the eye ring in weeks 0, 5 and 10 (Fig. S1). A standard grey strip was placed close to the head to control for any subtle variation in illumination between images. For each image, we calculated the red (R), green (G), and blue (B) components of the eye ring using Adobe Photoshop (v 7.0) by selecting a square of 1 cm² of only the pigmented area (see the procedure in Lopez Antia et al., 2013). The same components were calculated for the grey reference to compensate for possible differences in colour between images. The average of the RGB components from the four pictures was then calculated. The intensity of red colouration (redness) was calculated as R divided by the average of R, G, and B. This was performed in weeks 0, 5 and 10.

2.7. General behaviour

The behaviour of individual birds in the aviaries was scored to derive a time budget, using cameras (Reconyx, UltraFireTM). After acclimatization for 30 min, 10 s-videos were recorded every minute for 2 h. The videos were then watched individually to score "active behaviour" (eating, moving, vigilant, grooming) and "passive behaviour" (sleeping, immobile) for each 10-s video. Only the first behaviour observed in video was recorded when several activities occurred within the 10 s. Video recordings were made in weeks 0, 5 and 10 at the same hour.

2.8. Body mass index

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

2.9. Clutch and egg sizes

The first eggs were laid in week 16 in 2017 and week 17 in 2018. Once the first egg was laid, all boxes were checked three times a week (on Monday, Wednesday and Friday) to collect eggs over the whole season (8 weeks). All eggs were measured (maximum length and diameter) using the same digital calliper and weighed to the nearest 0.1 g using an electronic balance (Scout Pro). In 2017 only, the eggs laid 4th and 8th in the laying sequence (laying ranks 4 and 8) for each female, were sacrificed to assess the thickness of eggshell (see Lopez-Antia et al., 2013 for the details of the method).

2.10. Statistical analyses

Prior to analyses, the normality and homoscedasticity of the variable distributions were checked and transformed appropriately if required (see below). The difference in reaction to PHA between the organic and conventional groups, sex and year was tested with a linear regression model (LM) with *F*-ratio statistics. The haematocrit, H/L ratio (log-transformed), carotenoid concentration (log-transformed), colour (log-transformed), body condition, inactivity (square root transformed) and egg volume were compared using Linear Mixed-Effects Models (LMM) including the treatment (organic versus conventional), sex, and year as fixed effects, week of measure as a repeated effect to account for repeated measurements (time effect in the statistical model). Bird identity was treated as a

random effect except for egg volume and intestinal parasite analyses, in which the random effect was the pair identity, and the models did not include the sex. Eggshell thickness was analysed similarly but the model did not include the year as data were available only for 2017. Generalized Linear Mixed-Effects Models with negative binomial error (NB-GLMM) were used instead of LMMs to analyse the variation in the white blood cell counts and intestinal parasites, but the statistical procedure was identical to that for the LMMs. Egg-laying chronology was analysed using Cox mixed-effects model, using the pair identity as a random effect. The LMMs, NB-LMMs and Cox model were associated with an analysis of deviance based on χ^2 -statistics for unbalanced design. For all tests, *P* values are given at 5% significance level threshold. All statistical tests were performed using R software (v. 3.5.0, R Core Team 2018) implemented with *nlme* for LMM, *glmmADMB* for NB-GLMM, *car* for deviance analyses, *smatr* for the scale mass index computation and *coxme* for Cox mixed-effects model.

3. Results

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

Firstly, for the physiological traits, we found that the intestinal parasite load in the conventional group increased significantly during the course of the experiment while that of organic pairs remained stable (treatment $\times$ time interaction: *P* = 0.01) (Fig. 1a, Table S3). Moreover, the haematocrit of the conventional group

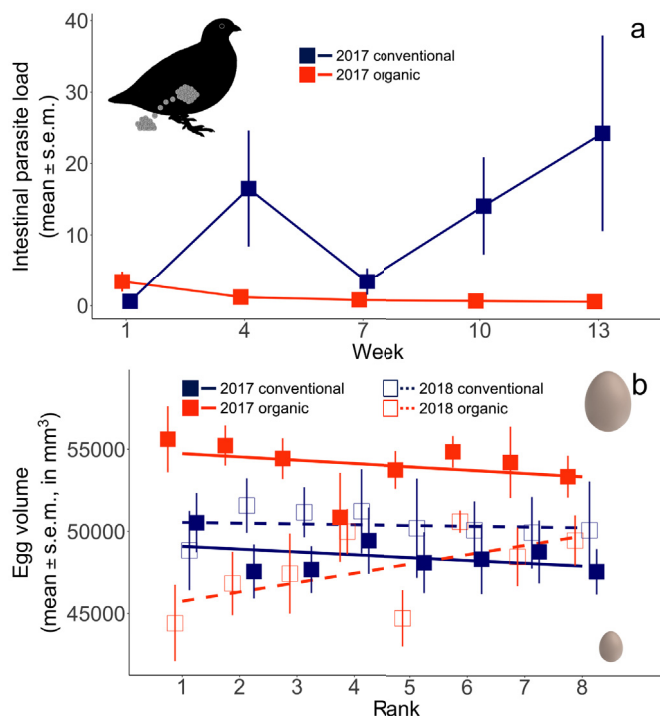


Fig. 1. (a) Number of intestinal parasites in both sexes for the organic and conventional groups during the course of the experiment in 2017 and (b) Egg volume laid by females for the control and pesticide groups according to the laying rank (1–8) in 2017 and 2018.

(both sexes, but particularly males) decreased significantly through the season, the trend being particularly pronounced at the end of the season, while it remained stable in the organic group (time $\times$ treatment $\times$ sex interaction: $P < 0.01$) for both years (Fig. 2a, Table S3). For both sexes, the WBC count (treatment $\times$ time interaction: $P < 0.0001$) and H/L ratio (treatment $\times$ time interaction: $P = 0.02$) significantly increased in the conventional group while there was a decrease in the organic group, for both years (Fig. 2b and c, Table S3). Finally, the inflammatory response to PHA was significantly higher in the conventional group with a more marked effect in 2018 than 2017 (Fig. 2d, Linear regression model, treatment: $F_{1,69} = 73.60$, $P < 0.0001$; year: $F_{1,69} = 123.69$, $P < 0.0001$) for both sexes (sex: $F_{1,69} = 1.52$, $P = 0.22$; treatment $\times$ sex interaction: $F_{1,69} = 0.41$, $P = 0.52$).

Secondly, for the behavioural traits, we found that eye-ring redness (carotenoid-based colouration) increased slightly for both males and females over the season in both years, but it increased and reached higher values in the organic males compared to the conventional males, especially in 2017 (time $\times$ treatment $\times$ year interaction: $P = 0.03$; Fig. 3a, Table S3). There was an even more marked difference for the concentration of plasma carotenoids for both years, which was similar for the two groups at the beginning of the season, but then decreased steadily in both sexes of the conventional group relative while it increased sharply in the organic group (time $\times$ treatment interaction: $P < 0.0001$; Fig. 3b, Table S3). In both sexes and years, the inactivity time of the organic group increased from 15% to about 40% (at week 10), while in the conventional group, it remained stable at about 15%; the difference was more marked for males than for females (time $\times$ treatment $\times$ sex interaction: $P < 0.01$, Fig. 3c, Table S3). Similarly, pesticide exposition (conventional group) affected body condition (Table S3). In males, the body condition of both groups declined slightly with time. For females, in the conventional group, the body condition

improved (especially after week 6), while for females in the organic group, the body condition remained stable (interaction time $\times$ treatment $\times$ sex: $P < 0.01$) (Fig. 3d). This effect on body condition was not the result of different daily food consumption rates as these were similar between groups (Table 1c).

Thirdly, for breeding, treatment did not affect the onset of laying (week 16 in 2017 and week 17 in 2018 for both groups) nor did it affect the clutch size in laying females (mean number of eggs $\pm$ s.e.m.: 12.17 ± 3.30 for the organic group versus 12.11 ± 1.67 for the conventional group in 2017 and 12.43 ± 2.26 versus 12.25 ± 2.86 in 2018, $N = 8$ females for both years; Wilcoxon rank sum test: for 2017, $W = 26$, $P = 0.95$, for 2018: $W = 28.5$, $P = 1.00$). This absence of an effect of pesticide exposition on laying was confirmed by a Cox mixed effects regression model for the egg laying dynamics (treatment: $\chi^2 = 1.17$, $df = 1$, $P = 0.28$; year: $\chi^2 = 0.0004$, $df = 1$, $P = 0.98$; treatment $\times$ year: $\chi^2 = 2.06$, $df = 1$, $P = 0.15$). However, pesticide exposure affected the egg volume which was lower in the conventional group than in the organic group, although this was only significant in 2017 (treatment $\times$ year interaction: $P < 0.01$; Fig. 1b, Table S3). On the other hand, eggshell thickness for the conventional group (mean $\pm$ s.e.m. in mm: 1.12 ± 0.02 and 1.15 ± 0.02 for the eggs with laying ranks 4 and 8 respectively) was greater than for the organic group (mean $\pm$ s.e.m. in mm: 0.99 ± 0.03 and 1.02 ± 0.04 , Table S3).

4. Discussion

Although our experimental approach consisted in providing untreated grains differing only in the source (grains from organic farms and grains from conventional farms), we were able to detect a suite of cascading consequences on life-history traits after only 10 weeks, which led to significant divergences between the two groups for every trait we monitored in both years despite modest

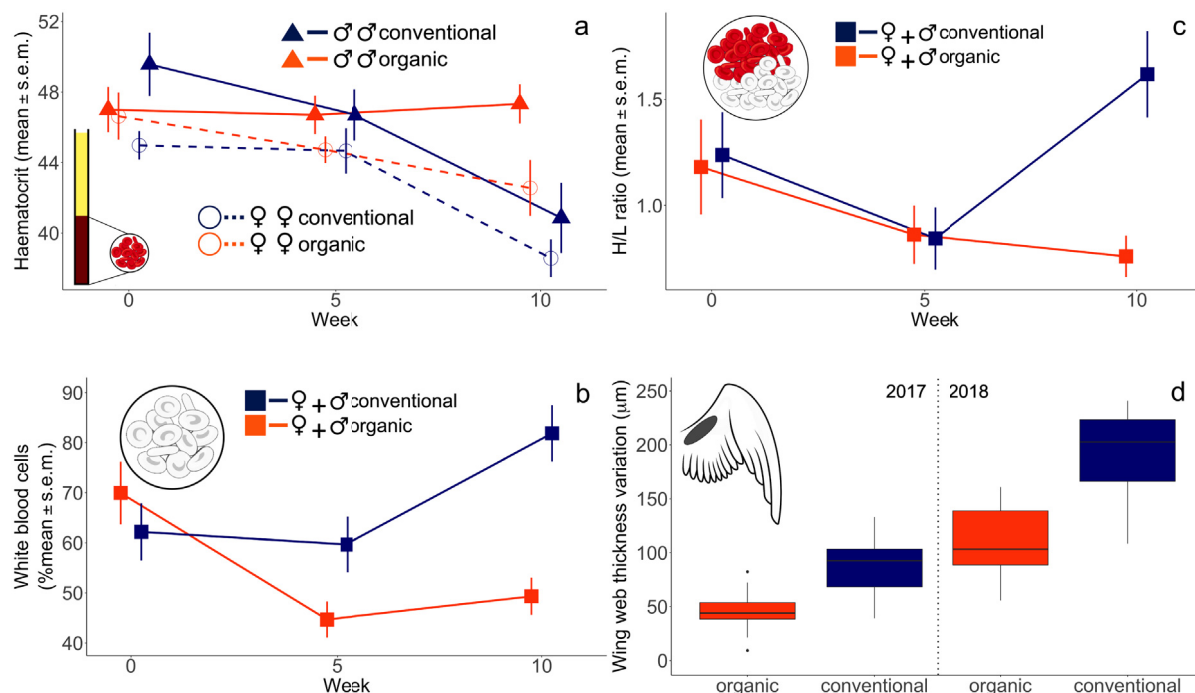


Fig. 2. Blood parameters and response to phytohaemagglutinin (PHA) for conventional and organic groups according to sex (or both sexes pooled). Average values are shown and given through the season (weeks 0, 5 and 10; see text). (a) Haematocrit (percentage of red blood cells in blood sample), (b) white blood cell count, (c) Heterophil to lymphocyte (H/L) ratio for 100 leucocytes as an index of physiological stress and (d) Cellular immune response (wing web swelling after PHA injection): the horizontal lines are the medians; the boxes enclose the middle two quartiles; the vertical lines are the 1.5 $\times$ interquartile ranges; the points are the outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

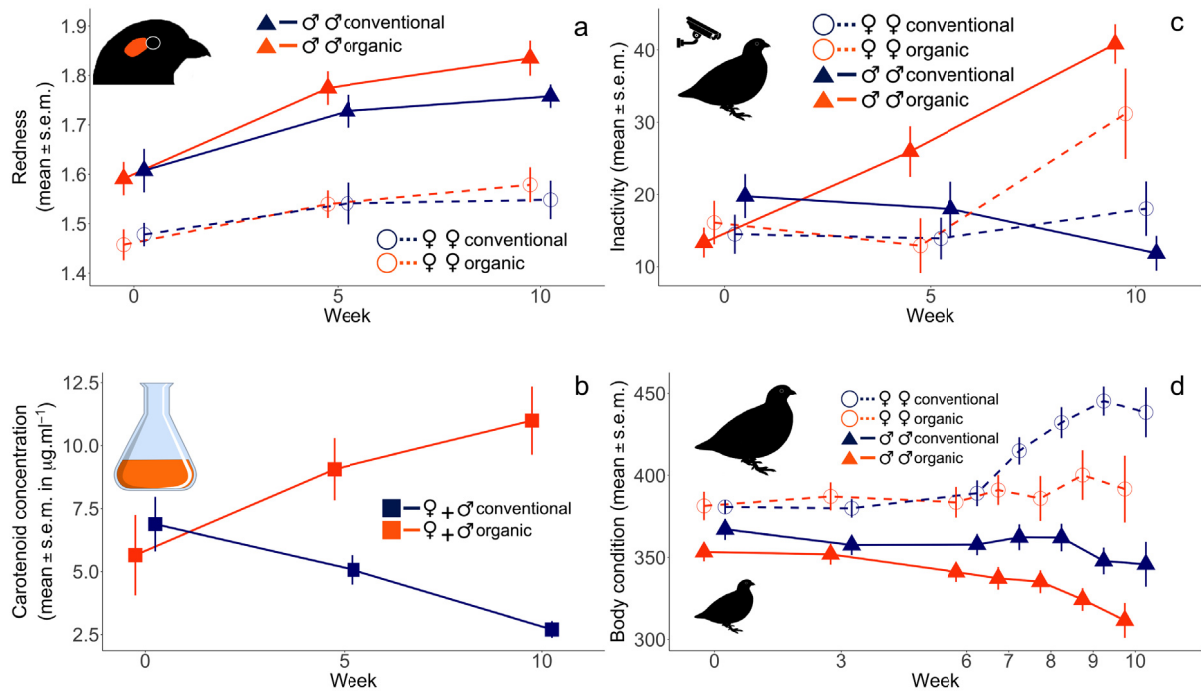


Fig. 3. (a) Intensity of red colouration around the eye, (b) Plasma carotenoid concentration, (c) Percentage of inactivity (time sampling every minute for 2 h) and (d) Body condition. Graphs for each group and sex (or both sexes pooled) through the season (weeks 0, 5 and 10 for a, b, c and weeks 0, 3, 6, 7, 8, 9, 10 for d; see text). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sample sizes. However, we acknowledge that organic and conventional systems differ in a set of parameters, which may affect birds in various pathways. Organic farming may provision more continuous and more abundant resources to birds across the landscape and throughout the season. Indeed, the ban of synthetic herbicides and mineral fertilizers increases floral, but also insect diversity or density (Bengtsson et al., 2005; Gabriel and Tschardt, 2007; Ponce et al., 2011). Moreover, organic farmland is often sown with a greater variety of crops than conventionally farmed land (Hole et al., 2005; Barbieri et al., 2017) and comprises larger areas of semi-natural elements (Gibson et al., 2007). These two processes however cannot affect our results since birds are hand-reared. Only grains origins differed in the two treatment groups since food pellets were identical, and the proportions and quantity of grains were also identical. Despite varieties differed between organic and the conventional diets, we detected no statistical difference in quality between the organic and the conventional diets, either for energetical content (Table 1b) or from producer's information regarding protein contents (Table 1a). Finally, feeding rates were similar between the two groups of partridges (Table 1c). Therefore, the only remaining difference in the grain's food provided to the two groups was the pesticide content: no detectable pesticide was found in organic food, but 1 to 4 pesticides were detected in the conventional food depending on the year. We are therefore confident that the differences detected in the behaviour and physiology between our two groups of partridges are the result of pesticide load, though we cannot exclude that another factor, unmeasured in this study, may further drive additional differences. The rapid development of different analytical techniques to assess simultaneously the detection and the quantification of different molecules of pesticides in avian blood samples (Rivera-Rodriguez et al., 2007; Taliansky-Chamudis et al., 2017; Byholm et al., 2018) will make it possible to quantify the prevalence of different molecules in wild grey-partridge in the near future.

Since the pesticide exposure did not increase mortality and the

divergence appeared gradually with the time between groups, this indicates sub-lethal effects. The main effects of pesticide exposure included: (i) less-developed carotenoid-based traits and lower concentration of plasma carotenoids, (ii) more active immune systems (stronger response to PHA, increased concentration of white blood cells), (iii) physiological stress (lower haematocrit, higher H/L ratio), (iv) higher intestinal parasitic load, (v) higher behavioural activity (males) and higher body condition (females) and (vi) lower breeding investment (smaller eggs, though with thicker eggshell). Despite a change in the source of the conventionally grown grains between the two years, hence, *a priori*, different pesticide loads, the differences between groups were consistent between the years. The effects were stronger in 2017 than in 2018, as the pesticide load was also higher in 2017 than in 2018 (Table 2). Twenty-three active substances were used during the cropping season in 2017 with 4 detected as residues versus 11 active substances in 2018, with one residue (Table 2). The sexes also reacted differently: pesticide exposure in males resulted in more active birds than in the control group, with no apparent cost for body condition, while females traded body condition and breeding investment since pesticide-exposed females improved their body condition at the expense of egg size. This sex difference could be due to pesticides since they can act as endocrine disruptors through different pathways associated with the hypothalamic–pituitary–gonadal axis (Grue et al., 1997). This includes endocrine processes, hence the secretion of steroid hormones, potentially affecting the production of sexual ornaments and/or the motivation to breed. For example, some pesticides may mimic the action of oestrogens (steroid hormones), resulting in an increase in the oestradiol concentration (associated with a decrease in testosterone) and leading to a decrease in adult fertility (Khalil et al., 2017). In some cases, pesticides may modulate the enzyme Cytochrome P450 aromatase responsible for the conversion of androgens to oestrogens (Saxena et al., 2015) and consequently the regulation of male sexual behaviour (Ubuka and Tsutsui, 2014).

Prolactin and oestradiol are also impacted and increased to different extents depending on the molecules involved (Mohanty et al., 2017; Pandey et al., 2017). The effects of pesticides on hormones have already been explored in the red-legged partridge, showing contrasting results (Lopez-Antia et al., 2013; 2015a). Such effects on sex-specific hormones in the grey partridge should be better assessed in future studies.

Partridges exposed to pesticides usually show physiological stress such as lower haematocrit, being indicative of anaemia, similarly to birds ingesting neonicotinoids (Mitra et al., 2011; Lopez-Antia et al., 2013). However, stress increased the activity of the immune system with a stronger response to PHA as well as an increase in the white blood cell count, an effect rarely reported so far (e.g., Lopez-Antia et al., 2013). Such an effect is well known for the human immune system structure and functions (Mokarizadeh et al., 2015) and an increase of the expression of the immune system was found with other contaminants such as mercury (Carey et al., 2006). More indices of quality of immune system such as the capacity of partridges to produce immunoglobulin should be designed in future studies to evaluate the delayed sublethal effects of pesticides on bird immunocompetence completely. Whatever the mechanisms involved, both a high level of expression of the immune system and the physiological stress may impact individual fitness like more damages by parasites and diseases (Van der Sluijs et al., 2015), a consequence that seems to be confirmed here by the increased intestinal parasitic load in the treated group compared to controls.

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

Pesticides are known in birds to perturb key reproductive hormones (Lopez-Antia et al., 2015a; Lv et al., 2016; Pandey et al., 2017), which may decrease egg production, delay egg-laying, reduce egg size and eggshell thickness (Hernández et al., 2008; Lopez-Antia et al., 2013, 2018; Liu et al., 2019). Here, we found mixed results: while females from the conventional group laid smaller eggs, which may affect chick survival (Blomqvist et al., 1997), their clutch size was similar to females from the organic group (see Lopez-Antia et al., 2015a for a similar result on red-legged partridges), and most surprisingly, they had actually thicker eggs. Pesticides such as organochlorides inhibit calcium pumps in the membrane of eggs and thus, reduce the transport of calcium carbonate from blood to the shell (Mitra et al., 2011), leading to the thinning of eggshells (Lopez-Antia et al., 2013; Gibbons et al., 2015). This result may suggest a trade-off between egg size and eggshell thickness: a reduced egg size may allow a higher investment in eggshell. We also found that pesticide-exposed birds had a higher body condition, an unexpected result

since the reverse has generally been found (Subapriya et al., 2007; Lopez-Antia et al., 2013). We suggest that the consumption of pesticides may have induced dysfunctions of metabolic pathways associated with fat storage, as shown in rats exposed to persistent organic pollutants (Ruzzin et al., 2010). There are potential costs associated with bird overweight, such as reducing their ability to escape predators (Binazzi et al., 2011).

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

5. Conclusion

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

Author statement

Moreau J: Funding acquisition, conception and design the study, acquisition of data, Formal analysis, drafting the article, final approval of the version, Monceau K: conception and design the study, Formal analysis, revising the article, final approval of the version, Crépin M: acquisition of data, Formal analysis, final approval of the version, Derouin Tochon F: acquisition of data, Formal analysis, final approval of the version, Mondet C: acquisition of data, Formal analysis, final approval of the version, Fraikin M: acquisition of data, Formal analysis, final approval of the version, Teixeira M: acquisition of data, Formal analysis, final approval of the version, Bretagnolle V: Funding acquisition, conception and design the study, drafting the article, final approval of the version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2021.116851>.

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Data accessibility

Once the paper has been accepted, data will be deposited in the Dryad.

Research ethics

All experiments complied with French laws on animal experimentation and all experimental protocols were approved by the Deux-Sèvres Committee of Animal Experimentation (APA FIS#9465–201703101551625). Experiments were conducted in the research facilities of the Zoodysee authorised animal park Villiers-en-Bois, France) on behalf of Bernard Ragot (79/D87/2015).

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