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Brood size is reduced by half in birds feeding on flutriafol-treated seeds below the recommended application rate

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Abstract

Despite the efforts of the European Commission to implement measures that offset the detrimental effects of agricultural intensification, farmland bird populations continue to decline. Pesticide use has been pointed out as a major cause of decline, with growing concern about those agrochemicals that act as endocrine disruptors. We report here on the effects of flutriafol, a ubiquitous systemic fungicide used for cereal seed treatment, on the physiology and reproduction of a declining gamebird. Captive red-legged partridges (*Alectoris rufa*; n=11-13 pairs per treatment) were fed wheat treated with 0%, 20% or 100% of the flutriafol application rate during 25 days in late winter. We studied treatment effects on the reproductive performance, carotenoid-based coloration and cellular immune responsiveness of adult partridges, and their relationship with changes in oxidative stress biomarkers and plasma biochemistry. We also studied the effect of parental exposure on egg antioxidant content and on the survival, growth and cellular immune response of offspring. Exposed partridges experienced physiological effects (reduced levels of cholesterol and triglycerides), phenotypical effects (a reduction in the carotenoid-based pigmentation of their eye rings), and most importantly, severe adverse effects on reproduction: a reduced clutch size and fertile egg ratio, and an overall offspring production reduced by more than 50%. No effects on body condition or cellular immune response of either exposed adult or their surviving offspring were observed. These results, together with previous data on field exposure in wild partridges, demonstrate that seed treatment with flutriafol represents a risk for granivorous birds; they also highlight a need to improve the current regulation system used for foreseeing and preventing negative impacts of Plant Protection Products on wildlife.

Key words: Pesticides; Azole fungicides; triazole; endocrine disruption; risk assessment.

Capsule: The ingestion of flutriafol treated seeds reduces partridge's reproductive output and represents a threat for granivorous bird populations.

1 Introduction

2 Recent studies have pointed out that the use of pesticides is a major cause of farmland bird
3 declines, either because of indirect effect on habitat and food supply or because of direct toxic effects
4 affecting the survival, health, or reproduction of birds (Geiger et al. 2010; Mineau and Whiteside, 2013;
5 Hallmann et al. 2014; Goulson, 2014). Reductions in winter food resources, due to the replacement of
6 overwinter cereal stubble and fodder crops by winter-sown cereal, is also an important factor
7 contributing to these declines (Siriwardena et al. 2000, 2007). These two components of agricultural
8 intensification converge in the problem of treated seeds. Winter cereal became a key component of the
9 diet of many granivorous birds (Browne & Aebischer 2003; Robinson 2004; Perkins et al. 2007) and
10 cereal seeds treated with pesticides (i.e. insecticides and fungicides) are frequently ingested by birds
11 (Lopez-Antia et al. 2016). As the ingestion of treated seeds results in the ingestion of large amounts of
12 pesticides in a short time (Prosser & Hart 2005), depending on the dose and the time of the exposure,
13 these may compromise survival or reproduction and therefore affect population dynamics (Mineau &
14 Whiteside 2013).

15 Pesticide effects on the endocrine system of non-target organisms are a growing concern. Many
16 fungicides used nowadays for seed treatment are proven or suspected to be endocrine disruptors
17 (reviewed in McKinlay et al. 2008; Maqbool et al. 2016). Triazoles are broad spectrum and systemic
18 fungicides used for plant, animal and human protection against fungal infection. Triazoles belong to the
19 Azole family, the second largest fungicide category according to the global market value (Lv et al. 2017).
20 Despite a widespread and massive use of these compounds, their toxicological and biological
21 consequences for non-target organisms such as wild birds remain largely unknown.

22 The mode of action of azole fungicides is the inhibition of one of the enzymes of the cytochrome
23 P450 (CYP) complex, the lanosterol-14 α - demethylase (CYP 51), which is essential for the production of
24 ergosterol, a basic component of the fungal cell membrane (Yoshida 1987). In animals, CYP 51 is

important for cholesterol synthesis and for steroid biosynthesis (Zarn et al. 2003; Goetz et al. 2007). In mammals, another target enzyme inhibited by some azole compounds, including many of those used in agriculture, is the enzyme aromatase (CYP 19), that converts androgens to estrogens (Middleton et al. 1986; Sanderson et al. 2002; Zarn et al. 2003). In birds, the inhibition of the aromatase enzyme by azole compounds was demonstrated by using homology models (Saxena et al. 2015). In general, the binding of azole compounds with their target enzyme is fairly unspecific, and so these compounds modulate the expression and enzyme activity of many cytochrome P450 proteins that regulate important metabolic functions (Ronis et al. 1994; Vinggaard et al. 2002; Taxvig et al. 2007; Goetz et al. 2007; Chambers et al. 2014; Lv et al. 2017). In mammals, agricultural azoles were often shown to disrupt the balance between androgens and estrogens, reduce fertility, alter sexual organ development or modify sexual behavior (Middleton et al. 1986; Zarn et al. 2003; Taxvig et al. 2007; Goetz et al. 2007; Lv et al. 2017), but studies on birds remain scarce (Ronis et al. 1994; Johnston et al. 1994; Matsushita et al. 2006; Grote et al. 2008; Lopez-Antia et al. 2013; Saxena et al. 2015).

Flutriafol is a systemic fungicide belonging to the triazole family, first mentioned for agricultural use in 1992 (Desprezloustau et al. 1992). This fungicide is widely used as cereal seed treatment and has been recently found in the crop and gizzard contents of hunted red-legged partridges (*Alectoris rufa*), demonstrating exposure in this declining farmland bird (Lopez-Antia et al. 2016). In a radiotelemetry study on grey partridges (*Perdix perdix*) combined with farmers' survey, a potential exposure to flutriafol was also pointed out as a concern (Millot et al. 2015). Despite the current extensive use of flutriafol for seed and foliar treatments, there is, to the best of our knowledge, only one peer reviewed publication that investigated toxicological effects of this compound in vertebrates: it reported an influence on estrogenic activity in human cells (Hurst and Sheahan 2003).

In December 2008, after the evaluation of the Draft Assessment Report (DAR 2006), the European Commission decided to withdraw the authorization of Plant Protection Products (PPPs)

containing flutriafol (Decision 2008/934/EC). The applicant industry made a resubmission application and sent further data in response to the issues identified in the DAR. After a new peer review focused on some issues of concern (EFSA 2010), flutriafol was included again in the list of approved substances, although a high long-term risk to insectivorous birds was identified (Regulation EU/540/2011). Thus, measures to mitigate such risk must be included in product authorizations issued by member states. The authorization of flutriafol at the EU level (EFSA, 2010) was based on a risk assessment for foliar application only, as proposed by the applicant. Consequently, the risk of the active ingredient for granivorous birds was not evaluated, while the authorization of PPPs containing flutriafol for cereal seed treatment remains as an exclusive responsibility of each member state (e.g. MAPAMA 2018 for Spain). We report here on an experiment that aims at filling this crucial knowledge gap and estimates the risks for wild seed-eating birds exposed to flutriafol. In order to understand the toxicity mechanisms of this compound and the risks for exposed wild birds, we designed an experiment considering an environmentally realistic exposure scenario and studied the effects of flutriafol-treated seed ingestion on body condition, biochemical, physiological and reproductive parameters of red-legged partridge.

Material and Methods

Experimental design

We conducted the experiment in the Dehesa de Galiana animal facilities (Ciudad Real, Spain). All the procedures were approved by the Universidad de Castilla-La Mancha's Committee on Ethics and Animal Experimentation (ref. 0909.01). We used 87 (36 pairs and 15 extra males) captive-born, one-year-old red-legged partridges, which were sexed genetically, following Fridolfsson and Ellegren (1999). One month before the experiment started (acclimatization period), partridges were placed in pairs, or individually in the case of the 15 extra males, in outdoor cages (95 x 40 x 42 cm) with *ad libitum* access

to tap water and commercial partridge feed (Partridge maintenance fodder, Nanta-Nutreco, Tres Cantos, Spain) mixed with wheat.

Pairs were randomly assigned to one of three treatments (control, low dose or high dose; 11, 12 and 13 pairs respectively). The extra males were assigned to the control (12 individuals) and the low dose (3 individuals) groups. Exposure began on January 19, 2012 and finished on February 13, 2012. This timing coincides with the late winter cereal sowing in central Spain, i.e. when potential exposure to treated seeds is closest to the onset of the breeding season (Casas et al. 2009). During this period, partridges were fed *ad libitum* with either treated wheat (low and high pesticide exposure groups) or with untreated wheat (control group). The duration of the experimental exposure (25 days) was adjusted to the maximum time unburied cereal seeds remain on the field surface (estimated at c. 21 days by Lopez-Antia et al. 2016). After this exposure period, partridges returned to a diet of untreated wheat and maintenance fodder. On February 14, we took 1 mL of blood (drawn by jugular venipuncture) from each partridge. Blood samples were kept refrigerated in heparinized tubes. We measured the hematocrit in a 75 μ L aliquot and centrifuged the remaining at $10,000 \times g$ for 10 min at 4 °C in order to separate and store at -80 °C the plasma from the cellular fraction (pellet). Plasma was used to monitor general biochemistry, vitamins and carotenoids and the cellular fraction was used to quantify several antioxidant and oxidative stress indicators (see below). We kept partridge pairs in their cages until the end of June in order to monitor their reproduction (see below).

Seed treatment and flutriafol exposure

We treated seeds with the commercial product Vincit Minima (flutriafol 2,5 % w/v, Cheminova Agro S.A.) to obtain a theoretical concentration of 0.0125 mg/g in the low dose group (20% of the Recommended Application Rate (RAR)) and 0.0625 mg/g in the high dose group (RAR). The low dose was equivalent to a diet containing 20% of coated seeds (i.e. similar to the cereal seed ingestion rate by wild

red-legged partridges in autumn; Perez and Perez (1981)). The high dose simulated the worst possible case in which partridge feed only on coated seeds during sowing. We used a hand sprayer (Apollo 5, EXEL gsa) to apply the flutriafol to wheat, and checked the flutriafol concentrations in the seeds by analyzing six replicate samples from the high dose treatment with LC-MS (see Lopez-Antia et al. 2013, 2016 for more details). The coefficient of variation (CV) for this analytical technique was 2.8%. We measured food consumption using the procedure described in Lopez-Antia et al. (2015b) with minor modifications (a detailed description is given in supplementary material). Exposure doses (in mg / kg body weight / day) during the experiment were estimated based on measures of flutriafol concentration in treated seeds, of food consumption during the exposure period (amount of seeds consumed by each partridge or couple), and of birds' body weight at the beginning of the experiment.

Survival and body condition of adult partridges

We checked partridges' survivorship daily during the experiment. We measured tarsus length and weighed each partridge before and after exposure and calculated a body condition index (scaled mass index (SMI); Peig and Green 2009) to investigate treatment effects on bird condition.

Immune response of adult partridges

We estimated the cell-mediated immune responsiveness of control and exposed birds using the phytohemagglutinin (PHA) skin test (see Mougeot et al. 2009 for details on the method). We performed the PHA test on February 15, two days after the end of the exposure period.

Reproduction

At the beginning of April (about one to two weeks before the expected beginning of the egg laying) the feed was switched to a fodder specific for reproduction (Partridge laying fodder, Nanta-

Nutreco, Tres Cantos, Spain) mixed with wheat. The first egg laying occurred on April 5. We collected eggs daily, and weighed and measured (maximum length and width) each before storing at 15 °C to temporarily prevent development and later incubate them. We withdrew the eggs laid in 4th, 8th, 12th and 16th position of the laying sequence of each pair and kept them at -80 °C in order to later analyze the vitamin and carotenoid contents in their yolk. The percentage of eggs that were laid and withdrawn did not differ between experimental groups ($F_{2,25}=2.88$, $p=0.075$). Daily egg collections continued after the experimental exposure until the end of the laying season (June 26). Throughout the study, we did a total of five consecutive incubation batches. Upon hatching, each chick was individually marked, weighed and measured (tarsus length). We recorded hatching date and measured again tarsus length and body mass at 8, 16, 24 and 32 days of age. We calculated chick body condition as for adults. We also conducted the PHA test on some chicks from the third and fourth incubation sets, excluding the first chick of each pair. The PHA test was performed as described for adults on selected chicks when they were 8 days old. Chicks were genetically sexed following Fridolfsson and Ellegren (1999). We examined unhatched eggs to determine fertility (based on the presence of an embryo or germinal disk) and measured Eggshell thickness (see Lopez-Antia et al. (2013) for details). A more detailed description of all these procedures is also provided as supplementary material.

Physiological parameters measured in adult partridges

We used red blood cell (RBC) homogenates to determine levels of antioxidants and oxidative stress indicators following the methodology described in Lopez-Antia et al. (2013; see also supplementary material). We specifically measured total (GSH) and oxidized glutathione (GSSG) levels, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity (relative to protein levels (per mg) and malondialdehyde (MDA) levels as an indicator of lipid peroxidation (Romero-Haro and Alonso-Alvarez 2014).

We determined plasma levels of retinol (vitamin A), α -tocopherol (vitamin E) and carotenoids (zeaxanthin and lutein) as described in Rodriguez-Estival et al. (2010). We extracted antioxidants from the egg yolk (Lopez-Antia et al. 2015a) and used the same analytical method as for plasma antioxidants. We used an A25 analyzer and reaction kits (BioSystems, Barcelona, Spain) to determine plasma biochemistry levels, specifically: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LHD), creatine phosphokinase (CPK), albumin, total protein, glucose, cholesterol, triglycerides, calcium, magnesium, phosphorus, creatinine, urea and uric acid.

Color measurements of adult partridges

When the exposure period ended, on February 14, we measured the red coloration of the beak and eye rings of partridges using a portable spectrophotometer (Minolta CM-2600 d; see Lopez-Antia et al. 2013 and supplementary material). Beaks and eye rings are pigmented by carotenoids, and that coloration is an indicator of health (Mougeot et al. 2009) and investment in reproduction (Alonso-Alvarez et al. 2012). We also quantified the percentage of eye-ring area pigmented by carotenoid (% eye-ring pigmented; Mougeot et al. 2009).

Statistical analyses

Considering the elevated number of physiological responses retrieved from partridges, and assuming that collinearity could exist among some of these variables, we used Principal Component Analyses (PCAs) to get information on associations between variables and perform analyzes on a reduced number of independent variables. Because of the limited volume of plasma collected from partridges, not all biochemical parameters could be measured in all individuals. In order to minimize the incidence of excluding individuals from the PCA because of a missing value, we run a first PCA including variables

for which we had data from the majority of individuals, namely body condition, hematocrit, wing web swelling, oxidative stress biomarkers, vitamins, carotenoids and ornamentation. A second PCA was run for plasma biochemistry variables including only the 45 individuals with a complete biochemical profile. Both PCAs revealed a low degree of association among variables (Table S1), so we kept original physiological variables for further analyses.

Differences in body condition between experimental groups throughout the experiment were analyzed using a General Linear Model (GLM) that included the initial and final body condition of each individual as repeated measure. We used a one-way ANOVA to test for differences in food consumption between experimental groups. We used GLM with experimental group and sex as fixed factors to test for flutriafol effects on all adult partridge traits (removing the sex $\times$ experimental group interaction from models when non-significant). We tested relationships between physiological variables and body condition, or between color measurements and carotenoid plasma levels using Pearson's bivariate correlations. When a variable was significantly correlated ($p < 0.05$) with body condition, we added body condition (SMI) as a covariate in the GLM to investigate if the treatment effect could be mediated by this condition. To test for flutriafol effects on egg and chick parameters, we performed mixed models that included the experimental group as fixed factor and the breeding pair as a random effect (to account for the non-independence of chicks from the same parents). For egg measurements, initial models also included the order of the egg in the laying sequence as a covariate and the interactions "fertilized" factor (yes or no) $\times$ experimental group and incubation set $\times$ experimental group. Interactions that were not significant were sequentially removed from models. We included chick condition upon hatching as a covariate in the analysis of response to PHA.

We used Generalized Linear Models (GLMz) with a quasi-Poisson distribution and the breeding pair as experimental unit (1 data per pair) to test for treatment effects on clutch size, brood size, days to the first laid egg, fertile egg rate, hatching rate, productivity rate, chick mortality rate and brood sex

ratio. For the rates we also performed GLMz with binary logistic distributions (number of events occurring in a set of trials) to consider overall values per treatment.

The significance threshold level was set at $p=0.05$ (p -values below 0.10 were considered as marginally significant). When significant or marginally significant treatment effect was found, we checked which dose caused the effects on marginal means using Tukey tests. Model residuals were checked for normality using Kolmogorov-Smirnov tests. To obtain a normal distribution of the model residuals, we square-root transformed the dependent variable “percentage of pigmented eye-ring area” and we log-transformed the following dependent variables: adult cellular immune response (wing web swelling), total protein, albumin, ALT, AST, LDH, calcium, phosphorus, cholesterol, triglycerides, CKP, uric acid, GPX, SOD, GSH, MDA, plasma lutein, plasma zeaxanthin, plasma α -tocopherol, plasma retinol, eye- and beak-chroma, egg α -tocopherol, egg lutein, egg zeaxanthin and chick cellular immune response.

Results

Food ingestion and treatment effects on adult partridges.

No partridge died during the exposure period, but one partridge from the control group, one from the low dose group and two from the high dose group died during the blood sampling procedure. There were no significant differences in the amount of food consumed between experimental groups (Table 1). Each partridge had a mean total consumption of 629.0 ± 17.7 g of wheat (25.2 g/partridge/day).

Flutriafol concentration in the high dose seeds averaged ($\pm$ SD) 44.33 ± 12.6 $\mu\text{g/g}$, corresponding to 70.8% of the theoretical dose. Assuming a similar ratio between the theoretical and real doses for the seeds treated at the low dose than for those treated at the high dose, mean flutriafol concentration in the low dose was estimated at 8.866 $\mu\text{g/g}$. Considering these average flutriafol concentrations per treatment, the amount of seeds eaten by each individual during exposure, and the body weight of each

partridge at the beginning of the experiment, the mean ($\pm$ SD) estimated daily ingestion of flutriafol per bird was estimated at 2.403 ± 0.523 mg/kg bw/day in the high dose treatment and 0.492 ± 0.116 mg/kg bw/day in the low dose treatment.

Body condition, hematocrit or immune response to PHA did not differ between experimental groups after the exposure period (Table 1). All plasma biochemical parameters are summarized in Table 1. Cholesterol level in plasma was reduced by the treatment but only in males ($F_{2,33}=4.54$, $p=0.018$). This reduction was significant in the low dose group (Figure 1). Triglyceride plasma levels were also reduced by the treatment ($F_{2,64}=4.28$, $p=0.018$) in both sexes and for both doses (Figure 1). Body condition was not correlated with any of these parameters. There were no differences between treatment groups in any other blood plasma parameter (Table 1).

Levels of oxidative stress parameters and antioxidant molecules in control and exposed partridges are summarized in Table S2. We found no significant differences between groups in any of these parameters. Plasma lutein levels were significantly lower in females than in males ($F_{1,82}=7.77$, $p=0.007$), irrespective of the treatment, and with no differences between experimental groups. Exposed partridges presented a reduced percentage of eye-ring pigmentation ($F_{2,81}=4.98$, $p<0.001$; Figure 2) compared with controls. Eye ring pigmentation was overall lower in females than in males ($F_{1,81}=11.74$, $p=0.001$), particularly in exposed birds, but the treatment effect was similar in both sexes (non-significant sex x treatment interaction). Post-hoc tests revealed that differences were only significant for the high dose group (post-hoc $p=0.007$). The percentage of eye-ring pigmentation was positively correlated with lutein ($t_{1,82}=2.66$, $p<0.01$) and zeaxanthin ($t_{1,82}=2.12$, $p<0.04$) plasma levels. The other color measurements (eye and beak hue or chroma) did not differ between treatment groups (Table S3).

Treatment effects on reproduction and indirect effects on offspring.

All the reproductive parameters of partridges are summarized in Table 2. Treatment had no effect on the number of laying females, but significantly affected clutch size: exposed females laid fewer

eggs than control females (Wald $\chi^2=6.06$, $df=2$, $p=0.048$; Table 2; Figure 3). Post-hoc tests revealed that differences were only significant for the high dose group (post-hoc $p=0.017$).

Regarding egg characteristics, there were no differences between experimental groups in their size, weight, shell thickness or antioxidant content (Table 2).

We found a marginally significant effect of flutriafol treatment on the ratio of fertile to total eggs (Figure 3). When considering overall values per treatment, the differences between groups were significant (Wald $\chi^2=6.85$, $df=2$, $p=0.03$; Table 2), with a reduced percentage of fertile eggs in the low dose group than in the control group (post-hoc $p=0.012$). This effect, together with the reduced clutch size, resulted in a 56-62% reduction in the brood size of exposed pairs (Wald $\chi^2=7.22$, $df=2$, $p=0.027$; Table 2; Figure 3), a difference that was significant at both exposure levels (Table 2). The hatching rate, brood sex ratio and chick quality indicators (condition at birth, growth, response to PHA, mortality) did not differ between experimental groups although a dose-dependent trend could be observed for sex-ratio (broods of exposed pairs tended to be more female-biased) and chick response to PHA (offspring from exposed pairs tended to have reduced responses).

4. Discussion

Adult partridges exposed to flutriafol through the consumption of treated seeds experienced physiological effects such as reduced levels of cholesterol and triglycerides, phenotypical effects such as a reduction in their eye ring pigmentation by carotenoids; and most importantly, severe adverse effects on reproduction: a reduced clutch size and fertile egg ratio, and an overall offspring production reduced by more than 50%.

With the collected data on food consumption, bird body weight and levels of pesticide in the seeds, we estimated a daily consumption of flutriafol of 2.403 and 0.492 mg/kg bw/day in the high and low dose groups, respectively. These levels are more than ten times lower than the “No Observed

Adverse Effect Level" (NOAEL; 35.8 mg/kg bw/day) based on reproductive parameters that the applicant industry calculated for another galliform bird (the bobwhite quail) during the product registration process (Draft Assessment Report of the EFSA on Flutriafol (DAR 2006)). The differences between the DAR and our study could be due to species-specific differences in sensitivity to the fungicide or a non-monotonic dose response to flutriafol. These non-linear dose relationships have been frequently described for endocrine disrupting chemicals (Welshons et al. 2003; Lagarde et al. 2015), with noticeable effects at NOAEL values, based on growth or reproduction performance parameters, in studies conducted for pesticide risk assessment.

Effects on adult partridges

We found reduced levels of cholesterol and triglycerides in exposed partridges. Previous studies on rats also detected an effect of epoxiconazole (a triazole pesticide) on blood cholesterol levels (Heise et al. 2015; Rieke et al. 2017), although in these cases cholesterol levels increased in exposed individuals. Another triazole pesticide, difenoconazole, was also found to increase levels of triglycerides in zebrafish (Mu et al. 2016). As food consumption did not differ between experimental groups in our experiment, we interpret that the observed effects on cholesterol and triglyceride plasma levels were a direct effect of flutriafol. The aforementioned effect of triazole pesticides, through the inhibition of the lanosterol-14 α - demethylase, on the metabolic pathway that leads to the biosynthesis of cholesterol (Zarn et al. 2003) may have knock-on effects on reproduction, because cholesterol is the substrate for the production of many other sterols, including sex steroid hormones. Moreover, some cholesterol precursors have been shown to modulate the development of male and female germ cells in mammals (Rozman et al. 2002, 2005).

Partridges fed seeds treated with the RAR for flutriafol presented a reduced percentage of eye ring area pigmented by carotenoids compared with controls. In red-legged partridges, this coloration

reflects the condition and health status of birds (Mougeot et al. 2009; Perez-Rodriguez et al. 2013) and is an important sexual signal that influences mate choice and reproductive investment (Alonso-Alvarez et al. 2012). Exogenous carotenoids (lutein and zeaxanthin) are the precursors for this red coloration displayed by partridges (Garcia-de Blas et al. 2014). These pigments must be acquired, mobilized and transported to the integuments, such as the eye-ring area, in order to produce the red colored signal. Previous works showed that lipoproteins bind to carotenoids allowing their transport to and deposition into integuments (McGraw and Parker 2006) so the expression of carotenoid-based ornaments depends on circulating cholesterol levels. We found that pesticide exposure reduced both circulating cholesterol levels and eye-ring pigmentation, suggesting a possible disruption of carotenoid transport and allocation to the eye ring area. In our experiment, plasma levels of these carotenoids were positively correlated with the percentage of eye ring pigmented (Pearson's correlation with transformed variables: lutein: $R=0.332$, $p=0.002$; zeaxanthin: $R=0.291$, $p=0.007$; $N=84$). Lutein and zeaxanthin are involved in several important physiological functions (i.e. oxidative stress protection and immune response; Pérez-Rodriguez et al. 2013). Pesticide exposed partridges may therefore have allocated more of their available pigments to compensate potential flutriafol pro-oxidant effects, to the detriment of using these to increase or maintain their coloration. We did not find strong statistical evidence for a pro-oxidant effect of flutriafol, although exposed partridges tended to have higher levels of lipid peroxidation (MDA) than controls (Table S2). An additional explanation for the reduced coloration of exposed partridges is the alteration of steroid hormone levels. Sexual hormones, especially testosterone, are known to modulate carotenoid allocation to ornaments (Mougeot et al. 2007; Martinez -Padilla et al. 2010; Perez-Rodriguez et al. 2013). Consistent with this potential mechanism, partridges fed with seeds treated with another triazole, difenoconazole, showed a reduced eye ring pigmentation and a concomitant reduction (although not significant) of their plasmatic sexual hormone levels (Lopez-Antia et al. 2013).

Effects on reproduction.

Exposed partridges laid fewer eggs than controls. Moreover, a greater proportion of these, compared with controls, were unfertile. This led to an overall breeding production reduced by 56-62% in exposed birds, even in the partridges fed with seeds treated with 20% of the RAR for seed treatment. Although the sample size is limited to 8-11 laying pairs per treatment group, such a large effect is a cause for concern.

The only data available on the effects of flutriafol on birds are those from the Draft Assessment Report of the EFSA on Flutriafol (DAR 2006), where a reproductive toxicity test was performed in bobwhite quail (*Colinus virginianus*; Arch 2005). Following OECD 206 guideline, birds were fed with food treated with 100, 300 and 1000 ppm for 22 weeks. As in our study, they found a reduction in the number of eggs laid, but only in the 100 and the 1000 ppm dietary concentrations (equivalent to 11.9 and 119 mg/Kg bw/day, considering the estimates on mean body weight and daily food intake). Due to a lack of effect in the 300 ppm dietary concentration, the effect in the 100 ppm level was dismissed and was considered to be unrelated to treatment. That study also found a reduced survival of hatchlings (considering the ones that hatched) in the three exposed groups, although this effect disappeared in the two lower dose groups when survival numbers were considered in relation to the clutch size or the female. In light with these results, the "No Observed Effect Concentration" (NOEC) estimated for bobwhite quail was in the 300 ppm dietary concentration (equivalent to 35.8 mg/Kg bw /day). In our study, we found similar effects on clutch size using much lower doses (12.5 and 62.5 ppm; 2.4 and 0.48 mg/Kg bw/day) and over a shorter exposure period. As mentioned above, these differences could be explained by species-specific sensitivities and/or to a non-monotonic dose response to flutriafol. Estrogenic activity of flutriafol has been previously reported (Hurts and Sheahan 2003), supporting the hypothesis that, like other endocrine disruptors (Welshons et al. 2003; Lagarde et al. 2015), flutriafol has

a non-monotonic dose response. This would also explain our results relative to overall fertility rate per treatment, where an effect was detected in the low dose but not in the high dose group.

There are a few studies on the effects of azole fungicides in mammals (Middleton et al. 1986; Vinggaard et al. 2002, 2005; Taxvig et al. 2007; Goetz et al. 2007), and even fewer in birds. Grote et al. (2008) found that epoxiconazole (a triazole fungicide), administered to Japanese quail (*Coturnix coturnix japonica*) at doses equivalent to 1.2, 6.6 and 68.5 mg/Kg bw/day during three weeks resulted in a clear impact on the testis at the two higher doses, although changes in hormone levels, fertility or laying rate were not detected. Lopez-Antia et al (2013) found that the triazole fungicide difenoconazole reduced the fertile egg rate of red-legged partridges when they were fed with seeds treated with the RAR and twice this RAR for seed treatment (equivalent to 3.2 and 6.0 mg/Kg bw/day) for 10 days. Although the effects were not significant, an alteration of sexual hormone levels was also apparent in difenoconazole exposed partridges (lower levels of testosterone in males and of estradiol in females (Lopez-Antia et al 2013)).

Azole compounds are known to inhibit the aromatase and lanosterol-14 α - demethylase (CYP 51; a target enzyme for azole fungicides), and to modulate the activity of many cytochrome P450 isoenzymes. Thus, the consequences of the exposure to these chemicals are difficult to predict. This complex pattern of induction, inhibition and suppression of CYP isoenzymes by azole compounds was previously reported in birds by Ronis et al. (1994).

Risk assessment

The registration of flutriafol as an active substance at the EU level did not include any assessment of its use as a seed treatment product, and therefore no data were available for the EU authorities relative to the risk assessment of this specific use. Assessments, however, should have been conducted in member states like Spain, where PPPs for seed treatment containing flutriafol are

approved (MAPAMA 2018). According to the guidance for pesticide risk assessment for birds and mammals in the EU (EFSA 2009), a risk from long-term exposures is estimated by dividing the NOAEL, as toxicity indicator, between the estimated daily dietary dose (DDD). This results in a toxicity to exposure ratio (TER), which must be >5 in order to consider negligible the risk from long-term exposure. Our results show that the NOAEL based on reproductive parameters should be lower than the estimated dose corresponding to the low exposure group (i.e. $\text{NOAEL} < 0.484 \text{ mg/kg b.w./day}$). This is below the DDD that would be considered in the first tier of the risk assessment, in which the realistic worst-case assumption of a diet containing 100% of freshly treated seeds is assumed. Such scenario would result in an DDD estimated directly from the application rate (i.e. $3.47 \text{ mg/kg b.w./day}$ with the intake rate and body weight of the animals used in the present study), and hence in a $\text{TER} < 5$. Therefore, the exposure estimates must be refined as part of a high tier assessment to obtain a more realistic DDD to be used in the calculation of a new TER.

Among the options provided in the guidance for pesticide risk assessment for birds and mammals in the EU (EFSA 2009), the most relevant refinement options would be to correct DDD based on i) the percentage seeds in the diet or ii) the availability of treated seeds in the field. Concerning percentage seeds in the diet, in a previous study, Lopez-Antia et al. (2016), analyzed the digestive contents of red-legged partridges hunted during the cereal sowing season (October to February), and found that cereal seeds accounted for 53.4% of the ingested biomass (reaching a maximum of 89.3% in some regions). Considering the nominal flutriafol application rate in cereal seeds (0.0625 mg/g), and the average values per individual of daily food intake (25.2 g) and body mass (459.3 g) from our study, a diet containing 53.4% or 89.3% of flutriafol treated seeds (i.e. $13.46\text{--}22.50 \text{ g seeds/day}$) would result in a DDD of 1.83 or $3.06 \text{ mg/kg b.w./day}$, respectively. Even if we compare the lowest DDD with the NOAEL resulting from our study ($< 0.484 \text{ mg/kg b.w./day}$) the TER would still remain far below 5, thus pointing to unacceptable risks of long-term exposure.

Regarding seed availability in the field, Lopez-Antia et al. (2016) estimated cereal treated seeds amounts in recently sown field to be between 0.6 and 2 g/m². This means that partridges could get their average daily intake of cereal seeds (13.46 g) in a maximum field surface area as low as 22.4 m². Lopez-Antia et al. (2016) also reported that treated seeds could remain up to 25 days in the surface of a given field. As not all fields in an area are sown at the same time, the chances for a partridge to exploit treated seeds as a food resource during 25 consecutive days are high. In summary, none of the most relevant exposure refinement approaches seems to lead to a reduction of long-term risk from flutriafol treated seeds large enough to make such risk acceptable for the red-legged partridge.

Conclusion

This experiment demonstrated that under realistic environmental conditions the ingestion of flutriafol treated seeds has important negative consequences on partridges' reproduction, with more than 50% reduction in the offspring. Whether these effects are attributable to endocrine disrupting effects of flutriafol or not remains to be demonstrated. The EU legislation for PPPs (Regulation No 1107/2009) establishes that "*active substances which are endocrine disruptors shall not be approved, unless there is negligible exposure in which case they may be approved under restricted conditions*". The question remains in determining the criteria by which PPPs are considered endocrine disruptors. The EU agencies EFSA and ECHA (European Chemical Agency) are currently working on a guidance document to identify endocrine disruptors. The implementation of that guidance could serve to determine, with the currently available data, whether flutriafol and other azole fungicides should be included within this category or not. The results of the present study, together with previous data on field exposure levels in wild partridges, provide evidence that the use of flutriafol, regardless of its potential endocrine disrupting activity, as cereal seed treatment represents a significant risk for granivorous bird populations.

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Figure 1. Mean ($\pm$ SE) plasma levels of cholesterol and triglycerides in the different experimental groups after the exposure period. Cholesterol levels are given for males and females separately. Sample sizes in each experimental group ranged between 20 and 26 partridges. Different letters indicate significant differences between treatment groups, cholesterol level in plasma was reduced significantly only in males

Figure 2. Mean ($\pm$ SE) eye-ring pigmentation (% of eye-ring area pigmented with carotenoids) according to treatment and sex. Different letters indicate differences between treatment groups irrespective of differences between sexes.

Table 1. Mean ($\pm$ SE) body condition, food haematocrit, cellular immune response (PHA, wing web swelling) and plasma biochemical parameters of adult red-legged partridges after the exposure period and according to treatment. Different letters indicate treatment doses significantly different at the $p < 0.05$ level according to Tukey test results (in the absence of letters, differences were not significant).

Parameter	Control	Low dose	High dose
Initial N	34	27	26
Mortality (%)	1	1	2
Body condition before	453 $\pm$ 5.8	456 $\pm$ 5.2	457 $\pm$ 6.8
Body condition after	452 $\pm$ 6.4	455 $\pm$ 5.4	454 $\pm$ 7.6
Food consumption/partridge	634.7 $\pm$ 36.17	632.7 $\pm$ 26.04	617.8 $\pm$ 24.56
Haematocrit (%)	38.9 $\pm$ 0.8	38.4 $\pm$ 0.9	39.3 $\pm$ 0.7
PHA-Wing web swelling (μ m)	739 $\pm$ 044	747 $\pm$ 50	721 $\pm$ 50
Albumin (g/L)	17.8 $\pm$ 0.4	16.8 $\pm$ 0.4	17.7 $\pm$ 0.6
Alanine aminotransferase (U/L)	16.1 $\pm$ 4.4	14.8 $\pm$ 2.8	8.2 $\pm$ 2.1
Aspartate aminotransferase (U/L)	230 $\pm$ 14	220 $\pm$ 9	199 $\pm$ 14
Calcium (mg/dL)	10.8 $\pm$ 0.5	10.3 $\pm$ 0.2	10.4 $\pm$ 0.2
Cholesterol (mg/dL)	269 $\pm$ 20	257 $\pm$ 16	239 $\pm$ 13
Creatine phosphokinase (U/L)	539 $\pm$ 69	671 $\pm$ 61	559 $\pm$ 70
Creatinin (mg/dL)	0.483 $\pm$ 0.05	0.427 $\pm$ 0.035	0.452 $\pm$ 0.03
Glucose (mg/dL)	391 $\pm$ 12	415 $\pm$ 9	405 $\pm$ 10
Lactate dehydrogenase (U/L)	640 $\pm$ 49	527 $\pm$ 50	527 $\pm$ 51
Phosphorus (mg/dL)	5.58 $\pm$ 0.41	5.42 $\pm$ 0.34	6.70 $\pm$ 1.04
Total protein (g/L)	45.7 $\pm$ 0.9	44.2 $\pm$ 0.8	44.6 $\pm$ 1.0
Triglycerides (mg/dL)	351 $\pm$ 17 A	290 $\pm$ 19 B	280 $\pm$ 19 B
Uric Acid (mg/dL)	3.88 $\pm$ 0.34	3.42 $\pm$ 0.22	3.30 $\pm$ 0.31

Table 2. Reproductive and immune response parameters (n, % or mean $\pm$ S.E.) of partridge pairs and chicks from the different experimental groups. Different letters indicate treatment doses significantly different at the $p < 0.05$ level according to Tukey test results (in the absence of letters, differences were not significant).

Parameter	Control	Low dose	High dose
Number of pairs	9	11	11
Number of laying females	8	11	9
Total number of eggs	110	75	58
Clutch size per laying female	12.2 $\pm$ 1.9 A	6.8 $\pm$ 2.1 AB	5.3 $\pm$ 1.6 B
Days to the first egg	24.9 $\pm$ 2.6	29.5 $\pm$ 5.8	32.8 $\pm$ 2.8
Egg mass (g)	17.0 $\pm$ 0.1	18.2 $\pm$ 0.2	17.4 $\pm$ 0.2
Egg length (mm)	38.1 $\pm$ 1.7	39.4 $\pm$ 0.2	38.6 $\pm$ 0.3
Egg width (mm)	28.8 $\pm$ 0.1	29.4 $\pm$ 0.1	28.9 $\pm$ 0.1
Shell thickness (μ m)	227 $\pm$ 4.1	215 $\pm$ 4.8	213 $\pm$ 6.1
Retinol in yolk (nmol/g)	510 $\pm$ 17	501 $\pm$ 22	543 $\pm$ 29
Tocopherol in yolk (nmol/g)	1159 $\pm$ 81	1317 $\pm$ 115	1226 $\pm$ 75
Lutein in yolk (nmol/g)	57.3 $\pm$ 10.3	66.9 $\pm$ 15.5	39.9 $\pm$ 5.3
Zeaxanthin in yolk (nmol/g)	39.9 $\pm$ 5.6	49.9 $\pm$ 10.0	32.3 $\pm$ 4.2
Fertile eggs (%)	Average/pair		
	85.7 $\pm$ 9.2	70.2 $\pm$ 12.4	63.0 $\pm$ 13.6
	Overall/treatment ²		
	79 $\pm$ 4.0 A	59 $\pm$ 6.4 B	76 $\pm$ 6.0 AB
Hatching rate (%) ¹	Average/pair		
	81 $\pm$ 5.4	76 $\pm$ 11.3	76 $\pm$ 13.5
	Overall/treatment ²		
	81 $\pm$ 4.8	83 $\pm$ 6.4	78 $\pm$ 7.3
Number of chicks	54	29	25
Brood size ³	6.0 $\pm$ 0.9 A	2.6 $\pm$ 0.9 B	2.3 $\pm$ 0.8 B
Chick body condition at birth	11.7 $\pm$ 0.2	12.2 $\pm$ 0.2	11.9 $\pm$ 0.4

Chick mortality rate (%)	Average/pair	34.4 ± 8.0	32.7 ± 11.3	38.7 ± 16.5
	Overall/treatment ²	32 ± 6.4	34 ± 8.8	16 ± 7.3
Female ratio (%)	Average/pair	58 ± 7.2	63 ± 9.2	77 ± 8.1
	Overall/treatment ²	54 ± 6.4	57 ± 9.0	64 ± 9.1
Wing web swelling (μm)		103 ± 18.5	88.2 ± 21.7	64.0 ± 12.0

¹Hatching rate of fertile eggs, ²Standard errors derived from marginal means of binary logistic models. ³

Some eggs (0-4 eggs/pair) were kept for analyzing and thus not incubated.

Fig. 3. Reproductive parameters (mean $\pm$ S.E) of partridges according to treatment group. Different letters indicate differences between experimental groups. For brood size, it should be considered that some eggs (0-4 eggs/pair) were withdrawn (a similar percentage in each group) for analyzing their content and thus not incubated.

Highlights

- Flutriafol-treated seed ingestion reduced levels of cholesterol and triglycerides.
- Flutriafol exposure reduced partridges' carotenoid-based coloration.
- Exposed females laid fewer eggs than control females.
- Exposed pairs tend to present a reduced fertile egg ratio.
- Overall offspring production was reduced by >50% in exposed pairs.