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Abstract

Drilled seeds are an important food resource for many farmland birds but may pose a serious risk when treated with pesticides. Most compounds currently used as seed treatment in the EU have low acute toxicity but may still affect birds in a sub-chronic or chronic way, especially considering that the sowing season lasts several weeks or months, resulting in a long exposure period for birds. Tebuconazole is a triazole fungicide widely used in agriculture but its toxicity to birds remains largely unknown. Our aim was to test if a realistic scenario of exposure to tebuconazole treated seeds affected the survival and subsequent reproduction of a granivorous bird, the red-legged partridge (*Alectoris rufa*). We fed captive partridges with wheat seeds treated with 0%, 20% or 100% of tebuconazole application rate during 25 days in late winter (i.e. tebuconazole dietary doses were approximately 0.2 and 1.1 mg/kg bw/day). We studied treatment effects on the physiology (i.e. body weight, biochemistry, immunology, oxidative stress, coloration) and reproduction of partridges. Exposed birds did not reduce food consumption but presented reduced plasmatic concentrations of lipids (triglycerides at both exposure doses, cholesterol at high dose) and proteins (high dose). The coloration of the eye ring was also reduced in the low dose group. Exposure ended 60 days before the first egg was laid, but still affected reproductive output: hatching rate was reduced by 23% and brood size was 1.5 times smaller in the high dose group compared with controls. No significant reproductive effects were found in the low dose group. Our results point to the need to study the potential endocrine disruption mechanism of this fungicide with lagged effects on reproduction. Risk assessments for tebuconazole use as seed treatment should be revised in light of these reported effects on bird reproduction.

Keywords: triazole fungicide, risk assessment, hatching success, endocrine disruption, farmland birds.

Capsule: A worst-case scenario of exposure to tebuconazole treated seeds reduces reproductive success in a declining farmland bird.

1. Introduction.

Pesticide use in agriculture is recognized as a main threat to farmland bird populations (e.g. Geiger et al. 2010; Mineu and Whiteside, 2013). A common application practice is to treat sowing seeds with pesticides. This practice poses a high risk to farmland birds that feed on these sown seeds when not properly buried and when other food resources become scarce (Lopez-Antia et al. 2016; Robinson and Sutherland, 2002; Siriwardena et al. 2007). Seeds treated with some compounds that are acutely toxic to birds have indeed resulted in multiple poisoning incidents in the past, due to the use of organochlorines, organophosphates and carbamates (e.g. Flickinger and King, 1972; Lelievre et al. 2001; ; Stone and Gradoni, 1985;), and more recently due to the use of neonicotinoids (e.g. Berny et al. 1999; Millot et al. 2017, Mineau and Palmer 2013). Most of these compounds are no longer used as seed treatments in the European Union (EU), but other products are still widely used. The autumn/winter sowing season of cereals within a given region is normally extended, lasting several weeks or months, so the consumption of treated seeds can result in a scenario of chronic exposure to high amounts of pesticides. Under such scenarios, long-term effects on birds can occur even when acute pesticide toxicity is low (Lopez-Antia et al. 2013, 2015b, 2018). Assessing long-term risks of pesticides is however complex because of the difficulty in estimating toxicity and exposure in an ecologically realistic way and extrapolating results to the population level (Bennet et al. 2005; Luttik et al. 2005; Mineau et al. 2005; Shore et al. 2005; Topping et al. 2020).

Cereal seeds (i.e. barley, oat, rye, triticale, wheat) used during autumn/winter sowing are often treated with fungicides, many of which are suspected to disrupt the endocrine system (Lv et al. 2017; McKinley et al. 2008; Warner et al. 2020). Triazole fungicides are systemic, broad-spectrum compounds frequently used as pesticides (FAOSTAT, 2020; Lv et al. 2017). The antifungal effect of these compounds is due to the inhibition of an enzyme of the cytochrome P450 (CYP) complex, the lanosterol-14a-demethylase (CYP 51), which is needed for the production of ergosterol, a basic component of the fungal cell membrane (Yoshida, 1987). CYP

51 is not exclusive to fungi; in animals, it is part of the metabolic pathway of biosynthesis of cholesterol, which, among other functions, is the precursor for the production of sex steroid hormones (Zarn et al. 2003). Moreover, the mechanism of action of triazole compounds is quite unspecific and they have the potential to regulate the expression and activity of multiple CYP proteins, important for metabolic functions such as xenobiotic metabolism and hormone synthesis and break down (Fernández-Vizcaíno et al. 2020; Taxvig et al. 2007; Walker, 1998; Yang et al. 2018; Zarn et al. 2003).

Tebuconazole is a triazole compound commonly used in agriculture (Kahle et al. 2008; Millot et al. 2015). It is highly persistent in soil and frequently found in water bodies (Berenzen et al. 2005; Kahle et al. 2008). Tebuconazole is often used as seed treatment, and seeds treated with this fungicide are frequently consumed by birds (Lopez-Antia et al. 2016). A study conducted in Spain during the cereal autumn sowing season showed that tebuconazole was the most commonly found pesticide both in sown seeds (30% of the analyzed seed samples) and in gizzards of hunted red-legged partridges (*Alectoris rufa*; 19% of the gizzards from 189 sampled birds contained tebuconazole residues; Lopez-Antia et al. 2016). Exposure to tebuconazole could also occur through the consumption of other food items after spraying, through the intake of contaminated water, or through the direct spraying of eggs (Bro et al. 2015; Millot et al. 2015).

Tebuconazole effects have been extensively studied in fish (Li et al. 2019, 2020; Macirella et al. 2019; Yu et al. 2013) and mammals (Lv et al. 2017; Roelofs et al. 2014; Taxvig et al. 2008; Trosken et al. 2004; Zarn et al. 2003). A main effect described in rodents is the disruption of steroid hormone levels (Lv et al. 2017; Roelofs et al. 2014; Trosken et al. 2004) with multiple consequences on reproduction, such as post implantation loss (Taxvig et al. 2008), increased gestational length, virilized females and feminized males (Taxvig et al. 2007) or reduced pup viability and body weight (Moser et al. 2001). In a recent study about effects on birds (Fernández-Vizcaíno et al. 2020), the exposure of red-legged partridges to seeds treated with tebuconazole (or other triazoles, i.e. flutriafol and prothioconazole) produced the overexpression of genes encoding for enzymes involved in the biosynthesis of sterols and steroid hormones, and reduced levels of plasmatic oestradiol.

In the EU, tebuconazole use as a fungicide was approved in September of 2009 (Commision Directive 2008/125/EC). A risk of tebuconazole on avian reproduction following exposure to tebuconazole treated seeds was identified as part of the process of registration of the active ingredient in the EU (EFSA 2014). However, such risk was associated to the ingestion of spring cereal seeds, whereas the consumption of cereal seeds sown during autumn or winter was deemed to have no impact on reproduction because of the long time from the ingestion of those seeds to breeding season. However, standard avian reproduction tests conducted as part of risk assessments might not be long enough to detect reproductive problems associated with the autumn or winter exposure to pesticide-treated seeds.

We conducted an experiment with the aim of finding out if a realistic scenario of exposure to tebuconazole treated seeds during winter sowing could affect the survival and reproduction of a granivorous bird. We recorded effects on the physiology (i.e. body weight, biochemistry, immunology, oxidative stress, coloration) and reproduction of the red-legged partridge, a medium-sized farmland bird well suited for the risk assessment of pesticides used for seed treatment (Lopez-Antia et al. 2016). Our aim was to better understand the toxicity mechanism of tebuconazole on birds, filling an important knowledge gap for the optimal assessment of its risk.

2. Material and methods.

Experimental design

We conducted the experiment at Dehesa de Galiana (Ciudad Real, Spain) under Ethics and Animal Experimentation (ref. 0909.01) permits granted by the Universidad de Castilla-La Mancha. Partridges (87 individuals of known sex) were housed in pairs (35 pairs), or individually (17 additional males were used) in outdoor cages (95 x 40 x 42 cm).

Each pair was randomly assigned to an experimental group (control, low dose or high dose; 11, 13 and 11 pairs respectively), and the additional males to the control (12 individuals) or high dose (5 individuals) group. Exposure was from January 19 to February 13, coinciding with the end of the winter cereal drilling period in central Spain, i.e. not long before partridges start breeding (Casas et al. 2009). Partridges were fed exclusively with treated wheat provided *ad libitum* (low and high pesticide exposure groups) or with untreated wheat also provided *ad libitum* (control group). The exposure duration (25 days) was decided considering sowing asynchrony among fields and the maximum permanence of seeds on the surface (i.e. more than 20 days for the complete disappearance; Lopez-Antia et al. 2016). After the exposure period, partridges returned to a diet of untreated wheat and maintenance fodder. The day after the exposure finished, 1 ml of blood was extracted from the jugular vein of each partridge. Hematocrit was measured in a 75 µl aliquot and the remaining volume was centrifuged at 10,000 × g for 10 min at 4 °C to separate plasma from the cellular fraction containing the red blood cells (RBC). Both samples were stored at -80 °C. We used the plasma fraction to measure biochemical variables, vitamins and carotenoids, and the RBC fraction to quantify several parameters indicative of oxidative status (see below). In order to assess the effects on reproduction, partridge were monitored until the beginning of July (see *Reproduction* subsection).

Treatment of the seeds and exposure to tebuconazole

Seeds were treated with TANSIL (tebuconazole 2,5 % w/v, Arysta Lifescience) at two application doses in order to obtain the theoretical concentrations (i.e. mg of tebuconazole / g of seeds) of 0.03 mg/g (high dose group) and 0.006 mg/g (low dose group), which correspond to the Recommended Application Rate (RAR) and 20% of the RAR respectively. The low dose simulated 20% of treated cereal seeds in diet, which is considered as a low proportion of cereal seeds in the diet of wild red-legged partridges during the sowing season (based on dietary studies performed in Spain; i.e. Lopez-Antia et al. 2016; Perez and Perez 1981). The high dose simulated 100% of treated seeds in diet, which is considered a worst-case assumption that, however, could be close to real exposure levels in some regions of Spain (Lopez-Antia et al. 2016). Wheat seeds were treated with tebuconazole using a hand sprayer (Apollo 5, EXEL gsa) and following the instructions in the product label. Tebuconazole concentration in seeds was quantified in six low dose diet samples using LC-MS (see supplementary material and Lopez-Antia et al. 2013, 2016 for more details). The coefficient of variation (CV) for the different replicates was 17.7%, mean (± SD) concentration measured was 4.0 ± 0.7 µg/g, an estimated 64.4% of the theoretical dose (6 µg/g). If we assume an equivalent ratio for the high dose treatment (theoretical vs. real dose), tebuconazole concentration in those seeds would be 19.3 µg/g. We measured food consumption (see Lopez-Antia et al. 2015c and supplementary material for more details) to estimate exposure doses (in mg /kg body weight / day) given the tebuconazole concentration in seeds, daily seed consumption (amount consumed by each partridge or pair), and partridges´ body weight at the beginning of the exposure period. We estimated a mean (±SD) tebuconazole daily ingestion of 0.242 ± 0.034 mg/kg bw/day for the low dose group and 1.102 ± 0.144 mg/kg bw/day for the high dose group.

Survival and condition of adult partridges

Partridges' survivorship was checked daily during the experiment. Measurements of each partridge (body mass and tarsus length) were taken at the beginning and at the end of the exposure period. Body condition was calculated using a scaled mass index (SMI; Peig and Green, 2009).

Immune response of adult partridges

We used the phytohemagglutinin (PHA) test to measure the cell-mediated immune responsiveness of partridges. We measure the skin swelling of the wing web after the intradermal injection of PHA, which produces a local infiltration of mononuclear cells (see Mougeot et al. 2009 for more details). The PHA test was performed two days after the end of the exposure period (February 15).

Biochemical response of adult partridges

Red blood cell (RBC) homogenates were used to measure antioxidants levels and oxidative stress parameters (Lopez-Antia et al. 2013; see also Supplementary Material). Specifically, we measured levels of total (GSH) and oxidized glutathione (GSSG), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities (relative to protein levels in the sample) and levels of malondialdehyde (MDA).

Plasma (Rodriguez-Estival et al. 2010) and egg yolk (Lopez-Antia et al. 2015a) levels of retinol (vitamin A), α-tocopherol (vitamin E) and carotenoids (zeaxanthin and lutein) were determined by high-pressure liquid chromatography coupled to diode array and fluorescence detectors. Plasma biochemistry parameters, 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, were measured using an A25 analyzer and the corresponding reaction kits for each parameters (Biosystems SA, Barcelona, Spain).

Carotenoid-based ornamentation

The day after the exposure finished (February 14), we quantified the eye ring and beak red coloration of adult partridges. These integuments are pigmented by carotenoids and their color reflects health status (Mougeot et al. 2009) and reproductive investment (Alonso-Alvarez et al. 2012). The percentage of the eye-ring area that presented carotenoid pigmentation was also measured (% eye-ring pigmented; Mougeot et al. 2009; Lopez-Antia et al. 2013). Details on the procedures used for color measurements are given as Supplementary Material.

Reproduction

One to two weeks before the estimated laying onset (beginning of April), we changed the food to wheat mixed with reproduction fodder (Partridge laying fodder, Nanta-Nutreco, Tres Cantos, Spain). The first egg was laid on April 24 and the last one on July 6. Eggs were collected daily, individually labeled, weighed and measured (maximum length and width), and incubated sequentially in five different batches (see Supplementary Material for details). The eggs laid in 4th, 8th,12th and 16th position of the laying sequence of each pair were removed and stored at -80ºC to measure the concentrations of vitamins and carotenoids in the yolk. We removed a similar proportion of eggs from each experimental group (χ^2=0.396, df=2, p=0.82). Chicks were weighed and measured (tarsus length) to calculate their body condition upon hatching and on days 8, 16, 24 and 32 post-hatching. Following the same protocol as for adults, we conducted a PHA test on a sample of chicks born during the third and fourth incubation sets, when these were 8 day-old. The sex of each chick and unhatched embryo was genetically determined as for adults (Fridolfsson and Ellegren 1999). We assessed the fertility of unhatched eggs and classified the developmental stage of unhatched embryos following Hamburger and Hamilton (1992). Egg shell thickness was measured following Lopez-Antia et al. (2013).

Statistical analyses

We compared the amount of food consumed in each experimental group using mixed models that included the cage number as a random effect. Changes in body mass and body condition from the beginning and the end of the exposure period were compared between experimental groups using repeated measures General Linear Models (GLM).

Due to the large number of physiological variables measured in adult partridges, we used Principal Component Analyses (PCAs) to explore possible collinearity between them. Two PCAs were run, one including the plasma biochemical variables (for which we had a lower sample size than for the other variables) and another one with the rest of the variables (i.e., body condition, hematocrit, response to PHA, antioxidant and oxidative stress biomarkers, vitamins, carotenoids and color measurements). PCAs indicated that variables were poorly associated with each other (Table S1), so further analyses were performed using each variable individually. These physiological variables were analyzed using GLMs with treatment and sex as fixed factors. We used Pearson's bivariate correlations to test if physiological variables were associated with body condition. When a variable was significantly correlated ($p<0.05$) with body condition, we added body condition in the GLM to explore if the effect of the treatment was mediated by condition. We also tested relationships between coloration, levels of carotenoids in plasma and cholesterol and triglycerides concentrations using Pearson's correlations.

For reproductive variables, we tested individual effects on egg and chick variables and pair-level effects on clutch size, brood size and days to the first laid egg. For egg and chick variables (i.e. egg weight and size, shell thickness, yolk vitamins and carotenoids, fertility and hatching rate, chick sex and survival, mass, condition and response to PHA), models included experimental group, egg laying order, fertility, incubation set, and their interactions as fixed factors, and pair identity as a random term. The body condition of hatchlings was also included in the models for PHA response. Treatment effects on fertility and hatching rate, chick sex and survival at 32 days of age were analyzed using mixed models with a binomial distribution of the variable and the breeding pair as a random effect. Potential effects of laying order, incubation set and egg characteristics (length, width, shell thickness) were also tested in these models by incorporating them as covariates.

Pair-level variables (i.e. clutch size, brood size and days to the first laid egg) were analyzed using Generalized Linear Models (GLMz) with a quasi-Poisson distribution. For clutch and brood size, the “days to the first egg” variable was included in the initial models to consider the effect of a delay in laying onset.

We used JMP Pro 14 and R 3.6.1 for statistical analyses. Dependent variables were transformed (log- or square-root) when necessary to obtain a normal distribution of the model residuals. We set the significance threshold level at p=0.05, but considered p-values between 0.05 and 0.10 to be marginally significant. Tukey tests were used for post-hoc analyses. We followed a backward model selection procedure, removing non-significant interactions first.

3. Results

Treatment effects on the food consumption and physiology of adult partridges.

Food consumption and other physiological variables, and plasma biochemical variables are summarized by experimental group in Table 1 and Table S2, respectively. No adult mortality occurred during exposure, but two female partridges (from the control and high dose group) died during blood extraction. All experimental groups consumed similar amounts of food (Table 1). Mean seed consumption was of 660 ± 12 g / partridge (26.4 g partridge /day).

A treatment effect was found for plasma levels of cholesterol ($F_{2,56}$=9.4; p=0.03, with an additive sex effect: p=0.005), triglycerides ($F_{2,55}$=10.3; p<0.001) and total proteins ($F_{2,56}$=4.2; p=0.02). Post-hoc tests showed that cholesterol and total proteins were significantly reduced in the high dose treatment (Figure 1) and triglycerides were reduced in both the low and high dose treatments compared with controls (Figure 1). Triglycerides concentration in plasma was correlated with body condition, but treatment effect on triglycerides was not explained by changes in body condition. No treatment effects were detected for the other physiological variables (Table 1).

Carotenoid-based coloration of the eye ring was reduced (a higher hue value indicating a reduced redness) in partridges from the low dose group compared with controls ($F_{2,80}$=3.6; p=0.03; Table 1). Eye ring hue also differed between sexes, but the treatment effect was independent of sex (Figure 1). The other coloration variables differed between sexes (males had a greater % of eye-ring pigmentation, eye chroma and beak chroma than females; Table S3) but were unaffected by treatments (Table 1). Circulating carotenoids (lutein and zeaxanthin) were not correlated with coloration variables but were positively correlated with cholesterol levels ($R \geq 0.39$; $p<0.001$).

Treatment effects on reproduction and nestlings.

Reproductive variables are shown in Table 2. The hatching rate was reduced in pairs exposed to tebuconazole (χ^2= 6.4 df=2, p=0.04). This reduction was only significant in the high dose group (post-hoc p=0.03; Table 3) for which the hatching rate was 23% lower than in controls (pair results; Figure 2). The best model also included a significant effect of the incubation set (p<0.001) and egg mass (p<0.01, positively correlated) on hatching rate. All embryos from unhatched eggs were in the final stages of development (stages 33 to 45), and most of them (47% in controls, 61% in low dose and 59% in high dose) were almost fully developed (stages 43 to 45).

We found a marginally significant effect of treatment (χ^2=5.5; df= 2; p=0.0647) on brood size, which was 1.5 times smaller in the high dose group than in controls (post-hoc p<0.05). The laying date of the first egg remained in the final model as a significant covariate (χ^2=7.2; df=1; p=0.007). There were no significant effects of tebuconazole treatments on any other reproductive variable or on the body condition, growth, sex ratio, immune response or survival of the chicks.

4. Discussion

Partridges feeding on tebuconazole treated seeds (high dose treatment, corresponding to the RAR) tended to lay fewer eggs, had a significantly reduced hatching rate and produced fewer chicks (reduced brood size) than controls. These results have important implications for tebuconazole risk assessment, because they reveal that seed treatment can adversely affect red-legged partridge reproduction. According to EFSA (2009), approved uses of pesticides in the EU should not affect avian reproductive output under a biologically relevant scenario. We also found effects of the fungicide exposure on some biochemical variables, altered at both doses (the RAR for seed treatment and 20% of the RAR), and an effect on sexual ornamentation that was only apparent in the low dose group. These physiological alterations may help understand the delayed reproductive effects that occurred in partridges feeding on RAR treated seed almost two months and a half after exposition. Our results, together with published literature on triazole fungicides, point to a potential disruption of steroid hormone homeostasis by the fungicide, with lagged effects on reproduction.

Effects on adult partridges

Partridges from the high dose group had reduced plasmatic concentrations of lipids (cholesterol, triglycerides) and of total proteins after exposure. Partridges from the low dose group also presented reduced levels of triglycerides in plasma. Exposed partridges in our experiment did not reduce food consumption or lose weight relative to controls, so the observed effects on plasma lipid content were likely due to a direct effect of tebuconazole. Triazole fungicides inhibit the activity of the lanosterol-14a-demethylase (CYP 51), an enzyme involved in cholesterol synthesis. In two similar experiments, red-legged partridges exposed to flutriafol, another triazole fungicide, also presented reduced plasmatic concentrations of cholesterol (Fernández-Vizcaíno et al. 2020) and of cholesterol and triglycerides (Lopez-Antia et al. 2018). However, Fernández-Vizcaíno et al. (2020) did not find alterations in lipid concentrations in red-legged partridges feeding on tebuconazole treated seeds (RAR level) during 11 days (Fernández-Vizcaíno et al. 2020).

Cholesterol is the substrate for the production of sex steroid hormones (Gao et al. 2018; Zarn et al. 2003), so these alterations of cholesterol levels by triazole fungicides could lead to hormonal disruption. An evaluation of steroid hormone profiles in exposed adults would help clarifying the effects of triazole fungicides on birds. In a recent experiment, red-legged partridges fed with tebuconazole treated seeds for 11 days were shown to have lower plasma estradiol levels than controls, supporting this hypothesis (Fernández-Vizcaíno et al. 2020).

In birds, triazole fungicides are known to inhibit and induce several cytochrome P450 enzymes (Riviere et al. 1984; Ronis et al. 1994; Saxena et al. 2015; Walker, 1983). Tebuconazole has similar effects in mammals (e.g. Lv et al. 2017; Roelofs et al. 2014; Taxvig et al. 2008; Yang et al. 2018). Despite this, most of the studied biomarkers (some of which are related to liver, renal or antioxidant functions, that are usually affected by pesticides) seemed to be unaffected in exposed partridges after 25 days of exposure. This suggests that adult partridges were able to compensate some of the effects originated by tebuconazole, and points to endocrine disruption as a possible explanation for the reproductive toxicity observed in this study. Previous exposures of partridges to seeds treated with triazoles also support this hypothesis (Lopez-Antia et al. 2013; Lopez-Antia et al. 2018; Fernández-Vizcaíno et al. 2020).

We found that tebuconazole exposure reduced the eye-ring redness of partridges, an effect that was only significant in the low dose group and similar in both sexes. Lutein and zeaxanthin are carotenoids used to color this ornament that indicates health status (Mougeot et al. 2009; Perez-Rodríguez et al. 2013) and reproductive investment in partridges: redder individuals invest more in reproduction than others (Alonso-Alvarez et al. 2012). Carotenoids are acquired from diet and transported through the bloodstream linked to lipoproteins, so a reduced concentration of cholesterol in plasma may reduce carotenoid transport (McGraw and Parker, 2006) and the carotenoid-based coloration of integuments such as eye rings. In our experiment we found significant correlations between cholesterol and circulating carotenoids, but not between circulating carotenoids and coloration. The effect of the low dose exposure on eye ring redness was likely indicative of a disruption of the regulation of carotenoid allocation to ornaments, which is modulated by carotenoid availability, steroid hormones and cholesterol-low and high density lipoproteins levels (mobilization and allocation; Furr and Clark, 1997; McGraw and Parker, 2006; Mougeot et al. 2007; Perez Rodriguez et al. 2013). Such effects may have been compensated for in the high dose group, but with adverse effects on reproduction (see below). Moreover, the group at the highest tebuconazole dose showed the highest lipid peroxidation level in blood and it is known that red-legged partridges under oxidative stress express a redder ornament coloration, possibly because this enhances de oxidative changes from the dietary xanthophylls (lutein and zeaxanthin) to the ornamental ketocarotenoids (papilioerythrinone and astaxanthin) as observed with diquat exposed partridges (García de Blas et al., 2016). In other experiments, exposure of partridges to flutriafol and difenoconazole similarly reduced eye ring coloration (% of eye-ring pigmentation) in males exposed to high doses of the fungicides (Lopez-Antia et al. 2013, 2018).

Reproductive effects

A reduction in hatching success (i.e. increased embryo mortality) was noted for eggs laid by tebuconazole-exposed partridges 60 days after the exposure finished. As tebuconazole's bioaccumulation potential is low (half-life in Japanese quail tissues was calculated to be shorter than 24 hours; Gross et al. 2020), these effects on embryonic survival are not expected to result from direct toxicity of maternally transferred tebuconazole to eggs. A possible explanation would be a potential disruption of steroid hormone homeostasis, what would lead to a series of tradeoffs that would ultimately result in a decreased investment in reproduction. A decreased investment is consistent with the tendency of exposed partridges to lay fewer eggs.

Steroid hormones are transferred by the mother to the egg (Schwabl, 1993) and are known to significantly affect many aspects of embryo development like hatchability, hatching time or muscular growth (Groothuis & Engelhardt, 2005; Saino et al. 2008). Other egg variables known to influence egg hatchability and viability, such as yolk vitamins and carotenoids, were not affected in our study. Egg mass was positively associated with hatching success regardless of the experimental group but was not affected by the treatment. Eggshell thickness, an endpoint typically measured in pesticide risk assessment that is related to hatching success (Mineau et al. 2005) did not differ between treatment groups. An unbalanced steroid hormone composition in eggs could explain for the reduced hatching success, and future studies could measure hormone levels in eggs to confirm this.

Effects of tebuconazole on reproduction have been reported for mammals, possibly mediated by the disruption of steroid hormones. Experimental exposures to tebuconazole in pregnant rats produced post-implantation loss (Taxvig et al. 2008), reduced pup viability and body weight (Moser et al. 2001), increase gestational length and led to the production of feminized males and virilized females (Taxvig et al. 2007). In birds, similar effects have been described for other triazole fungicides. Epoxiconazole reduced spermatid numbers in Japanese quails exposed during three weeks to a dose of approximately 65 mg/Kg bw/day, but with no apparent effect on hormone levels, fertility or reproductive outcome (Grote et al. 2008). The exposure of red-legged partridges to a difenoconazole dose of approximately 3.19 mg/Kg bw/day during 10 days prior to breeding caused an important reduction on fertility rate, and reduced egg size (Lopez-Antia et al. 2013). Finally, the exposure of red-legged partridges to flutriafol under similar conditions as in the current study (i.e. 25 days of exposure in late winter) produced, at both exposure doses (0.49 and 2.4 mg/Kg bw/day, corresponding also to 20 and 100% of the RAR, respectively), a marked reduction in clutch size and fertility rate, and an overall 50% brood size reduction (Lopez-Antia et al. 2018). Fernández-Vizcaíno et al. (2020) fed partridges with seeds treated with four different formulations containing triazoles as active ingredients (flutriafol, prothioconazole, tebuconazole and a mixture of the latter two), and found reproductive effects only for the flutriafol exposure, specifically a delay in the laying onset and a tendency to a reduced clutch size. In this experiment, the exposure period was shorter (11 days) and was done in November/December, two months earlier than the current study, highlighting the importance of considering the timing of exposure relative to breeding onset. Altogether, our study and previous ones (Lopez-Antia et al. 2018 and Fernández-Vizcaino et al. 2020), highlight that flutriafol treated seed consumption poses a greater threat than tebuconazole to granivorous bird populations.

Ecological relevance and risk assessment

We found reproductive effects in the high dose partridges, with a mean tebuconazole exposure dose estimated at 1.1 mg/kg bw/day. This dose is much lower than the "No Observed Effect Level" (NOEL; 5.8 mg/kg bw/day) reported with the northern bobwhite (*Colinus virginianus*) and used for the long-term risk assessment of the product in the EU (EFSA 2014). According to the risk assessment procedure, the ratio between the toxicity endpoint (i.e. the NOEL for the long-term risk assessment of birds) and the Estimated Theoretical Exposure (ETE), known as the toxicity to exposure ratio (TER), must be higher than five for the risk to be considered negligible. An exposure to the high dose (this study) would result in a TER>5, despite the referred NOEL was obtained after a much longer exposure than that used in our study (OECD 1993). Thus, the risk associated with this exposure level would have been considered acceptable from a regulatory point of view; however, it caused a significant reduction of the reproductive output. The trigger value of 5 for long-term risk assessment of birds has been criticized several times in the past (reviewed in Luttik et al. 2005) and our study reinforces the idea that this threshold is insufficient because it largely depends on the NOEL for the selected toxicity endpoint (i.e. effect on reproduction).

In the current study we detected reproductive effects in red-legged partridges consuming 100% of newly sown seeds during 25 days. To assess the risk of tebuconazole for partridges in a more ecologically realistic way, a first refinement factor would be to assess the percentage of treated seeds in partridges´ diet. With the purpose of adding a refined exposure scenario to our study, we included the low dose treatment, in which partridges consumed seeds treated with a 20% of the RAR and that caused no reproductive effects. This low-dose scenario would be a proxy of 20% of treated seeds in the diet, a percentage consistent with published data about diet composition of red-legged partridges along the year (Perez y Perez, 1980). However, a geographically wide analysis of crops collected on hunted red-legged partridges in Spain during cereal sowing (Lopez-Antia et al. 2016) estimated an average 53.4% of winter cereal seed biomass in partridges´ diet, that varied between 89.3% and 26.5% depending on the region. This indicates that the absence of reproductive effects resulting from the exposure refinement based on an all-year diet would underestimate the real risk, and that the high exposure dose used in the present study would be close to a realistic worst-case scenario. Furthermore, the ratio of treated seeds in the diet in other farmland bird species can be higher than in partridges, e.g. corn bunting is estimated to feed almost exclusively (98%) on cereal seeds in winter (Perkins et al., 2007; Robinson, 2004).

A crucial factor in pesticide risk assessment is the exposure time. It is common practice in long-term risk assessment to refine exposure estimates using the time-weighted average (TWA), which accounts for the potential degradation/dissipation of the compound in food items (e.g. seeds) over time. This refinement factor should be used only when the toxic effect is considered to be caused by a long-term exposure, and not if the toxic effect is considered to be caused by a short-term exposure (EFSA 2009). However, it is difficult in practice to distinguish between effects resulting from long- or short-term exposures, and so the TWA for a 21-day exposure period is used by default to refine estimates of long-term exposure of birds to pesticides (EFSA 2009), which is proven to underestimate the risk in many cases (Bennet et al. 2005; Shore et al. 2005; EFSA 2009). Moreover, for treated seeds, asynchronous sowing should be considered (in central Spain, sowing lasts from October to December for winter cereals, and from late January to February for spring cereals). Within their home range, that includes many different fields, birds may encounter recently sown treated seeds during several months, and thus TWA should not be used. In that respect, it would be useful to assess if birds actively select recently drilled fields. To date, there is evidence for positive selection and no selection of newly drilled fields (Bonneris et al. 2019; Lennon et al. 2020).

Finally, EFSA (2009) only request a test for effects on reproduction if birds are likely to be exposed during the breeding season. Based on this, long-term risk of tebuconazole treated seeds for granivorous birds was underrated for autumn/winter-sown cereals (EFSA 2014). Our work showed that exposure 60 days before laying does affect breeding performance and thus, exposure outside the reproductive phase should be consider in risk assessments.

Conclusion

Results from this study and previous ones indicate that farmland birds may be exposed to tebuconazole concentrations that negatively affect reproductive success through the ingestion of treated seeds. Current long-term risk assessments may therefore be underestimating the risks of using tebuconazole as seed treatment. Given that tebuconazole-treated seeds are widely available to birds during sowing and have been shown to be frequently consumed (Lopez-Antia et al. 2016), such underestimation may put at risk many farmland bird species that rely on sown seeds to survive during winter.

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Table 1. Mean (± SE) body condition, food consumption, haematocrit, cellular immune responsiveness (PHA, wing web swelling) and oxidative stress biomarkers, vitamins and carotenoid levels of adult red-legged partridges after the exposure period and according to treatment.

Variable	Control	Low dose	High dose
Sample size (N of partridges)	34	26	27
Body condition before treament*	453 ± 5.8	452 ± 5.5	455 ± 5.4
Body condition after treatment	452 ± 6.4	443 ± 7.0	447 ± 7.3
Food consumption/partridge (g total)	667 ± 30	677 ± 11	634 ± 11
Haematocrit (%)	38.7 ± 0.8	37.6 ± 0.7	38.5 ± 1.1
PHA-Wing web swelling (µm)	739 ± 044	693 ± 60	673 ± 57
RBC GPX (IU/mg prot.)	0.587 ± 0.02	0.570 ± 0.02	0.544 ± 0.02
RBC SOD (IU/mg prot.)	1.33 ± 0.06	1.34 ± 0.09	1.32 ± 0.06
RBC GSH (µmol/gr)	6.89 ± 0.27	6.10 ± 0.27	6.83 ± 0.27
RBC MDA (nmol/gr)	4.07 ± 0.3	4.35 ± 0.4	5.71 ± 0.6
Retinol (µM)	11.7 ± 0.3	12.2 ± 0.5	11.8 ± 0.5
Tocoferol (µM)	6.53 ± 0.4	6.32 ± 0.4	5.56 ± 0.3
Lutein (µM)	0.589 ± 0.06	0.445 ± 0.02	0.462 ± 0.03
Zeaxanthin (µM)	1.34 ± 0.2	1.12 ± 0.1	1.05 ± 0.1
% eye-ring pigmentation*	93.3 ± 0.7	91.0 ± 0.9	91.1 ± 1.1
Eye hue*	0.567 ± 0.01 **A**	0.588 ± 0.01 **B**	0.572 ± 0.01 **AB**
Eye Chroma*	36.4 ± 1.2	38.5 ± 1.7	36.1 ± 1.7
Beak hue	0.480 ± 0.01	0.499 ± 0.01	0.496 ±0.01
Beak chroma*	25.5 ± 1.0	24.0 ± 1.2	25.0 ± 1.2

*Significant differences between sexes (exact values for each sex are given in supplementary material)

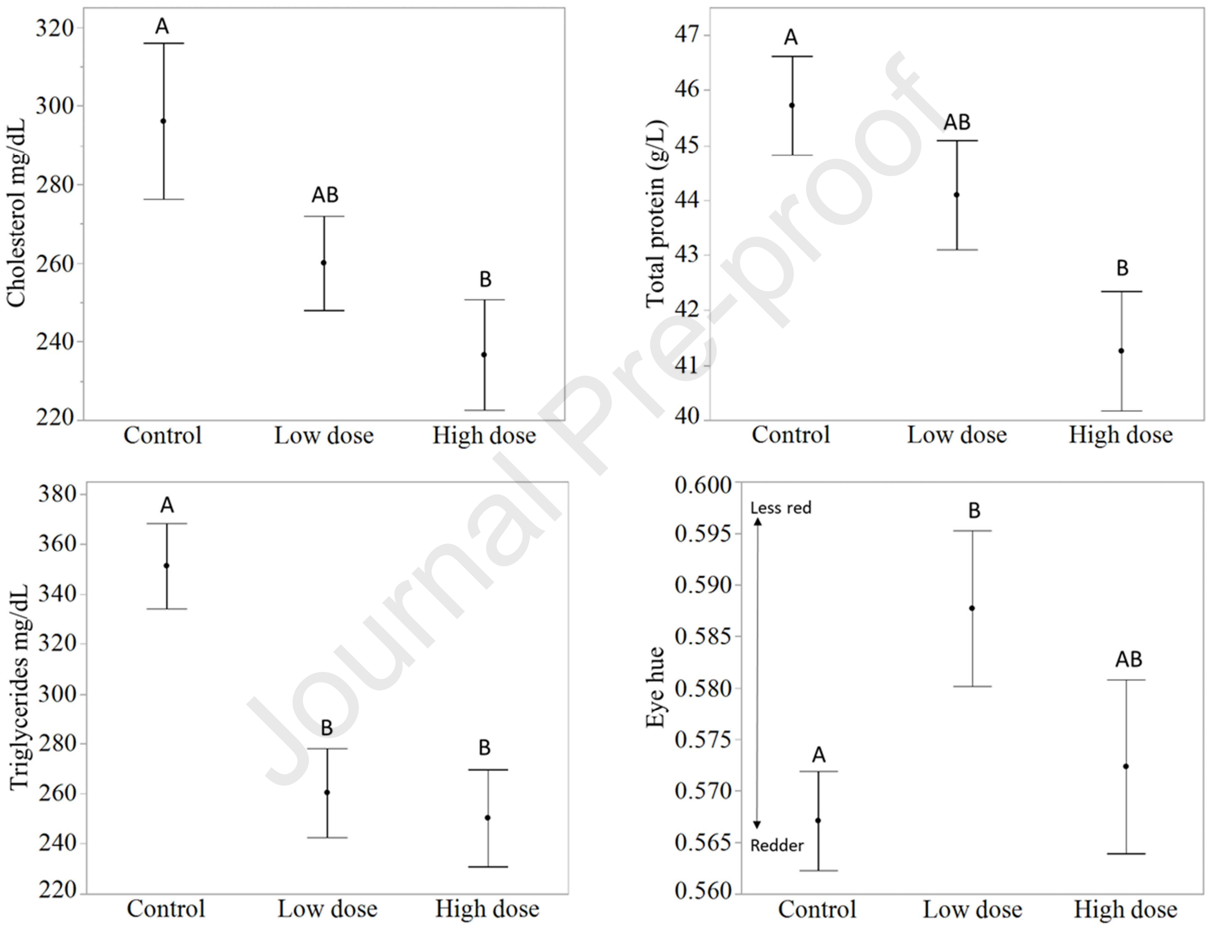

Figure 1. Mean (± SE) plasma levels of cholesterol, total proteins, triglycerides and eye ring hue values in the different experimental groups at the end of the exposure period. Sample sizes in each experimental group ranged between 15 and 22 partridges for biochemical parameters and between 24 and 35 for the eye hue. Note that eye hue values are inversely related to redness (the lower the hue, the redder the eye ring). Different letters indicate significant differences between treatment groups (Tuckey HSD differences).

Table 2. Reproductive and immune response variables (n, % or mean ± S.E.) of partridge pairs and chicks from the different experimental groups. Different letters indicate significant differences among treatments ($p<0.05$ level) based on Tukey test results.

Variable		Control	Low dose	High dose
Number of pairs		9	12	10
Number of laying females		8	11	9
Total number of eggs		110	126	98
Clutch size per laying female		12.2 ± 1.9	10.5 ± 2.0	9.8 ± 1.6
Days to the first egg		24.9 ± 2.6	26.8 ± 3.5	25.0 ± 3.3
Egg mass (g)		17.0 ± 0.1	17.7 ±0.1	17.9 ± 0.2
Egg length (mm)		38.1 ± 1.7	38.7 ± 0.1	39.1 ± 0.2
Egg width (mm)		28.8 ± 0.1	29.1 ±0.1	29.3 ± 0.1
Shell thickness (µm)		227 ± 4.1	216.2.6	209 ±3.9
Retinol in yolk (nmol/g)		510 ± 17	446 ± 18	518 ± 21
Tocoferol in yolk (nmol/g)		1159 ± 81	1260 ± 50	1230 ± 77
Lutein in yolk (nmol/g)		57.3 ± 10.3	44.8 ± 5.3	57.0 ± 10.4
Zeaxanthin in yolk (nmol/g)		39.9 ± 5.6	36.0 ± 3.2	42.3 ± 5.7
Fertile eggs (%)	Average/pair	85.7 ± 9.3	87.2 ± 4.8	74.9 ± 10.2
	Overall/treatment[1]	79 ± 4.4	90 ± 3.0	80 ± 4.5
Hatching rate of fertile	Average/pair	82.5 ± 5.4	67.4 ± 8.1	59.4 ± 8.9
eggs (%)	Overall/treatment[1]	81 ± 4.8 **A**	74 ± 4.7 **AB**	63 ± 6.1 **B**
Number of chicks		54	65	39
Brood size[2]		6.0 ± 0.9 **A**	5.4 ±1.2 **A**	3.9 ± 0.9 **B**
Chick body condition at birth		11.7 ± 0.2	11.8 ± 0.1	12.3 ±0.2
Chick mortality rate	Average/pair	34.4 ± 8.2	37.9 ± 7.1	37.1 ± 13.0

(%)	Overall/treatment[1]	31 ± 6.3	43 ± 6.1	26 ± 7.0
Female ratio (%)	Average/pair	58 ± 7.2	62 ± 7.2	49 ± 11.0
	Overall/treatment[1]	54 ± 6.4	59 ± 5.6	59 ± 6.7
Wing web swelling (µm)		103 ± 18.5	95.1 ± 23.4	92.3 ± 8.8

[1] Mean calculated at the individual (egg or chick) level. Standard errors derived from marginal means of binary logistic models. [2] Some eggs (0-4 eggs/pair) were kept for analyzing and thus not incubated.

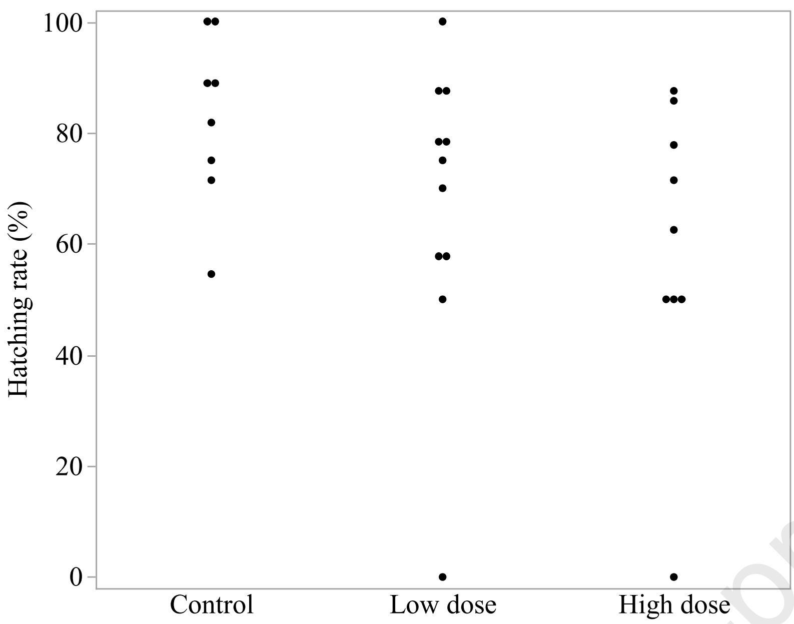

Figure 2. Hatching rates (%) of red-legged partridge clutches laid by pairs in the different experimental groups.

Highlights

- Tebuconazole-treated seed ingestion has lagged effects on bird reproduction
- Exposure reduced cholesterol and triglyceride levels, and affected ornamentation
- Hatching rate was reduced in exposed pairs
- Brood size was 1.5 times smaller in exposed pairs
- Current assessments underestimate long-term risks for farmland birds