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Experimental exposure of red-legged partridges (*Alectoris rufa*) to seeds coated with imidacloprid, thiram and difenoconazole

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Abstract Pesticide coated seeds are commonly used in agriculture, and may be an important source of food for some birds in times of scarcity, as well as a route of pesticide ingestion. We tested the lethal and sub-lethal effects of treated seed ingestion by the red-legged partridge (*Alectoris rufa*), a game bird of high socio-economic value in Spain. One year-old partridges ($n = 42$ pairs) were fed for 10 days in spring (prior to breeding) with wheat treated with difenoconazole (fungicide), thiram (fungicide) or imidacloprid (insecticide), using two doses for each pesticide (the one recommended, and its double to represent potential cases of abuse of pesticides). We investigated the direct and indirect effects on the body condition, physiology, immunology, coloration and subsequent reproduction of exposed partridges. For the latter, eggs were collected, measured and incubated and the growth and survival of chicks were monitored. Thiram and imidacloprid at high exposure doses produced mortalities of 41.6 and 58.3 %, respectively. The first death was observed at day 3 for imidacloprid and at day 7 for thiram. Both doses of the three pesticides caused sublethal effects, such as altered biochemical parameters, oxidative stress and reduced carotenoid-based coloration. The high exposure doses of imidacloprid and thiram also produced a decrease in cellular immune response measured by the phytohemagglutinin test in males. Bearing in mind the limitation of the small number of surviving pairs in some treatments, we found that the three pesticides reduced the size of eggs and imidacloprid and difenoconazole also reduced the fertilization rate. In addition, both thiram and imidacloprid reduced chick survival. These experiments highlight that the toxicity of pesticide-treated seeds is a factor to consider in the decline of birds in agricultural environments.

Keywords Breeding success · Carotenoid-dependent coloration · Fungicide · Immune response · Insecticide · Oxidative stress

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Introduction

The use of pesticides is increasingly recognized as a key factor for explaining population declines of farmland animals (Geiger et al. 2010). While the main and most immediate effect of pesticide use on avian wildlife is related to a reduction of food availability for birds or their prey (Wilson et al. 1999), direct effects as a consequence of intoxication may also compromise bird survival or reproduction (Mineau 2005), thereby contributing to population declines. The most common route of exposure of birds to pesticides is through ingestion of contaminated food, soil or water. For granivorous species, the ingestion of seeds treated with pesticides could be a risk of direct and severe intoxication.

Seeds treated with pesticides are widely used in agriculture, as they reduce the risks of exposure for farmers by eliminating the need for spraying (Prosser et al. 2006). However, treated seeds may put at risk granivorous farmland birds, as they constitute a potential food source for

these animals. Treated seeds are sometimes not properly buried or more often spilled from sowing machines, and therefore accessible to feeding farmland birds, representing a risk of pesticide intake. Seed coating practice became widespread during early 20th century, when the first seeds treated with organomercurial products appeared on the market. Since then, their use has been constant, and several cases of bird mortality related to treated seed ingestion have been reported (e.g. Stanley and Bunyan 1979). For some of the more toxic active ingredients used in seed coating, a bird feeding on treated seeds could get a lethal dose in less than a day (Hart 1990). Seed coating was responsible for up to 50 % of incidents on vertebrate wildlife caused by approved pesticide use in different European countries (de Snoo et al. 1999). While reports on risk of seeds treated with organochlorine or anticholinesterasic insecticides for birds are relatively common, most of the pesticides currently used for seed coating are based on active ingredients of lower toxicity and persistence. To date, little is known about the potential effects that these compounds have on seed-eating birds when they are directly ingested.

The aim of this study was to analyse the effects of treated seed ingestion on the physiology and fitness (short-term survival and reproduction) of a game bird of high socio-economic value, the red-legged partridge (*Alectoris rufa*). We studied the direct and delayed effects of a 10-day long exposure to seeds treated with two fungicides (thiram and difenoconazole) and an insecticide (imidacloprid).

Imidacloprid is one of the most commonly used insecticides worldwide belonging to the family of neonicotinoids. It binds to specific nicotinic acetylcholine receptors, thus interfering with the transmission of nerve impulses. Oral median lethal doses (LD50) for birds available in the literature vary from 31 mg/kg in the Japanese quail (*Coturnix japonica*) to 152 mg/kg in the bobwhite quail (*Colinus virginianus*) (Tomlin 2004/05). In birds, exposure to this insecticide has direct effects on biochemical and oxidative stress parameters (Balani et al. 2011) as well as on the immune system (Siddiqui et al. 2007). The ingestion of seeds treated with imidacloprid has also been linked with mortalities of wild birds in the field (de Snoo et al. 1999; Bro et al. 2010). Thiram is a dithiocarbamate fungicide known to negatively affect avian growth, physiology and reproduction (Guitart et al. 1996; Subapriya et al. 2007). Oral LD50s for birds have been found to vary from 673 mg/kg in the ring-necked pheasant (*Phasianus colchicus*) to more than 2,800 mg/kg in the mallard duck (*Anas platyrhynchos*) (Hudson et al. 1984). Thiram has also been found to be teratogenic when injected into the egg (Korhonen et al. 1982). Difenoconazole is a broad-spectrum fungicide belonging to the chemical class of azoles. To the best of our knowledge, no major deleterious effects of this active ingredient have been reported so far on birds. The available information points to a low toxicity of difenoconazole on birds, with oral LD50s higher than 2,000 mg/kg in both mallard duck and Japanese quail (KEM 2006). Besides seed treatment, these three products are also used as foliar spray applications in fruit crops and orchards, and imidacloprid and thiram can also be dissolved in the irrigation water (MMAMRM 2010). Apart from the LD50 values calculated for avian species commonly used in laboratory testing (quails, mallards), knowledge on the effects that these pesticides have on birds comes from studies on poultry. To date, nothing is known about their impact on wild birds, which are likely exposed to coated seed ingestion. In order to elucidate the toxicity mechanisms of ingestion of seeds treated with these pesticides, there is therefore a crucial need for investigating sublethal effects in adult partridges feeding on seeds, as well as possible delayed reproductive effects (i.e. egg or offspring quality potentially affected by maternal exposure to pesticides).

Materials and methods

Selection of pesticides and exposure doses

For the experiment, we purchased the following pesticides: (1) Score® 25 EC (difenoconazole 25 % w/v, Syngenta Agro, Madrid, Spain), (2) Pormasol® Forte (thiram 80 % w/w, Bayer CropScience, Alcácer, Spain) and (3) Escocet® (imidacloprid 35 % w/v, Bayer CropScience, Alcácer, Spain). The first two products are not specifically for seed treatment because difenoconazole- and thiram-based products for this purpose were not available at the time we purchased the pesticides.

Seeds were sprayed with different amounts of each commercial product to obtain nominal target concentrations, for that we used spray bottles at our facilities. We used two application doses for each pesticide; the lowest dose corresponding to the one recommended for cereal seed coating according to the current Spanish regulations (MMAMRM 2010). The highest dose was twice this recommended dose, and was used in order to assess the effects of potential abuses in pesticide application. The target concentrations for each pesticide were thus as follows; (1) difenoconazole (3 %): 200–400 cm^3/100 kg of wheat seeds (0.06–0.12 mg/g), (2) thiram (50 %) 350–700 cm^3/100 kg of wheat seeds (1.75–3.5 mg/g) and (3) imidacloprid (35 %) 200–400 cm^3/100 kg of wheat seeds (0.7–1.4 mg/g). Aliquots of each treatment were stored at -20 °C until analysis for the confirmation of the exposure doses.

Analysis of coated seeds: confirmation of exposure doses

The concentration in the treated seeds was confirmed in six aliquots of each treatment. One gram of seeds was extracted with 20 mL acetonitrile during 1 min of vortex, 5 min of sonication and 1 min again of vortex. The extract was filtered through a syringe nylon filter of 0.2 μm. After appropriate dilution with acetonitrile, the extract was analyzed by LC-ESI-MS with an analytical system formed by Agilent 1100 series chromatograph and Agilent 6110 Quadrupole LC/MS with a multimode source (MM). The nitrogen for mass detector was supplied with a high purity nitrogen generator (Whisper 2-50, Ingeniería Analítica, Sant Cugat, Spain). The chromatographic column used was Zorbax Eclipse XBD-C18 (4.6 × 150 mm, 5 μm) with a precolumn Zorbax Eclipse XBD-C18 (4.6 × 10 mm 5 μm). The injection volume was 20 μL. The chromatographic conditions of analysis consisted in a gradient elution of two phases (A: formic acid 0.01 % in water; B: formic acid 0.01 % in methanol). The initial conditions were 95 % A and 5 % B, reaching 50 % A and 50 % B at min 8.5, 10 % of A and 90 % of B at 25 min. This was maintained until min 28, returning to the initial conditions by min 29. The flow rate was 0.8 mL/min. Pesticides were detected using positive ion monitoring with the following MM-ESI source settings. Nebulizer pressure was set at 35 psi, drying gas flow was 12 L/min, drying gas temperature was 250 °C, vaporizer temperature was 200 °C, capillary voltage was 3,500 V and charging voltage was 1,000 V. The monitored ions were 175, 256 and 257 for imidacloprid, 72, 73, 88 and 240 for thiram, and 406, 408 and 459 for difenoconazole. Fragmentation voltage was 100, except for ion 175 of imidacloprid (150) and ion 459 of difenoconazole (175). Stock solutions of pesticide standards were purchased in acetonitrile at a concentration of 10 μg/mL form Dr. Ehrenstorfer (Augsburg, Germany). Calibration curves were performed with concentrations of the three pesticides ranging from 0.25 to 2 μg/mL in acetonitrile. The recovery of the analytical procedure was calculated with six replicates of non-treated wheat (1 g) spiked with 3 μg of each pesticide. The obtained recoveries were 100.2 ± 2.4 % for difenoconazole, 108.8 ± 1.8 % for thiram, and 106.7 ± 2.8 % for imidacloprid.

The concentrations measured (mean ± SD) in the treated seeds at low and high doses were 0.052 ± 0.006 and 0.098 ± 0.006 mg/g for difenoconazole, 1.077 ± 0.308 and 3.254 ± 0.354 mg/g for thiram, and 0.519 ± 0.081 and 0.869 ± 0.170 mg/g for imidacloprid. These concentrations were lower than the theoretical values (between 62 and 93 % of the target values), probably because part of the pesticide was lost in the containers used to treat the seeds. Captive partridges ate on average 25 g of wheat/day (authors' unpublished data) and weighed on average 407 g. We therefore estimated the following daily ingestion doses for the lowest and highest application doses, respectively: 3.19–6.02 mg/kg/day for difenoconazole, 66.2–199.9 mg/kg/day for thiram and 31.9–53.4 mg/kg/day for imidacloprid.

Experimental design and sample collection

The experiments were conducted in the Dehesa de Galiana experimental facilities (Ciudad Real, Spain). All experimental protocols were approved by the Universidad de Castilla-La Mancha's Committee on Ethics and Animal Experimentation. For the full set of experiments we used a total of 42 breeding pairs of captive-born, 1 year-old red-legged partridges obtained from the farm of the regional government of Castilla-La Mancha (Chinchilla, Albacete, Spain). The sex of individuals was determined genetically following Griffiths et al. (1998). The partridges were housed in pairs in outdoor cages (95 × 40 × 42 cm) and acclimatized to the facility during 15 days before starting the experiments. Commercial partridge feed (Partridge maintenance fodder, Nanta-Nutreco, Tres Cantos, Spain) mixed with wheat and tap water were provided ad libitum. Each pair was randomly assigned to one of the seven experimental groups (six groups corresponding to the two application doses of each pesticide, plus a control group that was shared among the three experiments in order to reduce the number of birds used for experiments). Sample size included six breeding pairs per group.

Prior to exposure, we took a blood sample from each partridge, in order to determine pre-treatment blood parameters and avoid initial differences. We extracted 1 mL of blood from the jugular vein, which was kept in heparinised tubes and centrifuged at 10,000×*g* for 10 min at 4 °C to separate plasma from the cellular fraction (pellet). Both plasma and pellet were stored separately at −80 °C for later analysis. Before centrifugation, an aliquot of each sample was taken to calculate the haematocrit.

The exposure began on March 15, 2010. Partridges were fed exclusively with treated wheat (low and high pesticide exposure groups), or with untreated wheat (control group). The exposure lasted for 10 days, at the end of which partridges returned to their usual diet of untreated wheat mixed with maintenance fodder. On March 24 (end of the exposure), we extracted blood again and processed it as described above (post-treatment sample). Partridges were kept in the cages throughout the spring and early summer in order to monitor reproduction (see below).

Survival and body condition of adult partridges

Survivorship was checked daily during the experiment. When a partridge died, we did a necropsy to detect gross

lesions or abnormalities in the main organs and systems. We initially measured tarsus length and weighed each partridge twice throughout the experiment: prior to and just after pesticide exposure (same days as blood collections). The body condition of partridges was calculated according to the scaled mass index proposed by Peig and Green (2009) and can be computed as:

$$M = M_i(L_0/L_i)^{b_{sma}}$$

where M_i and L_i are the body mass and the linear body measurement of individual i respectively; b_{sma} is the scaling exponent estimated by the standardised major axis (SMA) regression of M on L, L_0 is the arithmetic mean value for the study population; M is the predicted body mass for individual i when the linear body measure is standardized to L_0. The scaling exponent b_{sma} can be calculated indirectly by dividing the slope from an ordinary least squares (OLS) regression (b_{ols}) by the Pearson's correlation coefficient r.

Physiological responses of adult partridges

We measured oxidative stress indicators in red blood cells (RBC) homogenates by spectrophotometry following the methods described in Reglero et al. (2009). We quantified lipid peroxidation levels, estimated as thiobarbituric acid reactive substances (TBARS), as well as total (GSH) and oxidized (GSSG) glutathione levels. We used Ransel and Ransod kits (Randox Laboratories, Cornella de Llobregat, Spain) to measure the activities of the glutathione peroxidase (GPX, EC 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1), respectively. Enzyme activities were calculated relative to mg of protein, using the Bradford method to quantify total proteins in the homogenates (Bradford 1976).

We determined the levels of retinol (distinguishing the free, alcoholic, form and the form esterified with fatty acids), α-tocopherol and carotenoids (zeaxanthin and lutein) in plasma using high performance liquid chromatography coupled to a photodiode detector and a fluorescence detector. The methods used for extraction and analysis are described in Rodríguez-Estival et al. (2010).

We measured the following biochemical parameters in plasma with an automatic spectrophotometer analyzer A25 using the reaction kits available for each enzyme or analyte (BioSystems, Barcelona, Spain): alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase (AST), gamma glutamyl transferase, lactate dehydrogenase, creatine phosphokinase, albumin, total protein, glucose, cholesterol, triglycerides, calcium, magnesium, phosphorus, creatinine, urea and uric acid.

We quantified testosterone and estradiol levels in plasma samples using enzyme-linked immunosorbent assay commercial kits (DRG Instruments GmbH, Marburg, Germany). We obtained calibration curves using the standards included in the kits and adjusting the obtained values to a four parameter logistic model with the software XLSTAT (Addinsoft SATL, Paris, France).

Carotenoid-based coloration of adult partridges

Prior to and just after pesticide exposure, we took digital photographs to measure the red, carotenoid-based coloration of the beak and eye ring of partridges as in Mougeot et al. (2009). For each bird, we took a digital picture of the left side of the head using the same digital camera (Nikon Coolpix 4500) and under standardized conditions. For each picture, we placed the same grey standard reference (Kodak Gray Scale, Kodak, New York) as a background. We analyzed images using Adobe Photoshop 7.0. From each picture, we measured the RGB components (the average intensity of Red, Green and Blue components of pixels) of the eye ring and beak, and the relative amount of the eye ring area pigmented by carotenoids (see Mougeot et al. 2009). The intensity of carotenoid-based red coloration, or RGB values (hereafter referred as to redness) was calculated as R divided by the average of R, G and B (Villafuerte and Negro 1998). RGB values of the grey reference were used to standardize all colour measurements and correct for possible differences in coloration between pictures.

Immune response of adult partridges

We estimated cell-mediated immune responsiveness using the phytohemagglutinin (PHA)-skin test. On the last day of exposure, we measured wing web thickness of each partridge with a micrometer (Mitutoyo Absolut 547-401) to the nearest 0.01 mm and injected intradermally the wing web with 100 μL of PHA in PBS (1 mg/mL dilution). The PHA is a lectin that causes an accumulation of T lymphocytes followed by an infiltration of macrophages (Goto et al. 1978), which manifests in a local inflammation that reaches its maximum 24 h after injection. After this 24 h period, we measured again the wing web thickness, and estimated the intensity of the T cell-mediated immune response as the difference between the final and the initial thicknesses.

Humoral immune response was estimated using a haemagglutination test after injecting an antigen (i.e. sheep red blood cells, SRBC) that stimulated the synthesis of specific antibodies. Two days after the end of the exposure, we collected blood from the jugular vein of a sheep housed in the same estate as the experiments were carried out. We calculated the haematocrit of this blood and diluted it in PBS to a final concentration of 20 % SRBC. We injected

intraperitoneally 100 μL of this dilution to each partridge. To analyse the antibody production, we took a blood sample 12 days after injecting the SRBC. After centrifugation, an aliquot of plasma was incubated at 56 °C for 30 min in order to inactivate the complement and improve agglutination visibility (personal observation). The haemagglutination test in vitro was conducted on blood from the same sheep used for injecting the partridges, and collected on the same day as the test was performed. After testing several SRBC concentrations, better visibility was achieved using a 0.5 % dilution. Serial dilutions of plasma were prepared in wells of a microtiter plate (50 μL final volume) to which we added 50 μL of the SRBC dilution. A well in each row where we added 50 μL of PBS instead of plasma was used as negative control. Plates were tilted 45° and incubated at 37 °C for 1 h. After incubation, we examined the plates for SRBC agglutination and estimated the antibody concentration as the $\log_2$ of the less concentrated plasma dilution causing agglutination.

Reproduction

Because some birds died during the experiments, those partridges that were alone in the cages were paired again, when possible, with mates exposed to the same treatment. We checked daily all cages to collect the eggs, which were measured (maximum length and width) and kept at 15 °C to avoid development. Every 15 days, we transferred all eggs collected thus far to an automatic incubation chamber (Masalles Valltrade, Sant Cugat del Valles, Barcelona, Spain) where they were incubated for 21 days at 37.7 °C, 45 % humidity and with constant movement. Then, we candled the eggs and those that were found to have developed were introduced in individual cages and moved to a birth chamber where they were incubated at 37.7 °C with constant humidity but without movement. We checked the birth chamber daily for hatchlings. We took note of date of birth, tarsus length and body mass of each hatchling. Chicks were individually marked and housed in closed rooms with a heat source, water and food (Partridge growth fodder, Nanta-Nutreco, Tres Cantos, Spain). We measured (tarsus length) and weighed all chicks upon 8, 16, 24 and 32 days of age, and calculated their body condition as for adults. The storage time (0–15 days) was included in the early statistical models and no effect on embryo and chick development was found.

Unhatched eggs were opened and examined to see whether they were fecundated or not (observation of the presence of embryo or germinal disk). To measure shell thickness of hatched and unhatched eggs, we collected three small shell pieces (aprox. 0.5 × 0.5 cm) from the equatorial region of each egg; we separated the inner membrane, dried the shell pieces and measured thickness with a micrometer to the nearest 0.001 mm. Eggshell thickness was calculated as the average thickness of the three measurements.

Statistical analyses

We analyzed the survival of adult partridges using Kaplan–Meier survival analyses and the Mantel-Cox test for pairwise comparisons among treatment groups. Previously, we confirmed the absence of sex-related differences in survival using a Fisher's exact test. We used general linear models (GLM) with the experimental dose and the sex as fixed factors in order to test for pesticide effects on body condition, blood parameters (i.e. haematocrit, oxidative stress indicators, plasma biochemistry, vitamins, carotenoids and sexual hormones), immune responses and carotenoid-based coloration.

For reproduction-related variables (i.e. egg biometry, fecundation and hatching rates, and chicken survival and growth rates) we used the individual (egg or chick) instead of the breeding pair (cage) as experimental unit to study differences among treatments because of the small number of breeding pairs remaining in some treatment groups after pesticide exposure.

Variation in egg measurements (length, width and shell thickness) was analyzed with Generalized Linear Models (GLMz) considering lineal distributions of the dependent variables with the pesticide dose as fixed factor and the female body condition as a covariate. Fertile egg rate, as well as hatching rates considering both only fertile eggs and total eggs laid were analyzed with GLMz considering a binary logistic distribution of the dependent variables and using the female body condition as a covariate. In the case of thiram, because all the fertile eggs used for calculation of the hatching rate at high dose were laid by the same female, we did not use the covariate for the analysis of this variable. The effects of maternal exposure to pesticides on chick survival was analysed through Mantel-Cox tests after building Kaplan–Meier survival curves for each treatment. In order to test for the influence of maternal body condition or egg biometry on chick survival, we also run a GLMz with the survival at day 32 as dependent variable considering a binary logistic distribution using the treatment as fixed factor and egg size and female body condition as covariates. Chick growth was analysed with a repeated measures analysis of the covariance using the chick body condition over time (measured at days 0, 8, 16, 24 and 32 after hatching) as dependent variable, treatment as a factor and the female body condition and the egg size as covariates.

When significant differences were obtained in GLM or GLMz analyses, we checked for the dose causing effects on marginal means through least significant difference (LSD)

tests. Normality of all variables included in parametrical analysis was checked by Shapiro–Wilk tests. Chick survival data were log-transformed to obtain a normal distribution. Plasma testosterone data could not be normalized, so the effects of pesticide on this variable were analysed using a Kruskal–Wallis non-parametric test. We considered p values below 0.05 as significant. However, given the small sample sizes per treatment, established to reduce the number of animals used in experimentation, we considered p-values below 0.1 as marginally significant and conducted post hoc analyses when a p-value within this range was obtained. Data were analyzed using SPSS 19.0.

Results

Direct adverse effects on adult partridges

We did not detect any difference among treatment groups in the pre-exposure sampling for body condition or blood parameters, and therefore only considered post-exposure measurements in the analyses.

Table 1 shows the average mortality rate, body condition, haematocrit and biochemical parameters for each experimental group at the end of the exposure to pesticides. High exposure doses of imidacloprid and thiram significantly reduced the survivorship of adult partridges ($p \leq 0.002$). For imidacloprid the first death at the high dose occurred on the third day of treatment and the last one 2 days after finishing the exposure period. For thiram the first death occurred on the seventh day of treatment and the last one occurred the day after finishing the exposure. No differences in mortality were found between sexes. Both doses of imidacloprid and the highest dose of thiram reduced body condition and haematocrit (all p-values ≤ 0.005), whereas difenoconazole did not produce any significant change in these parameters.

All pesticides and doses influenced some of the measured biochemical parameters. Difenoconazole caused marginally significant effect ($p \leq 0.055$) in phosphorus and total protein levels. Post hoc tests revealed that both doses of the fungicide decreased plasma levels of these two parameters in a significant manner as compared to controls (Table 1). Difenoconazole exposure also reduced γ-glutamyl transferase levels also in a marginally significant manner ($p \leq 0.056$), although in this case LSD test did not show any significant effect of any particular dose. Both doses of thiram significantly reduced blood levels of calcium, cholesterol, magnesium and albumin, while the high thiram dose also reduced total protein concentration in plasma (all p-values ≤ 0.034). Finally, both doses of imidacloprid reduced albumin, alkaline phosphatase, calcium, cholesterol and total protein levels. The high dose of the insecticide also affected levels of aspartate aminotransferase (all p-values ≤ 0.020).

With regards to the variables related to the oxidative stress measured in erythrocytes, plasma vitamins and carotenoids (Table 2), partridges that consumed seeds treated with any thiram dose or with the highest difenoconazole dose showed increased SOD activity ($p \leq 0.025$). Imidacloprid reduced the activity of GPX ($p = 0.009$) at both doses and produced a marginally significant decrease in total GSH concentration ($p = 0.065$); LSD test revealed this effect was significant at the high insecticide dose (Table 2). Exposure to the low difenoconazole dose decreased retinyl palmitate levels in plasma ($p = 0.029$). Neither imidacloprid nor thiram affected vitamin levels, and none of the tested pesticides significantly affected circulating carotenoid levels.

Cellular but not humoral immune response was affected by treated seed consumption, although this effect was only detected in males (Fig. 1). The inflammatory response to PHA was impaired in male partridges that had been exposed to seeds treated with the highest doses of thiram and imidacloprid ($p \leq 0.009$) when compared to controls. We did not detect any immunotoxic effect for difenoconazole.

For carotenoid-based coloration, we did not find any significant treatment effect on beak or eye ring redness. However, we found significant differences among experimental and control groups in the percentage of pigmented eye ring, which was reduced after exposure to high doses of thiram, difenoconazole and imidacloprid ($p \leq 0.005$). These effects were mainly due to changes in the eye ring pigmentation of males, as in females this response was observed only in the imidacloprid experiment (Fig. 2).

Effects on reproduction and indirect effects on offspring

Plasma levels of sexual hormones did not differ significantly from the controls in any of the exposed groups and none of the treatments influenced average clutch size (Table 3). However, eggs laid by females previously exposed to seeds treated with any of the pesticides were smaller, in terms of length, than those laid by control females ($p \leq 0.007$), although in the case of thiram this effect was only detected at the high exposure dose (Table 3). Egg width was only reduced, as compared to controls, after maternal exposure to thiram-treated seeds ($p < 0.001$). Eggshell thickness decreased after exposure to both thiram doses and to the low dose of imidacloprid ($p \leq 0.001$). We also detected a marginally significant increase of eggshell thickness in eggs laid by females exposed to difenoconazole ($p = 0.062$) that was observed only at the high fungicide dose (Table 3). Maternal body condition was negatively associated with egg length in the

Table 1 Mean (±SE) body condition, haematocrit and plasma biochemical parameters of red-legged partridges at the end of the exposure period (day 10) in each experimental group

Parameter	Control	Difenoconazole		Thiram		Imidacloprid	
		Low dose	High dose	Low dose	High dose	Low dose	High dose
Mortality (%)	0.0	0.0	0.0	0.0	**41.6***	8.3	**58.3****
N[a]	12	12	12	12	8	11	5
Body condition[b]	417.2 ± 25.3	417.2 ± 20.1	417.5 ± 27.6	412.9 ± 69.8	**386.5 ± 60.4***	**388.6 ± 53.0****	**357.1 ± 89.3****
Haematocrit (%)	43.11 ± 0.75	40.44 ± 1.12	41.52 ± 1.07	39.91 ± 1.32	**35.21 ± 2.03****	**38.71 ± 1.01***	**34.13 ± 3.00****
Albumin (g/L)	18.45 ± 0.65	16.00 ± 1.31	16.67 ± 0.55	**16.25 ± 0.62***	**13.13 ± 1.16****	**13.00 ± 0.60***	**9.33 ± 1.89****
Alkaline phosphatase (U/L)	759.0 ± 172.0	810.7 ± 151.4	950.0 ± 79.0	834.7 ± 76.2	605.7 ± 122.9	**392.5 ± 66.7***	**204.2 ± 58.0***
Alanine aminotransferase (U/L)	18.92 ± 2.71	21.09 ± 2.91	28.25 ± 5.37	31.08 ± 5.20	30.88 ± 5.59	33.20 ± 5.35	27.33 ± 10.53
Aspartate aminotransferase (U/L)	263.8 ± 31.5	263.2 ± 29.9	293.8 ± 28.4	255.0 ± 40.6	236.9 ± 50.2	223.4 ± 34.1	**72.3 ± 36.9****
γ-Glutamyl transferase (U/L)	6.92 ± 1.40	4.82 ± 0.98	3.00 ± 0.71	5.50 ± 1.76	5.57 ± 2.01	6.80 ± 3.87	3.17 ± 1.62
Creatine phosphokinase (U/L)	926.9 ± 138.78	601.80 ± 54.82	775.58 ± 112.06	737.18 ± 118.70	822.20 ± 269.98	969.00 ± 177.70	
Creatinin (mg/dL)	0.41 ± 0.02	0.36 ± 0.02	0.37 ± 0.02	0.38 ± 0.03	0.38 ± 0.02	0.37 ± 0.02	0.38 ± 0.03
Urea (mg/dL)	6.22 ± 0.58	5.73 ± 0.92	6.03 ± 0.65	5.01 ± 0.66	8.48 ± 1.59	10.62 ± 2.18	9.78 ± 1.28
Calcium (mg/dL)	23.60 ± 2.04	19.30 ± 1.80	20.35 ± 1.87	**18.14 ± 0.80***	**16.19 ± 0.94***	**16.08 ± 1.01****	**15.54 ± 1.28****
Cholesterol (mg/dL)	385.4 ± 41.9	307.2 ± 36.8	306.9 ± 30.3	**277.4 ± 19.9***	**229.3 ± 27.0***	**232.0 ± 17.5****	**211.5 ± 43.8****
Glucose (mg/dL)	393.5 ± 25.6	409.4 ± 20.4	380.4 ± 45.9	419.4 ± 22.6	376.9 ± 22.5	405.7 ± 14.0	394.8 ± 43.6
Magnesium (mg/dL)	3.84 ± 0.50	2.79 ± 0.20	3.29 ± 0.40	**3.00 ± 0.12***	**2.56 ± 0.14***	2.62 ± 0.17	3.16 ± 0.14
Phosphorus (mg/dL)	7.53 ± 1.12	**4.71 ± 0.60***	**5.22 ± 0.43***	7.23 ± 0.87	5.32 ± 1.06	4.40 ± 0.52	6.78 ± 1.27
Total protein (g/L)	75.39 ± 5.73	**63.69 ± 3.58***	**59.38 ± 2.26***	64.77 ± 3.84	**48.90 ± 4.07***	**51.34 ± 3.51***	**35.48 ± 6.84****
Triglycerides (mg/dL)	178.3 ± 22.1	142.8 ± 12.7	165.6 ± 21.2	201.1 ± 28.0	148.1 ± 33.2	146.8 ± 27.5	108.3 ± 13.7
Uric acid (mg/dL)	8.17 ± 1.69	5.09 ± 0.50	7.03 ± 0.68	10.28 ± 0.50	15.38 ± 3.86	11.16 ± 2.23	23.74 ± 9.44

Bold values indicate groups significantly different from controls

[a] *N* per treatment for mortality calculation = 12 in all cases

[b] See text for details about method of estimation of body condition

* Significantly different from controls at the $p \leq 0.05$ level

** Significantly different from controls at the $p \leq 0.01$ level

Table 2 Mean (±SE) oxidative stress biomarker, vitamin and carotenoid levels at the end of the exposure period (day 10) in each experimental group

Parameter	Control	Difenoconazole		Thiram		Imidacloprid	
		Low dose	High dose	Low dose	High dose	Low dose	High dose
N	12	12	12	12	7	11	5
RBC TBARS (nmol/g)	28.0 ± 1.4	27.6 ± 1.2	25.9 ± 1.3	28.8 ± 1.3	24.9 ± 1.2	26.6 ± 0.9	29.2 ± 1.7
RBC GSH (μmol/g)	6.17 ± 0.64	5.51 ± 0.56	5.53 ± 0.41	5.83 ± 0.48	5.25 ± 0.59	5.77 ± 0.45	**3.51 ± 0.93***
RBC GSSG (μmol/g)	0.49 ± 0.38	0.49 ± 0.04	0.52 ± 0.03	0.57 ± 0.05	0.57 ± 0.75	0.61 ± 0.05	0.4 ± 0.06
RBC GPX (IU/mg prot.)	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.00	0.1 ± 0.01	0.12 ± 0.007	**0.08 ± 0.01****	**0.09 ± 0.01***
RBC SOD (IU/mg prot.)	0.61 ± 0.05	0.62 ± 0.03	**0.75 ± 0.04***	**0.93 ± 0.08***	**0.93 ± 0.09***	0.53 ± 0.058	0.47 ± 0.02
Retinol (μM)	13.11 ± 1.28	13.13 ± 0.73	13.56 ± 1.29	14.05 ± 1.30	14.56 ± 1.30	12.18 ± 1.38	7.98 ± 1.94
Retinyl palmitate (μM)	0.26 ± 0.06	**0.09 ± 0.01***	0.19 ± 0.04	0.20 ± 0.05	0.25 ± 0.06	0.22 ± 0.05	0.32 ± 0.08
Tocoferol (μM)	14.94 ± 1.66	13.39 ± 1.27	15.08 ± 1.41	14.72 ± 1.94	17.37 ± 1.81	20.46 ± 3.96	14.31 ± 6.00
Lutein (μM)	4.63 ± 0.78	4.06 ± 0.31	4.33 ± 0.36	5.13 ± 0.44	6.34 ± 0.66	7.17 ± 0.97	4.82 ± 1.33
Zeaxanthin (μM)	10.28 ± 3.58	7.34 ± 0.81	7.76 ± 0.91	9.58 ± 1.07	11.27 ± 1.46	11.17 ± 1.56	10.42 ± 2.48

Bold values indicate groups significantly different from controls

* Significantly different from controls at the $p \leq 0.05$ level

** Significantly different from controls at the $p \leq 0.01$ level

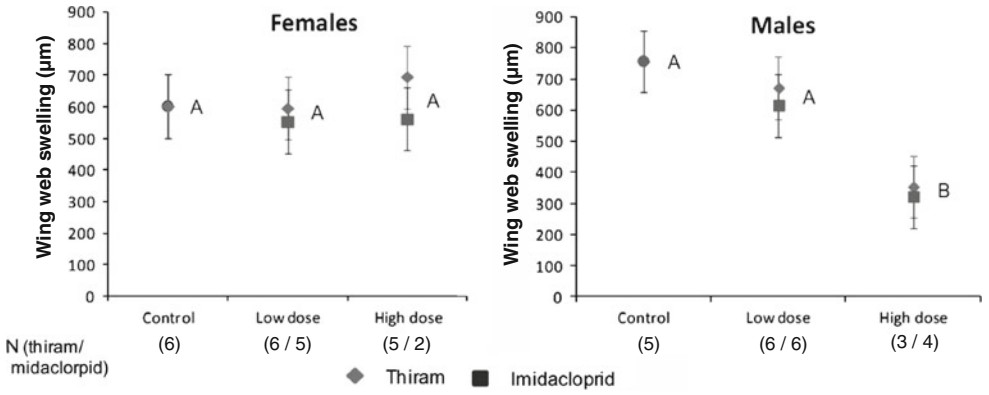

Fig. 1 Mean (±SE) cellular immune responsiveness (wing web swelling after PHA injection) as a function of treatment and sex in the thiram and imidacloprid experiments. Different letters indicate significantly different groups (LSD: $p < 0.05$)

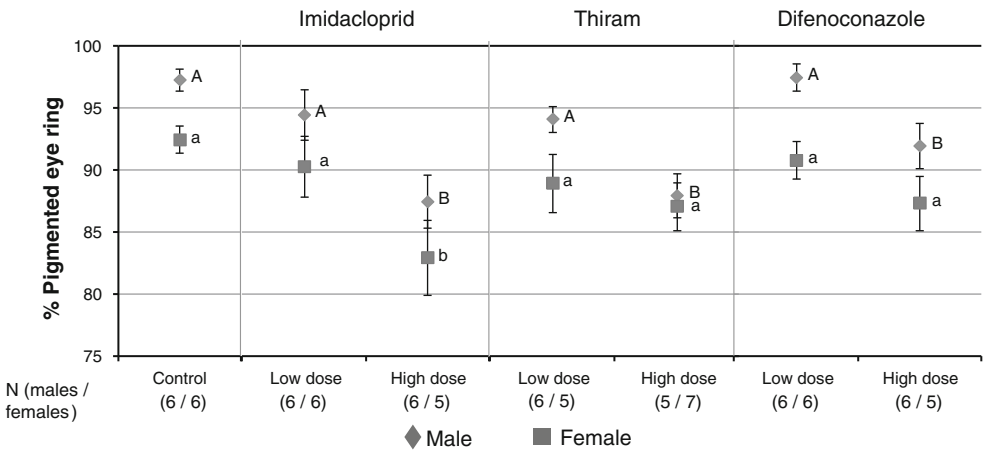

Fig. 2 Mean (±SE) percentage of eye ring area pigmented by carotenoids as a function of treatment and sex. Different letters indicate significantly different groups (LSD: $p < 0.05$)

Table 3 Mean (± SE) sexual hormone levels and reproductive parameters in each experimental group

Parameter	Control	Difenoconazole		Thiram		Imidacloprid	
		Low dose	High dose	Low dose	High dose	Low dose	High dose
N (males/females)	6/6	6/6	6/6	6/6	3/5	6/5	3/2
Testosterone (males) (ng/mL)	0.73 ± 0.57	0.21 ± 0.06	0.19 ± 0.03	0.55 ± 0.22	0.20 ± 0.15	0.21 ± 0.06	0.19 ± 0.03
Testosterone (females) (ng/mL)	0.10 ± 0.04	0.19 ± 0.07	0.10 ± 0.07	0.25 ± 0.07	0.27 ± 0.09	0.19 ± 0.07	0.10 ± 0.07
Estradiol (males) (pg/mL)	13.8 ± 4.1	18.7 ± 2.4	20.7 ± 3.8	29.2 ± 10.6	16.5 ± 5.3	18.7 ± 2.4	20.7 ± 3.8
Estradiol (females) (pg/mL)	48.4 ± 5.9	25.5 ± 7.0	37.4 ± 13.8	38.3 ± 7.8	33.4 ± 8.5	25.5 ± 7.0	37.4 ± 13.8
Number of breeding pairs	6	5	5	3	2	5	2
Number of laying females	4	4	2	3	2	2	2
Total number of eggs	40	78	40	26	10	40	34
Clutch size per female	6.7 ± 4.2	15.6 ± 4.9	8.0 ± 5.2	8.7 ± 4.6	5.0 ± 3.0	8.0 ± 5.4	17.0 ± 3.0
Egg length (mm)	40.64 ± 0.29	**38.92 ± 0.25****	**39.21 ± 0.28****	41.62 ± 0.24	**39.01 ± 1.50***	**39.45 ± 0.18****	**39.15 ± 0.20***
Egg width (mm)	29.54 ± 0.11	29.31 ± 0.10	29.13 ± 0.16	**30.04 ± 0.27***	**28.31 ± 0.77***	29.7 ± 0.09	29.55 ± 0.10
Shell thickness (mm)	0.205 ± 0.003	0.202 ± 0.003	**0.213 ± 0.003***	**0.199 ± 0.002***	**0.182 ± 0.005****	**0.190 ± 0.003****	0.204 ± 0.002
Fertile eggs (%)	85.0	**37.2****	**50.0***	57.7	60.0	**55.0***	**73.5***
Hatching rate of fertile eggs (%)	76.4	72.4	65.0	93.3	66.7	90.9	88.0
Hatching rate of total eggs (%)	65.0	26.9**	32.5**	53.8	40.0	50.0	64.7
Number of chicks	26	21	13	20	22	14	4
Chick body condition at birth[a]	13.12 ± 0.49	11.73 ± 0.54	12.44 ± 0.83	13.36 ± 0.88	10.59 ± 1.82	10.04 ± 0.65	11.31 ± 0.67
Chick mortality (%)	34.61	44.44	53.85	**71.42***	33.33	**70.00***	**76.19***
Chick mean survival time after hatching (days)	24.0 ± 2.2	22.0 ± 2.6	19.5 ± 3.4	**16.8 ± 3.2***	24.0 ± 8.0	**14.9 ± 2.6****	**13.8 ± 2.4****

Bold values indicate groups significantly different from controls

[a] See text for details about method of estimation of body condition

* Significantly different from controls at the $p \leq 0.05$ level

** Significantly different from controls at the $p \leq 0.01$ level

imidacloprid and difenoconazole assays ($p \leq 0.034$) and positively associated with egg width in the case of thiram, as well as to eggshell thickness in the two fungicide experiments ($p \leq 0.024$).

The fertile eggs rate was reduced by difenoconazole and imidacloprid ($p \leq 0.008$) when compared to controls. The hatching rate of fertile eggs was not affected by any treatment (Table 3) or by maternal body condition in imidacloprid and difenoconazole experiments. The hatching rate of total eggs was only affected by difenoconazole treatments ($p < 0.01$).

Chick survival rate was significantly lower when parents had been previously exposed to any imidacloprid dose ($p = 0.007$) as compared to controls, and the same was observed for thiram although this effect was only marginally significant ($p = 0.062$). LSD test revealed a significant difference in chick survival between control and low thiram dose groups (Table 3). Neither the maternal body condition nor any of the egg quality parameters were significantly related to chick survival.

Chicks from the imidacloprid and low thiram dose treatments showed lower body condition upon hatching than controls, although none of the pesticides significantly affected this variable when the effects of maternal body condition and egg size were introduced as covariates in the analyses (Table 3). Egg length was positively correlated to chick body condition ($p = 0.037$) whereas no treatment or covariate effects were detected for chick growth rate.

Discussion

Among the tested pesticides, imidacloprid caused more deaths, followed by thiram, whereas difenoconazole was not lethal to partridges. These results agree with reported LD50 values of these compounds for birds, as well as with reported cases of poisoning due to the ingestion of seeds treated with imidaclorprid in wild birds (partridges, pigeons and ducks) (de Snoo et al. 1999; Bro et al. 2010) and with thiram in farmed birds (Guitart et al. 1996). To our knowledge, no mortality has been reported in wild birds as a consequence of ingestion of difenoconazole-treated seeds.

Considering the estimated daily ingestion of imidacloprid (31.9–53.4 mg/kg at low and high exposure doses, respectively) and the oral LD50 of the insecticide known for other Galliforme (Japanese quail; 31 mg/kg), the mortality rates observed in the present study (8.3 and 58.3 % at low and high doses, respectively) seem to be below what could be expected. This might be attributed to inter-specific differences in sensitivity to imidacloprid exposure. However, the daily ingestion was calculated on the basis of a 25 g wheat/day intake, and the addition of pesticides may have reduced this daily food intake, and therefore the amount of pesticide ingested. This possibility is supported by the loss of body mass observed in partridges fed with imidacloprid- and thiram-treated seeds. In fact, autopsies performed on dead partridges revealed signs of emaciation in most individuals treated with these two products. Such reduction in food intake might be due, on the one hand, to the potential repellent effect of both pesticides (Avery et al. 1993; Werner et al. 2010). We have observed that partridges given the choice between untreated and treated seeds clearly rejected the treated seeds when they were coated with thiram, but did not show any preference between untreated seeds and seeds treated with imidacloprid (unpublished data). On the other hand, food intake reduction could be attributed to a toxic effect of the pesticides that made partridges feel sick and stop eating as Li et al. (2007) and Subapriya et al. (2007) observed in studies with chicken exposed to thiram.

The decrease in plasma levels of total proteins, albumin, cholesterol, calcium and magnesium, along with the decrease in haematocrit in partridges exposed to thiram and/or imidacloprid also appear to be symptoms of starvation. The decrease in total protein and albumin may be due to increased protein catabolism because of starvation, which is usually accompanied by an increase in uric acid levels (Lewandowski et al. 1986). Although we did not find such increase, the plasma level of uric acid in partridges feeding on seeds treated with the high dose of imidacloprid (23.7 mg/dL) was above the 20 mg/dL threshold considered as pathological (Lewandowski et al. 1986). Reduced cholesterol levels also typically mirror a loss of body condition in birds (Alonso-Alvarez et al. 2002). Reduced levels of calcium and magnesium in pesticide-exposed animals could be indirectly related to lower plasma albumin levels, as this protein is responsible for blood transport of these elements (Dacke 2000). Hypocalcaemia appears as a common effect in birds exposed to thiram (Edwards 1987), although in some cases it is not accompanied of a reduction in plasma albumin levels (Subapriya et al. 2007).

Exposure to imidacloprid reduced plasma level of AST in partridges. This effect has not been described before in birds and the information from the few studies conducted in mammals is contradictory. Unlike what we observed in partridges, Bhardwaj et al. (2010) found high levels of AST in rats treated with imidacloprid. In contrast, Aydin (2011) found that a chronic exposure to thiacloprid, another neonicotinic insecticide, caused a suppressive action on the enzyme in rats. Unlike imidacloprid and thiram, difenoconazole hardly caused any effect on body condition or biochemical parameters; the fungicide only reduced total protein concentration and phosphorus levels, which in birds has a poor diagnostic value (Lewandowski et al. 1986).

The production of free radicals and the consequent imbalance in the oxidant-antioxidant system is among the

main ways by which pesticides cause tissue damage (Banerjee et al. 2001). Therefore, the study of oxidative status constitutes a key tool in the identification of sublethal effects of a toxic on an organism. We have detected an increase in SOD levels in erythrocytes of animals treated with thiram and difenoconazole, although only at high exposure doses for the latter. SOD is the first line of antioxidant defence as it neutralizes the superoxide anion (O_2^-) (Knight 2000). To the best of our knowledge, this is the first study in which the oxidative stress caused by difenoconazole is assessed in wild birds. Regarding thiram, Li et al. (2007) found a decline in SOD activity of liver cells after dietary exposure in chicken. Whereas this result is contradictory to ours, we must take into account that the antioxidant levels and composition vary among species, tissues and cell types (Costantini 2008) as well as a function of age, sex, environmental conditions and diet (Banerjee et al. 2001). In fact, other studies measuring the effects of thiram on SOD in non-avian species have detected increased enzyme levels after exposure to the fungicide (Grosicka-Maciąg et al. 2008). The interaction of thiram with SOD could be due to the potential activity of the fungicide as a metal chelator, since the enzyme in the cytoplasm is Cu/Zn-dependent (Marikovsky 2002). Most studies indicate an effect of thiram on the activity of the antioxidant system, which would indicate that the fungicide is responsible for the production of free radicals forcing cells to react in order to neutralize them.

Imidacloprid also resulted in changes in the antioxidant defences, reducing both the activity of GPX and the levels of total GSH in erythrocytes. The same effects have been reported in rat liver (Kapoor et al. 2010), although imidacloprid-related increases of GPX in the same organ have also been detected in other cases (EL-Gendy et al. 2010). Immediately after the exposure to a toxicant, antioxidant enzymes tend to increase their activity; however, the activity may not be sustained because of enzyme depletion, either by decreased synthesis or by oxidative inactivation of the enzyme (Muthukumaran et al. 2008). GSH is the major intracellular antioxidant molecule and acts as substrate of several enzymes that mitigate or prevent the harmful effects of free radicals (Banerjee et al. 2001). For example, GPX uses GSH to transform H_2O_2 into H_2O (Knight 2000). The observed decrease in GSH levels could be related to this role as a substrate of antioxidant enzymes.

Although specific mechanisms of immunotoxicity by any pesticide remain poorly known (Galloway and Handy 2003), cells and organs of the immune system are known to be sensitive to damage by free radicals (Banerjee et al. 2001; Galloway and Handy 2003). This may explain the reduced cellular immune responsiveness (PHA skin test response) caused by imidacloprid and thiram, the two compounds with greater potential for causing oxidative stress. Accordingly, difenoconazole, the only compound for which immunotoxic effects were not detected, had fewer effects on oxidative status of exposed partridges. The immunotoxic effects of imidacloprid have been reported in a few cases, although no study has so far related the exposure to the insecticide to a depleted cellular immune response. Gatne et al. (2006) found a lowered humoral immune response of mice, whereas Balani et al. (2011) observed a decrease in the total number of leukocytes in poultry chicken as a consequence of imidacloprid exposure. Virtually no studies have analysed effects of fungicides on the immune system (Voccia et al. 1999) and, to our knowledge, there is nothing specifically described for thiram in this matter; only Pruett et al. (1992) reported a decrease in NK cells in rats after skin absorption of other dithiocarbamate, sodium methyldithiocarbamate.

Interestingly, the effect of pesticides on the cellular immune response affected only males. This may be because of higher testosterone levels in males—a sexual hormone that is required for expression of sexual signals but associated with immunosuppressive effects—an increased metabolic rate and the consequent production of free radicals (Alonso-Alvarez et al. 2009). In this context, none of the pesticides altered the normal levels of sex hormones, which means that the testosterone levels, and thus the potential for immunosuppressive effects, remained higher in males than in females even after pesticide exposure.

The carotenoid-based coloration of partridges, and in particular the percentage of eye-ring pigmentation, was reduced by higher doses of the three tested pesticides. In the cases of the two fungicides this effect was sex-dependent, and particularly marked in males, which invest more in carotenoid-based signalling than females (Villafuerte and Negro 1998). This kind of ornamentation is highly dependent on body condition and very sensitive to changes in the immunological and oxidative status of male partridges (Alonso-Alvarez et al. 2008; Mougeot et al. 2009). These observations, together with the aforementioned effect of testosterone on metabolic rate, make males more sensitive to energetic constrains caused by pesticide exposure, which ultimately reflects in a stronger effect on carotenoid-dependent coloration in males than in females.

We have found negative effects of the three pesticides on several reproductive parameters, including some indirect effects of imidacloprid and thiram on chick survival. However, these observed results have been obtained with a small number of surviving pairs, and therefore the obtained data must be taken with caution. Further research with lower doses of exposure and a larger number of birds is currently being developed in order to confirm or reject some of the preliminary results obtained here. These indirect effects on chicks were likely mediated via maternal effects, because offspring were never exposed themselves

to the pesticides. Physiological effects on adults such as reduced fitness, oxidative stress, immune suppression or carotenoid-dependent coloration may have further effects on reproduction. For example, a correlation between circulating carotenoids and clutch size, or a negative relation between hatching success and lipid peroxidation have been described in red-legged partridges (Bortolotti et al. 2003). At a phenotypic level, changes in carotenoid-based sexual traits may also affect reproduction in birds (e.g. Giraudeau et al. 2011). In terms of reproduction, the results of the present study constitute, to the best of our knowledge, the first published report about the effect of imidacloprid on vertebrates and about the effect of thiram and difenoconazole on birds.

We have found that some of the reproductive effects of pesticide exposure appeared at low doses but not at high ones (i.e. imidacloprid effects on shell thickness and thiram effects on chick survival). The lack of effect at the highest exposure doses may be due to several reasons; first, because most mortality occurred at higher doses, the surviving individuals may have been more resistant to pesticide toxicity. This is in agreement with the observation that stronger effects at lower doses were found for imidacloprid and thiram, the two compounds that caused significant mortality at high doses. Second, the potential rejection of treated seeds, especially those treated with thiram, may have led to a lower ingestion of seeds treated with high concentrations of pesticides. Partridges exposed to high doses might have suffered from anorexia, but this effect would be less severe than the intoxication caused by the ingestion of seeds treated with the lower dose.

Thiram-exposed females laid abnormally small eggs, narrower and with a thinner shell than controls. This effect of thiram has been described in previous studies on poultry after accidental ingestion of seeds treated with the fungicide (Guitart et al. 1996). Some of these effects were also found in eggs laid by difenoconazole- and imidacloprid-exposed females, but, to our knowledge, no study had related the exposure to these pesticides to egg quality. Apart from affecting egg biometry, difenoconazole and imidacloprid also reduced fertile eggs rate when compared to controls. These two effects are likely interrelated in the red-legged partridge, since lighter and smaller eggs are more likely to be unfertile (Cabezas-Díaz and Virgós 2007).

Egg size is related to chick condition at hatching, which is in turn related to chick survival (Blomqvist et al. 1997). Our results confirm that these parameters are correlated, but this relationship does not explain by itself the differences among pesticide treatments in chick survival. The effects of pesticides on offspring could also be mediated by effects on egg quality, in terms of amounts of carotenoids and antioxidants, hormones or enzymes (Berthouly et al. 2007; Cucco et al. 2008). The negative effects of maternal exposure to pesticides on chick body condition—indirectly through a reduction of egg size—are important when extrapolating the results to field conditions; in the wild, where animals may suffer from food limitation and continued predation risk, these parameters might have a greater influence on chick survival than what we can estimate from our experimental approach.

This experimental study suggests that pesticide abuses could represent a serious threat of intoxication and mortality in an important game bird such as the red-legged partridge. The results also highlight the importance of considering sub-lethal effects of coated seed ingestion by farmland birds, even at recommended doses of pesticide use, in terms of their physiological and reproductive effects. Moreover, imidacloprid and thiram are also applied with the irrigation water on crops often used by partridges such as vineyards and olive groves. As it occurs with treated seeds, irrigation water with dissolved agrochemicals can represent as significant risk for wildlife (i.e. nitrates; Rodríguez-Estival et al. 2010). Although we have used as model organism one of the bird species potentially more exposed to coated seed ingestion, similar effects as those reported for the red-legged partridge could occur in other threatened birds that share the same habitats and eat seeds, such as the pin-tail sandgrouse (*Pterocles alchata*), black-bellied sandgrouse (*Pterocles orientalis*) or great bustard (*Otis tarda*). Whereas our results suggest that treated seeds, under certain circumstances and regarding specific active ingredients, may constitute a risk for farmland birds, we must take into account that potential exposure was not characterized in the present study. Next steps should include an evaluation of the real exposure to coated seed ingestion by wild birds, including feeding behaviour analyses and estimation of food intake rates. Nevertheless, the negative effects documented here could well be enhanced under natural conditions because of additional stressing factors such as predation, food scarcity or lack of optimal breeding and sheltering habitats.

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References

Alonso-Alvarez C, Ferrer M, Velando A (2002) The plasmatic index of body condition in yellow-legged Gulls *Larus cachinnans*: a food-controlled experiment. Ibis 144:147–149

Alonso-Alvarez C, Pérez-Rodríguez L, Mateo R, Chastel O, Viñuela J (2008) The oxidation handicap hypothesis and the carotenoid allocation trade-off. J Evol Biol 21:1789–1797

Alonso-Alvarez C, Pérez-Rodríguez L, Garcia JT, Viñuela J (2009) Testosterone-mediated trade-offs in the old age: a new approach

to the immunocompetence handicap and carotenoid-based sexual signalling. Proc R Soc B Biol Sci 276:2093–2101
Avery ML, Decker DG, Fischer DL, Stafford TR (1993) Responses of captive blackbirds to a new insecticidal seed treatment. J Wildl Manag 57:652–656
Aydin B (2011) Effects of thiacloprid, deltamethrin and their combination on oxidative stress in lymphoid organs, polymorphonuclear leukocytes and plasma of rats. Pestic Biochem Physiol 100:165–171
Balani T, Agrawal S, Thaker AM (2011) Hematological and biochemical changes due to short-term oral administration of imidacloprid. Toxicol Int 18:2–4
Banerjee BD, Seth V, Ahmed RS (2001) Pesticide-induced oxidative stress: perspectives and trends. Rev Environ Health 16:1–40
Berthouly A, Helfenstein F, Richner H (2007) Cellular immune response, stress resistance and competitiveness in nestling great tits in relation to maternally transmitted carotenoids. Funct Ecol 21:335–343
Bhardwaj S, Srivastava MK, Kapoor U, Srivastava LP (2010) A 90 days oral toxicity of imidacloprid in female rats: morphological, biochemical and histopathological evaluations. Food Chem Toxicol 48:1185–1190
Blomqvist D, Johansson OC, Götmark F (1997) Parental quality and egg size affect chick survival in a precocial bird, the lapwing *Vanellus vanellus*. Oecologia 110:18–24
Bortolotti GR, Negro JJ, Surai PF, Prieto P (2003) Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. Physiol Biochem Zool 76:367–374
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
Bro E, Decors A, Millot F, Soyez D, Moinet M, Berny P, Mastain O (2010) Intoxications des perdrix grises en nature. Nouveau bilan de la surveillance «SAGIR». Faune Sauvag 289:26–32
Cabezas-Díaz S, Virgós E (2007) Adaptive and non-adaptive explanations for hatching failure in eggs of the red-legged partridge *Alectoris rufa*. Ardea 95:55–63
Costantini D (2008) Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238–1251
Cucco M, Guasco B, Malacarne G, Ottonelli R, Tanvez A (2008) Yolk testosterone levels and dietary carotenoids influence growth and immunity of grey partridge chicks. Gen Comp Endocrinol 156:418–425
Dacke CG (2000) The parathyroids, calcitonin and vitamin D. In: Whittow GC (ed) Sturkie's avian physiology. Academic Press, San Diego, pp 473–488
de Snoo GR, Scheidegger NMI, de Jong FMW (1999) Vertebrate wildlife incidents with pesticides: a European survey. Pest Manag Sci 55:47–54
Edwards HM Jr (1987) Effects of thiuram, disulfiram and a trace element mixture on the incidence of tibial dyschondroplasia in chickens. J Nutr 117:964–969
EL-Gendy KS, Aly NM, Mahmoud FH, Kenawy A, El-Sebae AKH (2010) The role of vitamin C as antioxidant in protection of oxidative stress induced by imidacloprid. Food Chem Toxicol 48:215–221
Galloway T, Handy R (2003) Immunotoxicity of organophosphorous pesticides. Ecotoxicology 12:345–363
Gatne MM, Ramesh Bhoir PS, Deore MD (2006) Immunotoxicity studies of imidacloprid in rats. Toxicol Int 13:89–92
Geiger F, Bengtsson J, Berendse F, Weisser WW, Emmerson M, Morales MB, Ceryngier P, Liira J, Tscharntke T, Winqvist C, Eggers S, Bommarco R, Pärt T, Bretagnolle V, Plantegenest M, Clement LW, Dennis C, Palmer C, Oñate JJ, Guerrero I, Hawro V, Aavik T, Thies C, Flohre A, Hänke S, Fischer C, Goedhart PW, Inchausti P (2010) Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. Basic Appl Ecol 11:97–105
Giraudeau M, Duval C, Czirják GÁ, Bretagnolle V, Eraud C, McGraw KJ, Heeb P (2011) Maternal investment of female mallards is influenced by male carotenoid-based coloration. Proc R Soc B Biol Sci 278:781–788
Goto N, Kodama H, Okada K, Fujimoto Y (1978) Suppression of phytohemagglutinin skin response in thymectomized chickens. Poult Sci 57:246–250
Griffiths R, Double MC, Orr K, Dawson RJ (1998) A DNA test to sex most birds. Mol Ecol 7:1071–1075
Grosicka-Maciąg E, Kurpios D, Czeczot H, Szumiło M, Skrzycki M, Suchocki P, Rahden-Staroń I (2008) Changes in antioxidant defense systems induced by thiram in V79 Chinese hamster fibroblasts. Toxicol In Vitro 22:28–35
Guitart R, Mateo R, Gutierrez JM, To-Figueras J (1996) An outbreak of thiram poisoning on Spanish poultry farms. Vet Hum Toxicol 38:287–288
Hart ADM (1990) The assessment of pesticide hazards to birds: the problem of variable effects. Ibis 132:192–204
Hudson RH, Tucker RK, Haegele MA (1984) Handbook of toxicity of pesticides to wildlife. Resource publication 153. United States Department of the Interior, Fish and Wildlife Service, Washington, DC
Kapoor U, Srivastava MK, Bhardwaj S, Srivastava LP (2010) Effect of imidacloprid on antioxidant enzymes and lipid peroxidation in female rats to derive its No Observed Effect Level (NOEL). J Toxicol Sci 35:577–581
KEM (2006) Draft assessment report. Difenoconazole, vol 3. Swedish Chemicals Inspectorate, Sundbyberg
Knight JA (2000) Review: free radicals, antioxidants, and the immune system. Ann Clin Lab Sci 30:145–158
Korhonen A, Hemminki K, Vainio H (1982) Application of the chicken embryo in testing for embryotoxicity. Thiurams. Scand J Work Environ Health 8:63–69
Lewandowski AH, Campbell TW, Harrison GJ (1986) Clinical chemistries. In: Harrison GJ, Harrison LR (eds) Clinical avian medicine and surgery. W.B. Saunders Company, Philadelphia, pp 192–200
Li J, Bi D, Pan S, Zhang Y (2007) Effect of diet with thiram on liver antioxidant capacity and tibial dyschondroplasia in broilers. Br Poult Sci 48:724–728
Marikovsky M (2002) Thiram inhibits angiogenesis and slows the development of experimental tumours in mice. Br J Cancer 86:779–787
Mineau P (2005) A review and analysis of study endpoints relevant to the assessment of "long term" pesticide toxicity in avian and mammalian wildlife. Ecotoxicology 14:775–799
MMAMRM (2010) Registro de productos fitosanitarios. Ministerio de Medio Ambiente y Medio Rural y Marino. http://www.magrama.gob.es/es/agricultura/temas/medios-de-produccion/productos-fitosanitarios/fitos.asp. Accessed 06 Mar 2010
Mougeot F, Pérez-Rodríguez L, Sumozas N, Terraube J (2009) Parasites, condition, immune responsiveness and carotenoid-based ornamentation in male red-legged partridge *Alectoris rufa*. J Avian Biol 40:67–74
Muthukumaran S, Sudheer AR, Menon VP, Nalini N (2008) Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicology 243:207–215
Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118:1883–1891
Prosser PJ, Hart ADM, Langton SD, McKay HV, Cooke AS (2006) Estimating the rate of poisoning by insecticide-treated seeds in a bird population. Ecotoxicology 15:657–664

Pruett SB, Barnes DB, Han YC, Munson AE (1992) Immunotoxicological characteristics of sodium methyldithiocarbamate. Fundam Appl Toxicol 18:40–47

Reglero MM, Taggart MA, Monsalve-González L, Mateo R (2009) Heavy metal exposure in large game from a lead mining area: effects on oxidative stress and fatty acid composition in liver. Environ Pollut 157:1388–1395

Rodríguez-Estival J, Martínez-Haro M, Martín-Hernando MP, Mateo R (2010) Sub-chronic effects of nitrate in drinking water on red-legged partridge (*Alectoris rufa*): oxidative stress and T-cell mediated immune function. Environ Res 110:469–475

Siddiqui A, Choudhary M, Goriya HV, Bhavsar SK, Thaker AM (2007) Evaluation of immunotoxic effect of short-term administration of quinalphos and imidacloprid in white leghorn cockerels. Toxicol Int 14:15–19

Stanley PI, Bunyan PJ (1979) Hazards to wintering geese and other wildlife from the use of dieldrin, chlorfenvinphos and carbophenothion as wheat seed treatments. Proc R Soc Lond B Biol Sci 205:31–45

Subapriya S, Vairamuthu S, Manohar BM, Balachandran C (2007) Growth performance studies in thiram toxicosis in broiler chicken. Int J Poult Sci 6:248–250

Tomlin CDS (2004/05) Imidacloprid (138261-41-3). In: The e-pesticide manual, version 3.1, 13th edn. British Crop Protection Council, Surrey

Villafuerte R, Negro JJ (1998) Digital imaging for colour measurement in ecological research. Ecol Lett 1:151–154

Voccia I, Blakley B, Brousseau P, Fournier M (1999) Immunotoxicity of pesticides: a review. Toxicol Ind Health 15:119–132

Werner SJ, Linz GM, Tupper SK, Carlson JC (2010) Laboratory efficacy of chemical repellents for reducing blackbird damage in rice and sunflower crops. J Wildl Manag 74:1400–1404

Wilson JD, Morris AJ, Arroyo BE, Clark SC, Bradbury RB (1999) A review of the abundance and diversity of invertebrate and plant foods of granivorous birds in northern Europe in relation to agricultural change. Agric Ecosyst Environ 75:13–30