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Imidacloprid-treated seed ingestion has lethal effect on adult partridges and reduces both breeding investment and offspring immunity ☆

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ABSTRACT

The ingestion of imidacloprid treated seeds by farmland birds may result in exposure to toxic amounts of this insecticide. Here we report on the effects that the exposure to the recommended application rate and to 20% of that rate may produce on birds feeding on treated seeds. Experimental exposure to imidacloprid treated seeds was performed on red-legged partridges (*Alectoris rufa*) ($n=15$ pairs per treatment group: control, 20% or 100% of the recommended application rate) during two periods that corresponded to the autumn (duration of exposure: 25 days) and late winter (10 days) cereal sowing times in Spanish farmlands. We studied effects on the survival, body condition, oxidative stress biomarkers, plasma biochemistry, carotenoid-based coloration, T-cell mediated immune response and reproduction of exposed adult partridges, and on the survival and T-cell immune response of their chicks. The high dose (recommended application rate) killed all partridges, with mortality occurring faster in females than in males. The low dose (20% the recommended application rate) had no effect on mortality, but reduced levels of plasma biochemistry parameters (glucose, magnesium and lactate dehydrogenase), increased blood superoxide dismutase activity, produced changes in carotenoid-based integument coloration, reduced the clutch size, delayed the first egg lay date, increased egg yolk vitamins and carotenoids and depressed T-cell immune response of chicks. Moreover, the analysis of the livers of dead partridges revealed an accumulation of imidacloprid during exposure time. Despite the moratorium on the use of neonicotinoids in the European Union, birds may still be at high risk of poisoning by these pesticides through direct sources of exposure to coated seeds in autumn and winter.

1. Introduction

In the last decades, farmland birds in Europe and North America have been suffering population declines at higher rates than birds from other habitats (EBCC, 2014). Farmland is being profoundly altered through agricultural intensification, posing a major challenge for biodiversity conservation today in many countries (Krebs et al., 1999). Recent studies have pointed out that a major cause of bird population declines is the use of pesticides, either because of indirect effects on habitat and food supply (Hallmann et al., 2014; Goulson, 2014) or because of direct toxic effects on the health of birds (Mineau and Whiteside, 2013). A greater probability of lethality in birds occurs when the ratio between the LD50 and the estimated field exposure dose is low (EFSA, 2009). Pesticides with higher LD_{50} or lower risk of exposure can produce a range of sub-lethal effects such as loss of physical condition, immunosuppression, neurological impairments or endocrine disruption (Fry, 1995). All these effects may ultimately affect survival or reproduction, and therefore impact on population dynamics.

Imidacloprid is a systemic insecticide belonging to the family of neonicotinoids and it is currently the first insecticide and the second agrochemical most used in the world (Jeschke et al., 2011; Goulson, 2013). This pesticide acts by binding to specific nicotinic acetylcholine receptors, thus interfering with the transmission of nerve impulses. Starting in December 2013, the European Union declared a moratorium on the use of three neonicotinoid insecticides (i.e. imidacloprid, thiamethoxam and clothianidin) for

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seed coating, soil treatment and foliar treatment due to its toxicity on pollinators, but their use for seed treatment of winter cereals, as well as after crop flowering and in crops harvested before flowering, continues to be approved (Regulation 485/2013). Imidacloprid oral acute LD_{50} for birds 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 the field, there are some documented cases of wild bird mortalities due to the ingestion of seeds treated with imidacloprid (Berny et al., 1999; Ibáñez et al., 2011; Bro et al., 2010; Mineau and Palmer, 2013).

Imidacloprid is predominantly used as seed coating in a large variety of crops (Jeschke et al., 2011; Goulson, 2013). In the 20th century seed coating was responsible for up to 50% of incidents on wildlife caused by approved pesticides in Europe (De Snoo et al., 1999). Since that, the niche market for insecticidal treated seed has tripled and neonicotinoids monopolized 77% of this market in 2005 (Elbert et al., 2008). Farmland birds are at risk of exposure to pesticide-treated seeds because sown seeds are not always properly buried in the field. The US Environmental Protection Agency estimated that about 1% of the drilled seeds remain accessible for granivorous vertebrates. In addition, occasional spillages during sowing activities (especially at field corners and extremes of sowing lines) may also be attractive for foraging birds and increase the risk of exposure to pesticides. Under such circumstances, treated seed ingestion can result in the intake of a high amount of a toxic pesticide over a short foraging time period. It is estimated that a farmland bird could get a lethal dose with the ingestion of less than five imidacloprid treated seeds (Goulson, 2013; Mineau and Palmer, 2013).

In a previous 10 day-exposure study performed in spring with adult red-legged partridges (*Alectoris rufa*), animals were exposed to seeds treated with the recommended application rate for cereal seed coating and with twice this rate (to assess the effects of potential abuses in pesticide application). The highest imidacloprid dose used in that study (twice the recommended application rate) was shown to reduce exposed partridge survival, whereas both doses produced physiological and biochemical changes, affected fertility and decreased offspring survival (Lopez-Antia et al., 2013). Similar adverse effects on biochemical, oxidative stress and immune system parameters have been reported in poultry (Siddiqui et al., 2007; Balani et al., 2011; Kammon et al., 2012; Gibbons et al., 2014). In the current work, we wanted to test if these effects still occur under a more realistic scenario, in which coated seeds represent only part (20%) of an adult partridge's diet. We also considered two exposure periods that correspond to the sowing seasons of autumn (long-cycle winter cereal) and late winter (short-cycle winter cereal) in Spanish farmlands. Moreover, our main interest was to study the impact of the insecticide on reproduction, as the published literature in this regard is limited to only one preliminary study (Lopez-Antia et al., 2013); in that experiment the reproductive results were obtained from a small number of breeding pairs and thus should be confirmed. In the current study, based on a larger sample size, we also studied new reproductive performance parameters (egg quality and content; offspring immunity), in order to obtain a better understanding of the indirect effects of imidacloprid on reproduction.

We hypothesized that exposure to field doses of imidacloprid would produce oxidative system imbalance and would impact on important health parameters, such as the immune system, or on the reproductive system (Banerjee et al., 2001; Sheweita et al., 2005; Agarwal et al., 2012). Carotenoid-based ornamentation has been proposed as a good indicator of oxidative damage and general health status (Pérez-Rodriguez and Viñuela, 2008) and is known to influence reproductive investment (Alonso-Alvarez et al., 2012). The simultaneous investigation of effects on all these variables should help us to better understand the range of effects and toxicity mechanism of imidacloprid.

2. Material and methods

2.1. Experimental design

The experiment was conducted in the Dehesa de Galiana experimental facilities (Ciudad Real, Spain). All experimental protocols were approved by the Committee on Ethics and Animal Experimentation of the University of Castilla-La Mancha. We used 96 (51 females and 45 males) captive-born, one year-old red-legged partridges from the experimental farm of the University of Castilla-La Mancha. The sex of individuals was determined genetically following Fridolfsson and Ellegren (1999). Partridges were housed in pairs in outdoor cages ($95 \times 40 \times 42$ cm^3) 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 three experimental groups (control, low dose or high dose) with a sample size of 15 pairs in each group. Six additional females were housed and assigned to one of the three treatments in order to have a replacement in cases of deaths by male aggressions.

The first exposure began on November 26, 2010 and the second one on March 4, 2011. During exposure periods, 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 first exposure lasted for 25 days and the second one for 10 days, which correspond to the duration of the two cereal sowing seasons in Spain (a longer season in autumn and a shorter one in late winter). At the end of the exposure periods, partridges returned to their usual diet of untreated wheat mixed with maintenance fodder. On December 21 and March 14 (after the first and the second exposure periods, respectively), we took a blood sample from each partridge. 1 mL of blood was drawn by jugular venipuncture, 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 samples were stored separately at −80 °C for later analysis. Before centrifugation, an aliquot of each sample was taken to calculate the hematocrit. Partridge pairs were kept in their cages throughout the spring and early summer in order to monitor reproduction (see below).

2.2. Seeds treatment and exposure doses

Seeds were treated with the commercial product Escocet® (imidacloprid 35% w/v, Bayer CropScience, Alcácer, Spain) using a professional hand sprayer (Apollo 5, EXEL gsa, Villefranche, France). We used two application doses; the highest dose corresponded to the recommended application rate for cereal seed coating according to the current Spanish regulations (MAGRAMA, 2013), and the lowest dose was set at 20% of the recommended application rate, which would represent an intake of 20% of treated seeds in the diet. This estimation of sowing seed occurrence in the diet of red legged partridge is based on data given by Perez y Perez (1981).

The doses applied to the seeds were 40–200 mL of imidacloprid (35%) per 100 kg of wheat seeds (theoretical concentration: 0.14–0.7 mg/g). In a recent study using the same application technique, we verified that the actual concentration of imidacloprid in seeds treated using the recommended application rate was 74% of the nominal concentration (Lopez-Antia et al., 2013).

Captive partridges ate on average 25 g of wheat per day (Lopez-Antia et al., 2014). Partridges from this experiment weighed on average 397 g. We estimated that the daily ingestion dose for the high and the low dose groups would be of 44 and 8.8 mg/kg/day, respectively. With these daily ingestion doses, partridges from the high dose group would reach the LD_{50} (31 mg/kg for the Japanese quail) in less than one day, and those from the low dose group ones in less than four days. These ingestion doses will, however, be modulated by the rejection of treated seeds due to a post-ingestion distress (Lopez-Antia et al., 2014).

2.3. Survival and body condition of adult partridges

During the experiment, we checked partridges' survivorship daily. We initially measured tarsus length and weighed each partridge four times throughout the experiment: prior to and just after each pesticide exposure. The body condition of partridges (mass corrected for size) was calculated according to the scaled mass index proposed by Peig and Green (2009).

2.4. Immune response of adult partridges

On January 24, about a month after finishing the first exposure period, we estimated cell-mediated immune responsiveness using the phytohemagglutinin (PHA)-skin test. For each partridge, we took three measures of the right wing web thickness at the injection site 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). PHA is a lectin that causes an accumulation of T-lymphocytes followed by an infiltration of macrophages, 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 (three measures), and estimated the intensity of the T-cell-mediated immune response as the change in average wing web thickness (difference between the final and the initial thicknesses).

2.5. 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. Reproductive parameters could be obtained for 24 reproductive pairs after the second exposure period (12 control pairs and 12 pairs in the low dose group). Breeding (egg laying) began one month after the end of the second exposure period. We checked all cages daily and collected eggs, which were measured (maximum length and width) and kept at 15 °C to temporarily prevent 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. The storage time (0–15 days) was included in the early statistical models but had no significant effect on embryo and chick development. Before transferring eggs to the incubation chamber, eggs laid in 4th, 8th and 12th position of the laying sequence of each pair were separated and kept frozen at −80 °C for analyzing egg yolk vitamins and carotenoids. On the 21st day of incubation, eggs were candled and those that were found to have developed were introduced in individual cages and moved to a hatching chamber where they were incubated at 37.7 °C with constant humidity but without movement. We checked the chamber daily to monitor hatching events. We took note of hatching date, 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. Some chicks from the third and fourth round of incubation were selected for the PHA test, excluding first chicks of each pair. PHA test was performed as has been described for adults. Chick sex was determined genetically following Fridolfsson and Ellegren (1999).

Unhatched eggs were opened and examined to determine fertility (observation of the presence of embryo or germinal disk). To measure shell thickness of hatched and unhatched eggs, we collected three small shell pieces (approximately 0.5×0.5 cm^2) from the equatorial region of each egg; we separated the inner membrane, dried the shell pieces and measured shell thickness with a micrometer to the nearest 0.01 mm. Eggshell thickness was calculated as the average thickness of the three measurements.

2.6. Imidacloprid levels in dead partridges and egg yolk samples

Imidacloprid concentrations in the crop contents of partridges found dead during the experiments were measured by LC–MS, as described for seeds in Lopez-Antia et al. (2013). The concentrations in liver and egg yolk samples were determined following the method described by Sanchez-Barbudo et al. (2012) with some modifications. A sample (1 g) was homogenized with anhydrous sodium sulfate (9 g) in a mortar, the homogenate was placed in glass tubes with Teflon caps. Then, 0.5 μg of thiacloprid (Sigma-Aldrich; Madrid, Spain) from a solution with 10 ng/μL was added as internal standard, as well as 15 mL of acetonitrile. The samples were stirred for 10 min in a horizontal shaker (SH30L; Finepcr, Seoul, Korea), followed by 5 min of sonication (Ultrasons-H Selecta; Abrera, Spain). The extracts were filtered and washed twice with 5 mL of acetonitrile, evaporated in a rotary evaporator (Büchi; Flawil, Switzerland) at 100 mbar and 40 °C and re-suspended in 2 mL of ethyl acetate:cyclohexane (1:1 v/v). Extract purification was done by gel permeation chromatography (GPC) at atmospheric pressure in a glass column with an internal diameter of 17.25 mm and filled with 43.5 cm of Bio-Beads S-X3 (Bio-Rad Laboratories; Madrid, Spain). The mobile phase was ethyl acetate: cyclohexane (1:1 v/v). The fraction corresponding to 0–60 mL was discarded. The 60–90 mL fraction was collected and evaporated with a rotary evaporator until an approximate volume of 1 mL, transferred to a vial and evaporated to dryness under nitrogen flow, re-suspended in 0.5 mL of acetonitrile and analyzed by LC–ESI–MS using the method described by Lopez-Antia et al. (2013) with some modifications. The monitored ions of imidacloprid were 256, 257 with fragmentation voltage at 100 and 175, at 170. The ion used for quantification was 256. The monitored ions of thiacloprid used as internal standard were 133.1, 253, 254, 255 and 256 with fragmentation voltage at 120 and 126, 127 and 128 with voltage at 160. The ion used for quantification was 253. The recovery of the analytical procedure was calculated with five replicates of blank liver of partridge (1 g), spiked with 0.5 μg of imidacloprid, and processed as the samples. The obtained recovery for imidacloprid was $82.1 \pm 2.3\%$. The limit of detection (LOD) was established at 5 ng/g by serial dilutions of the standard and a signal to noise ratio (S/N) > 3 and the limit of quantification (LOQ) was established at 16.7 ng/g of sample (S/N > 10).

2.7. Biochemical responses of adult partridges

We measured oxidative stress indicators in red blood cells (RBC) homogenates with an automatic spectrophotometer analyzer A25 (BioSystems, Barcelona, Spain) following the methods described in Reglero et al. (2009). We quantified the total (GSH) and oxidized glutathione (GSSG) levels. We used Ransel and Ransod kits (Randox Laboratories, Cornellà 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 measured using the Bradford method to quantify total proteins in the homogenates (Bradford, 1976). The coefficients of variation (CV) of these analytical techniques calculated with sample replicates were 3.1% for SOD ($n=6$) and 2.0% for GPx ($n=3$). Moreover, the analyses of control samples provided by the manufacturer were always within the certified range of SOD and GPX activities. For GSH and GSSG techniques we calculated a CV with sample replicates ($n=6$) of 3.1% and 6.0%, respectively.

We measured lipid peroxidation as levels of malondialdehyde (MDA) in the RBC homogenates. For this, MDA-tiobarbituric acid adducts were measured as described in Romero-Haro and Alonso-Alvarez (2014) with an Agilent 1100 Series High Performance Liquid Chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) coupled with a fluorescence detector (FLD) and a 5 μm ODS-2 C-18 (4.0×250 mm^2) column, maintained at 37 °C. The CV of this analytical technique was 8.0% ($n=10$).

We determined the levels of the following antioxidants molecules: retinol (distinguishing the free, alcoholic, form and the form esterified with fatty acids), α-tocopherol and carotenoids (zeaxanthin and lutein), in plasma and eggs yolk using HPLC coupled to a photodiode detector (DAD) and a FLD. The method used for extraction and analysis of plasmas antioxidants molecules is described in Rodríguez-Estival et al. (2010). For all the antioxidant molecules in plasma we calculated a CV with samples replicates ($n=14$) of $\leq 8.6\%$. For the egg yolk the method used for extraction was the same as for plasma, but a single extraction with dichloromethane was made. The analytical methods were exactly the same for both sample types. For all the antioxidant molecules in the egg yolk measured in samples replicates ($n=6$) we obtained a CV $\leq 12.2\%$.

Plasma biochemistry was determined spectrophotometrically using the reaction kits available for each enzyme or analyte (Bio-Systems, Barcelona, Spain): 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. For these techniques, a control sample provided by the kit manufacturer was analyzed and results were always within the certified range.

The measure of brain acetylcholinesterase (AChE) was performed according to the Ellman's method with the modifications described by Hill and Fleming (1982). Activities were compared with values obtained with six samples of control partridges that died of natural causes.

2.8. Color measurements of adult partridges.

The red coloration of the partridges' beak and eye rings is carotenoid-based and relates to individual health (Mougeot et al., 2009) and investment in reproduction (Alonso-Alvarez et al., 2012). Eye ring and beak color were measured before (February, 24) and after (March, 14) the second exposure using a portable spectrophotometer (Minolta CM-2600 d; Tokyo, Japan). For each study bird, we took three measurements of both the eye ring and beak color. Reflectance spectrum was determined from 360 to 700 nm wavelength at 10-nm intervals. Most of the variability in the reflectance of carotenoid-based colors of red-legged partridge ornaments is distributed in the red part of the spectrum (625–700 nm; Pérez-Rodriguez and Viñuela, 2008; Alonso-Alvarez and Galvan, 2011), and therefore covered by the spectrum range of measurements. We extracted and processed the reflectance data using the SpectraMagic™ NX software (Konica Minolta). For each color measurement, we obtained "*L*", "*a*", "*b*" values ("Lab" color space) that were used to calculate the hue and chroma of each measured trait. Color measurements (eye ring and beak hue and chroma) were highly repeatable (repeatability calculated following Lessells and Boag (1987); all R-values > 0.92; all $P < 0.001$). We used the average values from the three measurements taken on each individual part for analyses of color variation. Color hue is inversely related to red shift (higher hue values describe more orange eye-rings or beaks, while lower hue values describe redder eye-rings or beaks). Chroma values are indicative of color saturation (the higher the chroma, the greater the color purity and perceived color intensity).

2.9. Statistical analyses

Adult partridge survival was analyzed using a Kaplan–Meier survival analysis and the Mantel–Cox test for pairwise comparisons among treatment groups and among sexes within groups. For blood parameters measured both during the first and second exposure (oxidative stress biomarkers, plasma biochemistry, vitamins and carotenoids), we first analyzed treatment effects using data from both exposure periods using mixed models, which included the individual as a random effect, and the season, dose and the interaction season × dose as fixed effects (in order to asses whether treatment effect differed between exposure seasons; see Table S3). We subsequently analyzed the data from each exposure period separately. We did so to report season-specific exposure effects and because not all parameters were measured for all birds in both seasons (hence, sample size for each separate season is greater than for analyses that combined both seasons). We used Generalized Linear Models (GLMz) with the experimental dose and the sex as fixed factors in order to test for imidacloprid effects on body condition, body weight, body weight loss, blood parameters (i.e. hematocrit, oxidative stress indicators, plasma biochemistry, vitamins and carotenoids), PHA response and carotenoid-based coloration of integuments. Initial models testing for treatment effects on adult partridge traits included the Sex × Treatment interaction, which was removed from the final models when non-significant. For body condition and body weight, we checked that there were no initial differences between groups. For analyses of carotenoid-based coloration after the second exposure, we included the initial color measurements (before exposure) as a covariate. For adult response to PHA, we used body condition as covariate. We used a quadratic correlation to analyze the relationship between imidacloprid levels in liver and number of survival days. To test for treatment effects on reproductive parameters, we performed GLMz using two databases: 1) Eggs and chicks as experimental units. We performed analyses with the pesticide dose as fixed factor and the breeding pair as a nested factor within the treatment group. Egg measurements (length, width, eggshell thickness, vitamin and carotenoid levels), chick survival days and chick condition at hatching were analyzed as linear distribution variables, while fertile egg rate, hatching rates (of fertile eggs and of total eggs) and chick mortality were analyzed as binary logistic distribution variables. Chick response to PHA was analyzed using the dose as a fixed factor and the chick hatching condition as a covariate. Differences in chick growth were tested with a general linear model of repeated measures with the dose as a fixed factor. 2) Breeding pair as the experimental unit. This data base was used to test for differences among treatment groups in mean clutch size, number of laying females, sex ratio and latency to first egg. The mean clutch size and the latency to the first egg were analyzed as linear distribution variables, the laying females were analyzed with a binary logistic distribution, and the sex ratio was also analyzed with a binary logistic distribution where we compared between doses the number of female chicks, weighed by the total number of chicks.

Statistical analyses were performed with IBM SPSS Statistics 19.0. Significance was established at $p < 0.05$, but results with $p < 0.1$ have been also commented and considered as marginally significant.

3. Results

3.1. Effects on adult partridges

Imidacloprid treatment at the high dose killed all partridges in 21 days, with lethality occurring earlier in females than in males ($\chi^2 = 7.74$, $p = 0.005$) (Fig. 1), the mean survival time for the high dose group was 6.7 ± 1.1 days for females and 12.7 ± 1.8 for males. The first deaths occurred on the third day of autumn treatment (10 partridges died this day, eight females and two males). This mortality rate in the high dose group (100%) was higher than in the low dose group (18.7%; $\chi^2 = 44.75$, $p < 0.001$) and in the control group (15.6%; $\chi^2 = 60.16$, $p < 0.001$) (Table S1). Imidacloprid was detected in crop and liver of partridges dying during the exposure periods (Table 1), and a positive quadratic relationship was found between the survival days during the exposure time and the concentration of imidacloprid in liver in the high dose group ($F_{2,16} = 9.57$; P = 0.0018; $r = 0.738$; Fig. 2). Insecticide levels (in the crop and liver of dead partridges) did not significantly differ between the low and high dose groups (Table 1). Partridges treated with the low dose of imidacloprid presented a reduced body condition after the first exposure (Wald $\chi^2 = 8.98$, $p = 0.003$), but not after the second exposure, compared with controls (Fig. 3). Body weights of exposed partridges were significantly lower than those of controls after both exposure periods (Wald $\chi^2 = 21.9$, $p < 0.001$ and Wald $\chi^2 = 12.8$, $p < 0.001$ respectively), and were also lower before the second exposure period (Wald $\chi^2 = 6.24$, $p = 0.012$; Table S1). When considering body weight loss, partridges from the low dose group lost significantly more weight than controls after the first exposure (Wald $\chi^2 = 7.83$, $p = 0.005$), and a similar, marginally significant trend was also found after the second exposure (Wald $\chi^2 = 3.68$, $p = 0.055$) (Table S1). Cellular immune responsiveness (inflammatory response to PHA) did not differ between controls and low dose adult partridges (Table S1).

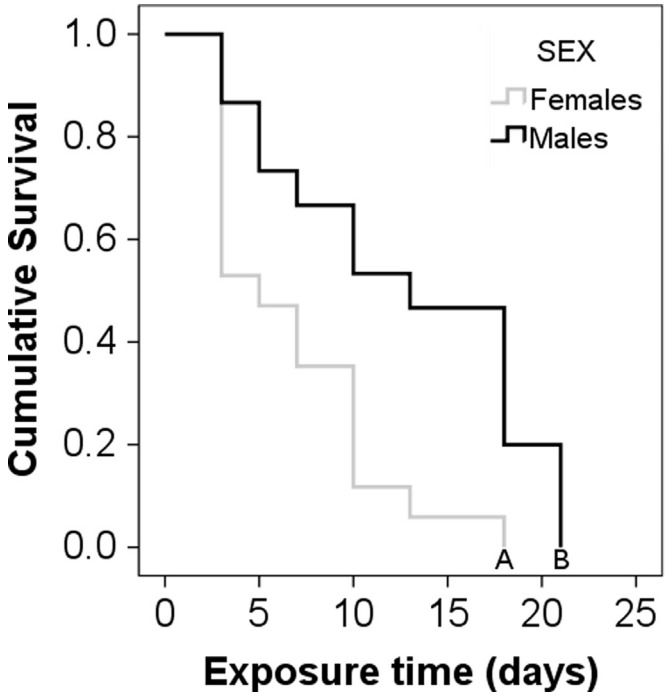

Fig. 1. Cumulative survival of male (black lines) and female (gray line) red-legged partridges exposed to the high dose of imidacloprid. Different letters indicate significant differences between sexes (Mantel–Cox test, $p \leq 0.05$).

Table 1
Levels (mean ± SE) of imidacloprid measured in the crop content and liver of dead partridges from the low and high dose groups.

Treatment	N[a]	Crop content (g)	Imidacloprid concentrations	
			Crop (μg/g)	Liver (ng/g)
High dose	19	5.9 ± 1.24	55.3 ± 17.9	82.6 ± 22.5
Low dose	3	1.32 ± 0.23	4.1 ± 13.0	56.0 ± 28.0
Control	4	1.41 ± 0.83	0.0 ± 0.0	

[a] Differences in sample sizes between groups are due to the higher mortality that occurred in the high dose group.

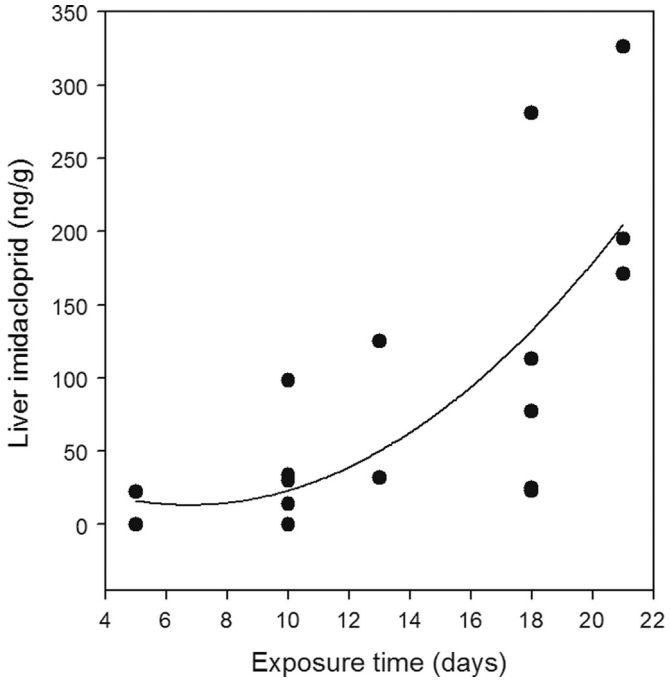

Fig. 2. Quadratic relationship between the exposure time (from initial exposure until death, in days) and the imidacloprid liver concentration (ng/g) in partridges from the high dose group ($F_{2,16} = 9.57$, $r = 0.74$, $p = 0.002$).

Regarding the biochemical parameters, mixed models including data from both seasons (Table S3) indicated significant seasonal differences for albumin, bilirubin, uric acid and total protein, aspartate aminotransferase and lactate deshydrogenase, but with no significant dose effect or dose by season interaction. However, for glucose and magnesium, there was a significant dose effects (with reduced levels in the low dose groups in both seasons), and for creatinine and creatine phosphokinase, there was a marginally significant dose × season interaction (Table S1). When analyzing data for each exposure period separately, glucose and magnesium reductions in the low dose group were non-significant during the first exposure, but were significant during the second exposure (Fig. 4, Table S1). Lactate dehydrogenase and creatine phosphokinase levels in plasma also appeared significantly reduced by the low dose of imidacloprid, but only after the second exposure (Fig. 4, Table S1). For creatinine, a marginally significant reduction in the low dose group was observed only during the first exposure (Table S1). Amongst these biochemical parameters, only glucose levels correlated with body weight loss after the second exposure period, ($r = 0.422$, $p = 0.04$, $n = 24$). The imidacloprid effect on

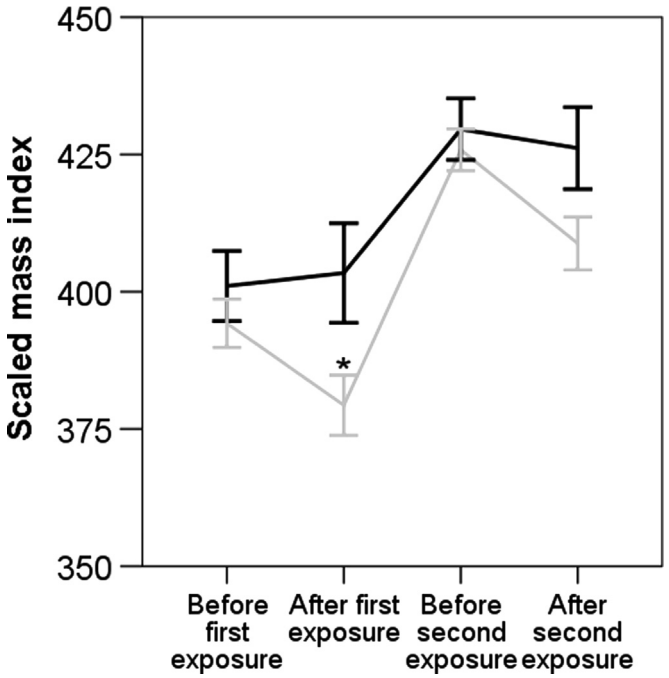

Fig. 3. Scaled mass index (mean ± SE) before and after both exposure periods in control (black line) and low dose (gray line) treated partridges. *Significantly different from controls at the $p \leq 0.05$ level.

glucose levels remained significant when body loss was included as a covariate in the model (Wald $\chi^2=11.56$, $p=0.001$).

Regarding the oxidative stress biomarkers, mixed models including data from both seasons (Table S3) indicated seasonal changes in SOD and GPX activity (Table S2), no significant season x dose interactions in any parameter, and a marginally significant effect of imidacloprid on SOD activity only. Analyses conducted for each exposure period separately indicated that partridges treated with the low dose of imidacloprid showed a significant increase in SOD activity in red blood cells after the first exposure (Wald $\chi^2=6.92$, $p=0.009$) and a non-significant effect in the same direction after the second exposure (Fig. 4).

Regarding antioxidants (vitamins and carotenoids) plasma levels, there was seasonal variation for retinol, lutein and zeaxathin (Table S2), but no significant dose effect or dose × season interactions were observed (Table S3).

No differences were found in brain AChE activity between partridges dead in the high dose group (18.8 ± 1.0 μmol/min/g) and control partridges that died of natural causes (15.9 ± 1.1 μmol/min/g).

Before the second exposure, eye ring and beak coloration did not differ between groups. After the exposure to the low dose of imidacloprid, the eye ring chroma was reduced significantly compared with controls (Wald $\chi^2=4.84$, $p=0.028$), while eye ring hue was unaffected (Fig. 5, Fig. S1). Beak redness (the inverse of hue) slightly increased in the low dose treated birds (Wald $\chi^2=3.19$, $p=0.026$), while beak chroma was unaffected by treatment (Fig. S1).

3.2. Effects on reproduction and indirect effects on offspring

All reproductive parameters are summarized in Table 2. The clutch size per laying female was significantly reduced in low dose treated partridges ($F_1=4.98$, $p=0.043$) and this group also laid the first egg later than the control group (Wald $\chi^2=4.42$, $p=0.036$; Table 2). Egg size, shell thickness, fertile egg rate and hatching rate did not differ between experimental groups (Table 2). Eggs laid by imidacloprid-low dose exposed females had higher levels of antioxidants in the yolk than those laid by control females: retinol (Wald $\chi^2=5.97$, $p=0.015$), α-tocopherol (Wald $\chi^2=9.29$, $p=0.002$), lutein (Wald $\chi^2=11.19$, $p=0.001$) and zeaxanthin (Wald $\chi^2=9.41$, $p=0.002$) (Fig. 6). Imidacloprid residues were not detected in the yolk of analyzed eggs ($n=11$).

Finally, there were no differences in chick survival, chick growth or sex ratio between experimental groups (Table 2). Chicks from imidacloprid low dose exposed partridges showed a reduced inflammatory response to PHA compared to those from control partridges (Wald $\chi^2=7.24$, $p=0.007$; Table 2). In this response, chick body condition at hatching was negatively associated with PHA response (Wald $\chi^2=6.91$, $p=0.009$), it is, chicks with lower body condition developed higher PHA response.

4. Discussion

The target dose of imidacloprid (the recommended application rate for cereal seed coating) killed all partridges, with mortality occurring more rapidly in females than in males. A sublethal dose of 20% of the recommended application rate reduced levels of some plasma biochemical parameters (glucose, Mg and LDH), increase SOD activity, produced changes in carotenoid-based integumental coloration, reduced the clutch size, delayed the first egg lay date, increased egg yolk vitamins and carotenoids and depressed chick response to PHA. Moreover, liver analysis revealed an accumulation of imidacloprid in this organ during exposure time.

4.1. Exposure estimation and adult survival

Imidacloprid treated seeds have proven highly lethal for partridges at the target dose, killing all exposed partridges in 21 days. We think that this worst case scenario, in which partridges feed only on treated seeds for 21 days, could be possible in times of scarcity, when there is no alternative food. Moreover, up to 31–47% of females died at the third day of exposure, and 50% of birds died after a week. The mortality we reported in this study for the high dose group was higher than that reported in a previous experiment (Lopez-Antia et al., 2013) using the same dose, in which mortality rate averaged 8% after 10 days of exposure. These differences may be due to the harsher weather conditions: the previous exposure was performed in spring, whereas in the current experiment the lower temperatures could have increased the treated seed ingestion (Chatelain et al., 2013). The crop content of dead partridges in the high dose group (Table 1) confirmed the results of previous cage studies that concluded that imidacloprid treated seeds were only partially refused by birds (Avery et al., 1993; Lopez-Antia et al., 2014). These crop contents indicated a mean consumption of 5.9 ± 1.2 g of treated seeds/partridge/day, which is similar to the mean consumption of seeds treated with the imidacloprid recommended application rate that we recorded in a recent study (Lopez-Antia et al., 2014). With this consumption rate, partridges would have ingested between 8.11 and 12.58 mg of imidacloprid/kg/day, reaching the LD_{50} (31 mg/kg for the Japanese quail) between day two and day four of exposure. This is consistent with the high mortality reported in the first three days of exposure and demonstrates that the partial rejection of treated seeds does not protect partridges from acute intoxication. This risk of acute intoxication could be even greater for smaller birds with higher ingestion rate by body mass and greater sensitivity to the insecticide than partridges. Mineau and Palmer (2013) estimated that a 15 g passerine bird, in the 5% tail of a species sensitivity distribution, will have a 50% chance of lethality from ingesting 3.9 imidacloprid-treated seeds. Intoxicated birds have been found

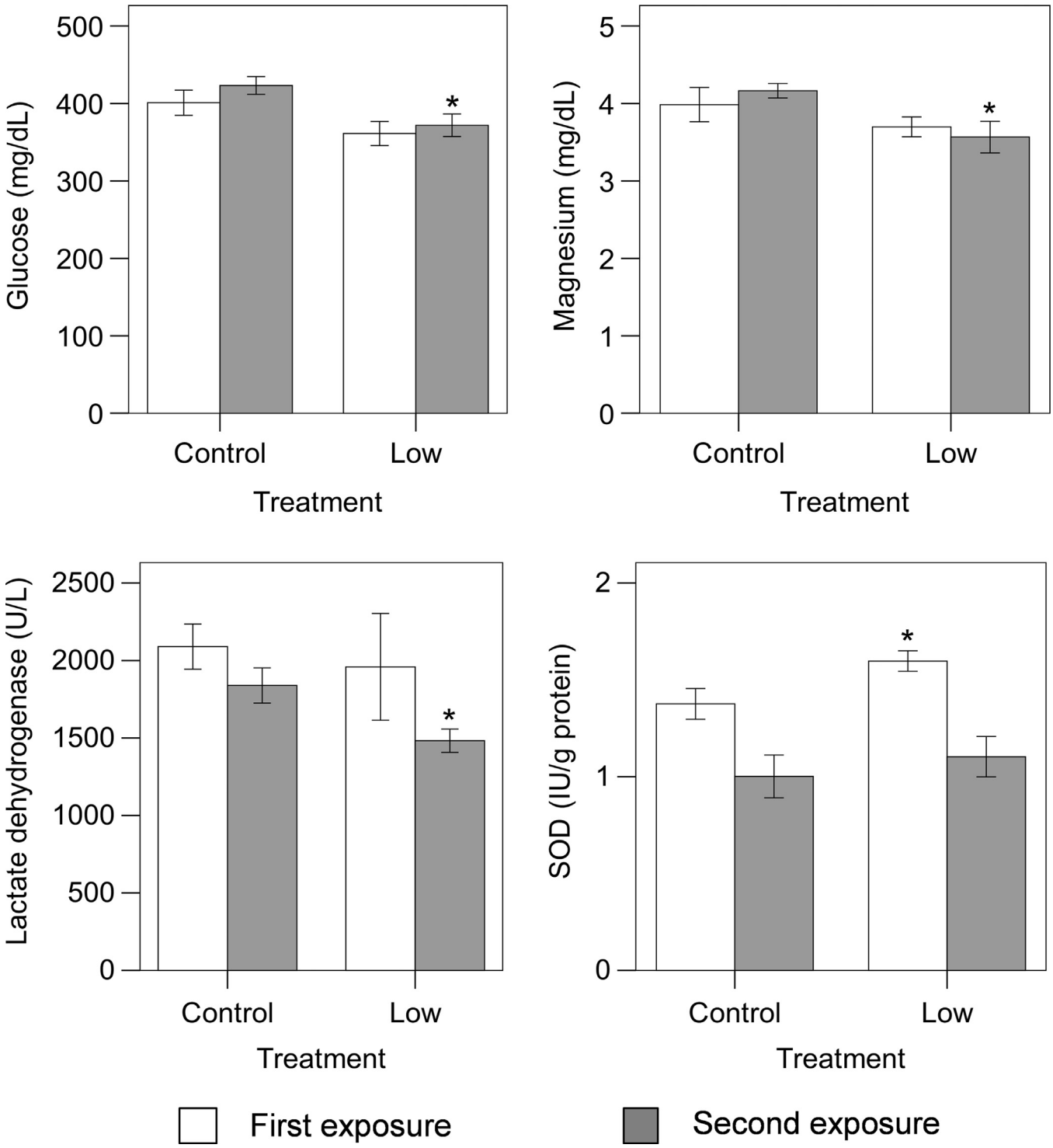

Fig. 4. Levels (mean ± SE) of glucose, magnesium and lactate dehydrogenase in plasma and SOD activity in red blood cells in control and low dose treated partridges after the first and the second exposure periods. (Sample sizes per treatment ranged between 21–26 partridges; *significantly different from controls at the $p \leq 0.05$ level).

in the field with imidacloprid treated seeds in their crop, which demonstrates that acute poisonings can occur (Berny et al., 1999; Ibáñez et al., 2011; Bro et al., 2010; Mineau and Palmer, 2013).

As we said above, with a normal food ingestion rate (25 g/day), the insecticide daily ingestion rate for partridges from the low dose group would be 8.8 mg/kg/day and partridges would reach the LD_{50} (31 mg/kg for the Japanese quail) in less than four days. In this group, three partridges died on the third day of exposure and another three died between days 10 and 14. Unfortunately we did not measure the food consumption, but given the rejection of imidacloprid-treated seeds is due to post-ingestion distress (Avery et al., 1993; Lopez-Antia et al., 2014), we may expect a normal food ingestion rate during the first days of exposure. Moreover we expected a great variation in treated food ingestion between individuals, as we have seen in previous studies (Lopez-Antia et al., 2014).

The mean daily survival rate in the high dose group was significantly lower in females than in males (Fig. 1). As far as we know, this study is the first that highlights such difference between sexes. In a previous experiment, no differences in survival

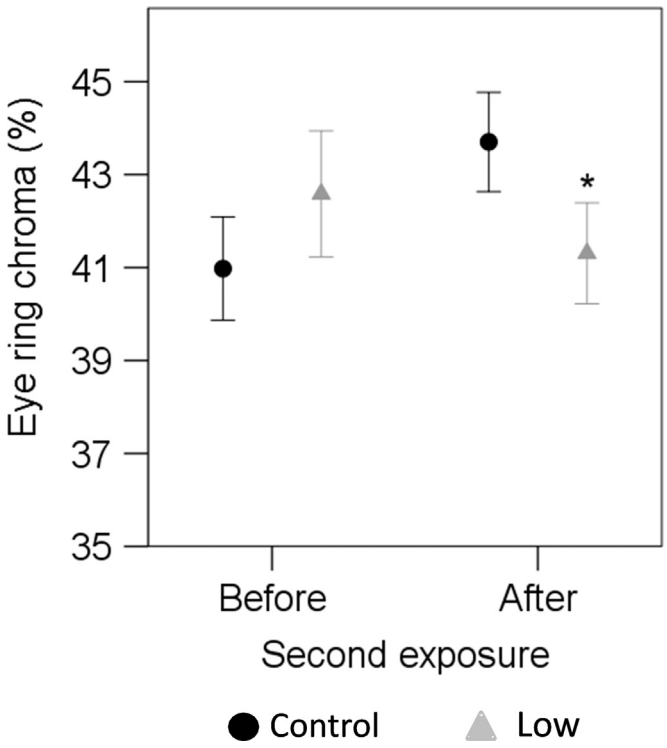

Fig. 5. Mean (±SE) chroma of the eye-ring of control and exposed (low dose group) partridges before and after the second exposure to imidacloprid ($N=25$ for control and 26 for low dose; *significantly different from controls at the $p \leq 0.05$ level).

Table 2
Reproductive parameters (n, % or mean ± SE) of partridge pairs from the control and low dose groups (there are no data from high dose group because all partridges died during the first exposure).

Parameter	Control	Low dose
Number of pairs	12	12
Number of laying females	7	9
Total number of eggs	138	83
Clutch size per laying female	19.7 ± 4.4	9.2 ± 2.3[a]
Days to the first egg	10.3 ± 4.2	21.0 ± 3.5[a]
Egg length (mm)	39.25 ± 0.11	39.84 ± 0.21
Egg width (mm)	29.87 ± 0.07	29.22 ± 0.11
Elongation index	1.31 ± 0.01	1.36 ± 0.01
Shell thickness of fertile eggs (mm)	0.225 ± 0.002	0.227 ± 0.003
Shell thickness of unfertile eggs (mm)	0.217 ± 0.003	0.207 ± 0.007
Fertile eggs (%)	70.2	82.8
Hatching rate of fertile eggs (%)	85.4	82.5
Hatching rate of total eggs (%)	72.9	75.8
Number of chicks	70	47
Chick body condition at hatching	5.88 ± 0.05	5.73 ± 0.09
% Female chicks[b]	0.50 ± 0.07	0.54 ± 0.12
Chick mortality (%)	35.71	41.66
Wing web swelling (mm)	0.34 ± 0.03	0.17 ± 0.09[a]

[a] Significantly different from controls at the $p \leq 0.05$ level.
[b] Number of female chicks/total number of chicks.

between sexes were reported (Lopez-Antia et al., 2013), probably because of the small sample size (6 pairs), and because of differences in the season and length of exposure between studies. This difference between sexes in survival time could be due to differences in sensitivity to the insecticide or differences in the insecticide ingestion rate (quantity of toxicant/body weight). Imidacloprid concentration in crop and liver did not differ significantly between sexes, but body weight was significantly lower in females than in males before the start of the experiment. We think that this differential mortality by gender needs further research given its importance for species demography.

Imidacloprid liver concentration was positively correlated with the number of days under exposure to the high dose until death (Fig. 2), which indicates that imidacloprid was accumulated in liver over time. To the best of our knowledge, this is the first paper that reports the accumulation of this pesticide in animal tissues. This finding can be useful for field studies and risk assessment, as levels of imidacloprid in the liver of birds could provide a measure of their exposure. We must remark that imidacloprid levels found in dead partridges did not differ significantly between low and high dose groups (Table 1). This is probably due to the dispersion of the data (SE was higher than the mean in the low dose). The mass of crop content was also very small, and in some cases the pesticide had probably been transferred into the crop mucosa, depending on the time in contact. Berny et al. (1999) analyzed crop contents and livers of partridges and pigeons found dead in the field, and the liver levels they found were even higher than those detected in partridges here (Table 1). Probably, these birds in the field had ingested a higher dose of imidacloprid or during more time, because birds have less capacity to avoid treated seeds in more unpredictable environments, as is the case of field scenarios (Lopez-Antia et al., 2014). The accumulation over time observed here for imidacloprid contrasts with the results obtained in rats treated with clothianidin, another neonicotinoid, which was not accumulated in tissues of exposed rats (Yokota et al., 2003).

4.2. Sublethal effects on adults

Mg, LDH and glucose levels in plasma were reduced in low dose-exposed birds after both exposure periods, but only significant so after the second exposure (Fig. 4). These changes were associated with a decrease in metabolism, which might be explained by a reduction in food consumption. However, some studies described a thyroid disrupting potential of imidacloprid (Bhaskar and Mohanty, 2014). Balani et al. (2011) also detected a decrease in plasma glucose after 14 and 28 days of administration of sublethal doses of imidacloprid to white leghorn chickens. Thyroid hormones are important mediators in basal metabolic rate, so the observed changes could indicate an effect on thyroid function that will need further research.

Some papers recently published have described a decrease of AChE brain activity after the exposure of rats to imidacloprid at sub-lethal doses (Vohra et al., 2014; Kapoor et al., 2014). By contrast, we did not observe any decrease in this enzyme activity in the brain of dead partridges, as compared with controls analyzed in our laboratory.

SOD activity in the red blood cells was increased significantly by imidacloprid, but only in the first exposure (Fig. 4). It is well know that the metabolism of some pesticides generates free radicals that have to be neutralized by the antioxidant system; SOD is the first line of this antioxidant defense because it acts against superoxide anion ($O_2^{\cdot -}$) that can be produced during phase I reactions of xenobiotic detoxification (Parkinson, 2001). As we found no changes in glutathione redox system or lipid peroxidation, we can conclude that SOD induction prevented further oxidative stress produced by this dose of imidacloprid in the partridges. Oxidative stress induced by imidacloprid has been studied in rats, and lipid peroxidation has been observed at sublethal levels during sub-chronic and chronic exposures (Kapoor et al., 2010; Mohany et al., 2012; Bal et al., 2012). Kapoor et al. (2010) also observed a decrease of GSH levels in liver, SOD activity in liver and brain and GPX activity in brain. In a previous work, Lopez-Antia et al. (2013) observed that partridges exposed at higher doses of

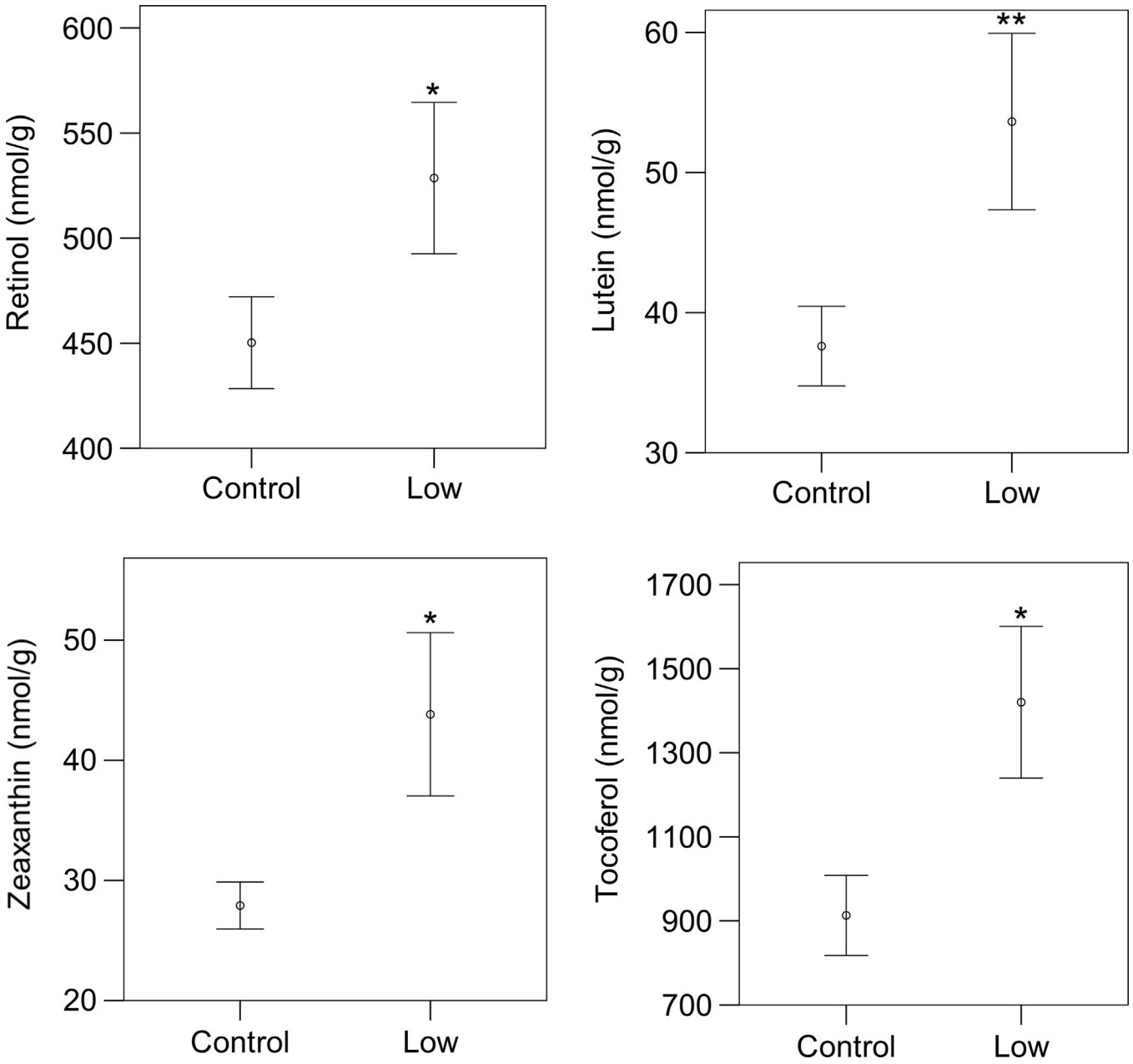

Fig. 6. Vitamin and carotenoid levels (mean ± SE) in the yolk of eggs laid by control and low dose treated partridges. ($N=22$ for controls and 11 for low dose; *significantly different from controls at the $p \leq 0.05$ level; **significantly different from controls at the $p \leq 0.01$ level.)

imidacloprid showed a reduction of GPX activity and levels of GSH in red blood cells, but no changes in SOD.

We found no significant differences in antioxidant plasma levels (carotenoid and vitamin) between controls and the low dose group (Table S2), probably because the oxidative imbalance was compensated by the increase in SOD activity. We found that the carotenoid based coloration of partridges was affected after the second exposure. Specifically, we observed a reduction in the chroma of the eye ring (Fig. 5). The chroma component of the color is defined as the strength of the color (saturation) and, therefore, our results indicated that the red color of the eye ring was less intense in low dose treated partridges than in controls (Fig. 5; Fig. S1). This reduction of the chroma can be interpreted as lower amounts of carotenoids (astaxanthin and papilioerythrinone) deposited in the eye ring of partridges (García-de Blas et al., 2014). We also observed a shift in the hue of the beak coloration, indicating a slightly redder beak in the low dose treated partridges (Fig. S1). This could be interpreted as a higher proportion of the redder carotenoid pigment, astaxanthin, which is produced from zeaxanthin by two oxidation steps (García-de Blas et al., 2014). In this sense, imidacloprid exposure may have favored this oxidative metabolism. Eye ring and beak coloration seem to be honest signals of condition and health in red-legged partridges (Pérez-Rodriguez and Viñuela, 2008; Perez-Rodriguez et al., 2013) and are also important sexual signals that influence mate choice and reproductive investment (Alonso-Alvarez et al., 2012). In our previous experiment (Lopez-Antia et al., 2013), we detected a reduction in the proportion of eye ring area pigmented by

carotenoids in partridges exposed to twice the recommended application rate. Here we detected more subtle changes in eye ring coloration at a lower dose.

4.3. Effects on reproduction and indirect effects on offspring

Regarding the reproductive parameters, pairs from the low dose group laid significantly fewer eggs than pairs from the control group, and also laid the first egg later (Table 2). Preliminary studies for the industry, revised by Mineau and Palmer (2013), already described effects on egg laying and hatching on mallards fed with a mash containing 240 ppm of imidacloprid. In mammals, some studies have observed adverse effects of imidacloprid on reproductive organs and germ cells. In seven day-old male rats, a sublethal exposure caused reductions in epididymis and vesicula seminalis weights, epididymal sperm concentration and testosterone levels (Bal et al., 2012). In female rats, a chronic exposure produced morphological alterations and changes in oxidative stress parameters in ovaries and altered levels of sexual hormones (Kapoor et al., 2011). Fertilization process and zygotes formation were adversely affected by imidacloprid in an in vitro study (Gu et al., 2013). Finally, another neonicotinoid, clothianidin, produced fragmentation of germ cells and a decrease in embryonic length in male quails after 30 days of exposure (Tokumoto et al., 2013). These effects are mainly attributed to oxidative damage caused by imidacloprid. The effect on the clutch size that we found in our experiment could be also due to a reduction in the body condition (although this reduction was not significant (Fig. 3)) or to the reduction of food ingestion (Mineau, 2005). A reduction in clutch size in the field may result in clutch abandonment, and a nesting delay could lead to reduced chances of re-nesting (Mineau, 2005).

We found that eggs laid by low dose treated partridges had higher levels of vitamins and carotenoids in the yolk. This could be due to the smaller number of eggs laid by these exposed females, which may have allowed greater allocation of such resources for each laid egg. In fact, if we include the clutch size as a covariate in our models, this variable significantly explains levels of vitamins and carotenoids in the egg yolk, and a treatment effect remained significant only for zeaxanthin levels in eggs. A trend for increased carotenoid plasma levels was also found in imidacloprid low dose exposed birds (in both males and females; Table S2) before laying, which may indicate that the accumulation of carotenoids was a response against the xenobiotic. Finally, although differences in chick mortality were not significant, we detected a reduction in the PHA response of chicks from pairs exposed to imidacloprid low dose (Table 2). In our previous study, we found a higher mortality in chicks from imidacloprid exposed pairs (Lopez-Antia et al., 2013), but we did not measure the immune response in these chicks. This reduction in cellular immune responsiveness may explain the higher chick mortality observed here. Immunotoxicity of imidacloprid has been described in poultry (Siddiqui et al., 2007; Kammon et al., 2012) and in adult red-legged partridges (Lopez-Antia et al., 2013), but this is the first study reporting an indirect immunotoxic effect on the offspring of exposed parents. This effect on chicks is not explained by a transfer of imidacloprid to the eggs, because residues were not detected in yolk, but other effects on egg quality may have occurred. In fact, the higher levels of carotenoids and vitamins in the eggs of treated birds could correspond to a compensatory mechanism against imidacloprid effects during yolk formation.

5. Conclusions

This study revealed that imidacloprid exposure not only have lethal effects, but also numerous deleterious sub-lethal effects on redox balance, secondary sexual traits and reproduction in a bird at sublethal levels equivalent to 20% of treated seed in diet during sowing periods. We also found a reduced cell-mediated immune response in the offspring not directly exposed to the toxic. In addition to seed treatment, imidacloprid is applied through the irrigation water and as foliar spray, after flowering and in crops harvested before flowering (MAGRAMA, 2013). Moreover, birds may be exposed at lower imidacloprid levels from the dust detached or the soil residues from the treated seeds at planting (Krupke et al., 2012) and by drinking water contaminated by soil leachate (Goulson, 2013). These harmful effects of imidacloprid at exposure doses according to the usage rates in the field are important, especially given the ubiquity of the product. Despite the current restrictions on use of neonicotinoids in the European Union (Regulation 485/2013), birds are still at a high risk of exposure to these pesticides through direct sources of exposure such as the ingestion of coated seeds.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2014.10.023.

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Supplementary material

Imidacloprid-treated seed ingestion has lethal effect on adult partridges and reduces both breeding investment and offspring immunity

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Table S1. Mean (± SE) body condition, body weight, haematocrit, wing web swelling and plasma biochemical parameters of partridges in each experimental group after both exposure periods.

Parameter	First exposure			Second exposure	
	Control	Low dose	High dose	Control	Low dose
Initial N	32	32	32	28	26
Mortality (%)	12.5	18.7	100**	7.1	3.8
N	28	26	0	26	25
Body condition	403 ± 9	367 ± 8**		426 ± 7	411 ± 8
Body weight	405 ± 9	357 ± 9**		433 ±8	400 ± 9**
Body weight loss	-2.0 ± 8.2	-35.2 ± 8.4**		-3.1 ±5.0	-17.9 ± 6.1†
Haematocrit (%)	34.9 ± 1.4	35.1 ± 1.2		42.6 ± 0.7	41.0 ± 0.8
Wing web swelling (mm)	0.52 ± 0.02	0.49 ± 0.02			
Albumin (g/L)	14.7 ± 0.8	14.6 ± 0.7		17.2 ± 0.6	17.1 ± 1.0
Bilirubin (mg/dL)	31.5 ± 5.0	26.7 ± 4.6		42.85 ± 4.09	51.57 ± 4.27
Alkaline phosphatase (U/L)	1981 ± 231	2460 ± 501		1790 ± 266	2309 ± 401
Alanine aminotransferase (U/L)	34.2 ± 6.9	23.7 ± 2.1		28.00 ± 3.7	31.13 ± 5.1
Aspartate aminotransferase (U/L)	295 ± 22	323 ± 44		264 ± 16	258 ± 12
Creatine phosphokinase (U/L)	656 ± 135	801 ± 118		663 ± 76	480 ± 37*

Creatinin (mg/dL)	0.529 ± 0.03	0.480 ± 0.01†	0.474 ± 0.02	0.480 ± 0.01
Uric acid (mg/dL)	1.74 ± 0.30	2.72 ± 0.58	6.33 ± 0.98	5.88 ± 1.07
Calcium (mg/dL)	11.8 ± 0.6	11.3 ± 0.5	12.3 ± 0.4	12.3 ± 0.6
Cholesterol (mg/dL)	216 ± 15	205 ± 17	203 ± 12	190 ± 11
Glucose (mg/dL)	401 ± 16	361 ± 16†	423 ± 11	372 ± 14**
Magnesium (mg/dL)	3.98 ± 0.22	3.70 ± 0.13	4.16 ± 0.94	3.57 ± 0.20**
Phosphorus (mg/dL)	5.77 ± 0.52	5.65 ± 0.54	6.47 ± 0.30	6.38 ± 0.42
Total protein (g/L)	43.6 ± 2.3	45.8 ± 1.7	49.4 ± 1.2	49.0 ± 1.7
Triglycerides (mg/dL)	224 ± 30	235 ± 27	202 ± 22	193 ± 14
Lactate dehydrogenase (U/L)	2090 ± 146	1960 ± 345	1839 ± 113	1483 ± 75**

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

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

†Difference with controls close to significance (p=0.055)

Table S2. Mean (± SE) values of oxidative stress biomarkers, vitamins and carotenoid levels in each experimental group after both exposure periods.

Parameter	First exposure		Second exposure	
	Control	Low dose	Control	Low dose
N	23	23	25	26
RBC MDA (nmol/gr)	5.97 ± 0.27	5.42 ± 0.27	5.78 ± 0.28	5.78 ± 0.28
RBC GSH (µmol/gr)	3.19 ± 0.27	3.34 ± 0.24	3.56 ± 0.22	3.34 ± 0.26
RBC GSSG (µmol/gr)	685.6 ± 72.6	527.2 ± 69.3	641.7 ± 80.6	708.1 ± 117.0
RBC GPX (IU/mg prot.)	0.402 ± 0.02	0.402 ± 0.03	0.559 ± 0.04	0.563 ± 0.05
RBC SOD (IU/mg prot.)	1.38 ± 0.08	1.60 ± 0.05*	1.00 ± 0.11	1.103 ± 0.10
Retinol (µM)	36.3 ± 3.4	34.5 ± 3.4	50.1 ± 2.3	50.0 ± 2.8
Tocoferol (µM)	27.3 ± 2.3	23.9 ± 2.1	26.5 ± 1.9	27.4 ± 1.7
Lutein (µM)	1.59 ± 0.17	1.53 ± 0.15	3.07 ± 0.26	3.55 ± 0.21
Zeaxanthin (µM)	1.53 ± 0.31	1.46 ±0.21	6.10 ± 0.50	7.15 ± 0.46

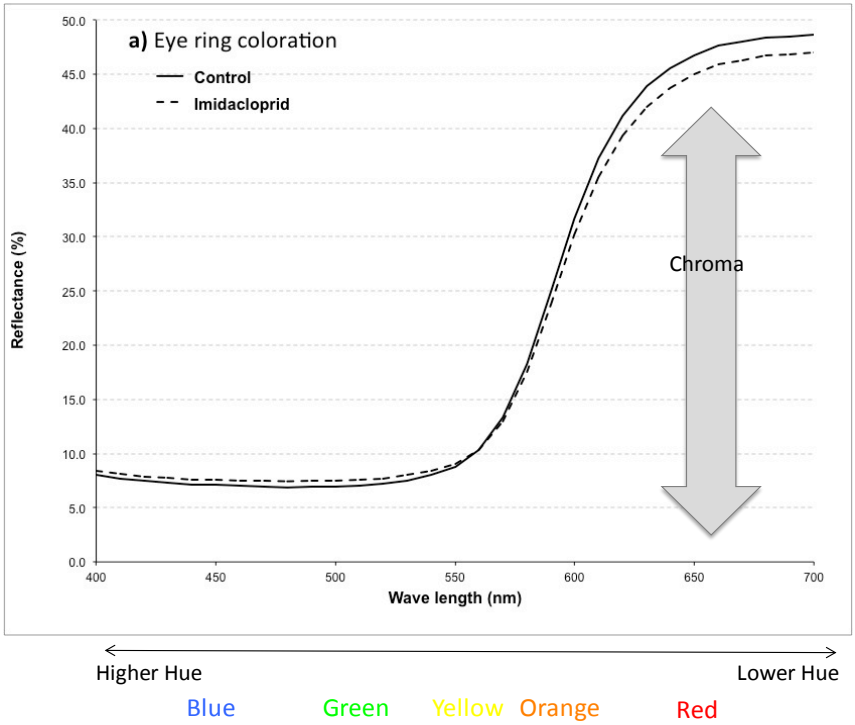
Bold values indicate groups significantly different from controls

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

Table S3. Results from the mixed models testing for treatment effects during both exposure periods. Explanatory variables included the season (first vs second exposure), the dose, and the interaction season x dose. All mixed models included the individual identity as a random effect, in order to account for repeated measures on the same individuals during the first and second exposure season.

Parameter		Season		Dose		Season × Dose	
	df	F	p	F	p	F	p
Haematocrit	1,98	41.4	<0.001	1.07	NS	0.174	NS
RBC MDA	1,101	1.65	NS	0.001	NS	0.205	NS
RBC SOD	1,92	26.4	<0.001	3.32	0.07	0.325	NS
RBC GPX	1,99	22.6	<0.001	0.00	NS	0.001	NS
RBC GSSG	1,93	0.49	NS	0.07	NS	1.28	NS
RBC GSH	1,96	1.59	NS	0.005	NS	0.644	NS
Retinol	1,93	27.2	<0.001	0.233	NS	0.145	NS
Tocoferol	1,93	1.57	NS	0.174	NS	0.870	NS
Lutein	1,93	78.15	<0.001	1.33	NS	1.29	NS
Zeaxanthin	1,93	174.4	<0.001	1.64	NS	1.72	NS
Albumin	1,90	8.54	0.004	0.04	NS	0.201	NS
Bilirubin	1,90	17.71	<0.001	0.129	NS	2.48	NS
Calcium	1,89	2.22	NS	0.173	NS	0.509	NS
Cholesterol	1,90	0.899	NS	0.897	NS	0.001	NS
Creatinin	1,89	2.30	NS	1.89	NS	3.61	0.06
Glucose	1,91	1.07	NS	11.2	0.001	0.000	NS

Magnesium	1,92	0.046	NS	7.00	0.01	0.691	NS
Phosphorus	1,92	1.58	NS	0.005	NS	0.000	NS
Triglycerides	1,91	1.91	NS	0.044	NS	0.323	NS
Uric acid	1,79	20.5	<0.001	0.124	NS	0.460	NS
Total protein	1,89	6.05	0.016	0.487	NS	0.181	NS
Alkaline phosphatase	1,78	0.034	NS	1.21	NS	0.028	NS
Alanine aminotransferase	1,88	0.000	NS	0.145	NS	1.12	NS
Aspartate aminotransferase	1,90	3.53	0.063	0.277	NS	0.487	NS
Creatine phosphokinase	1,85	5.13	0.026	0.000	NS	3.49	0.065
Lactate dehydrogenase	1,93	27.2	0.021	1.89	NS	0.525	NS



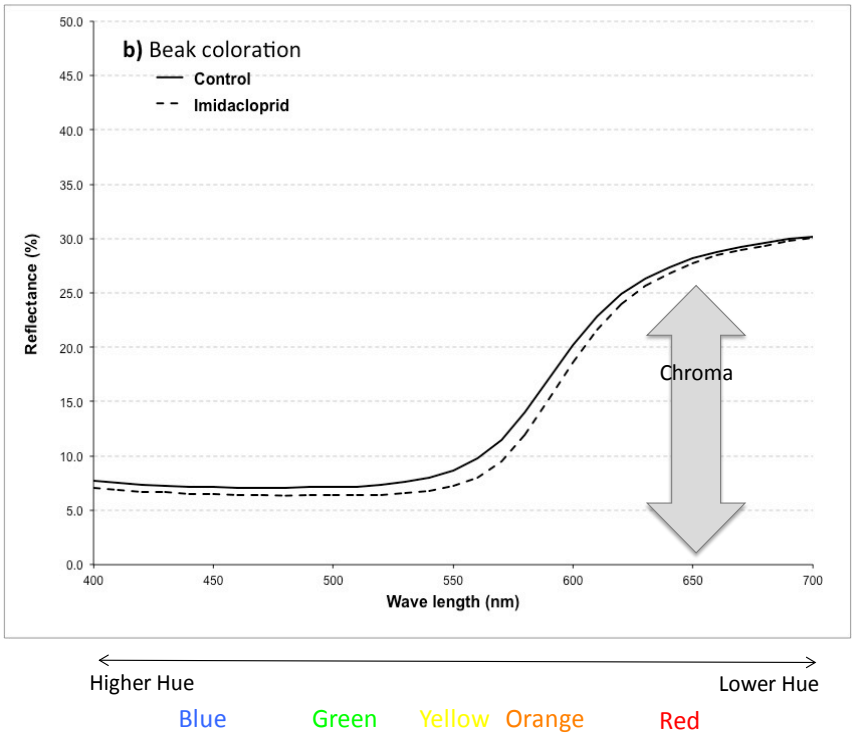


Fig.S1. Reflectance spectra (% reflectance over the 400-700 nm wavelength range) of the eye ring (a) and beak (b) of control (solid lines) and exposed (low dose- dotted lines) partridges after the second exposure period, in spring. From these spectra (raw

data), we calculated the color hue (the wavelength of the inflection point of the reflectance curve) indicative of the perceived color (lower hue = redder trait; higher hue = oranger traits) and the chroma (the % reflectance in the red color range, i.e. 600-700nm) indicative of color saturation (higher % reflectance = more saturatation).