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High prevalence of the neonicotinoid clothianidin in liver and plasma samples collected from gamebirds during autumn sowing

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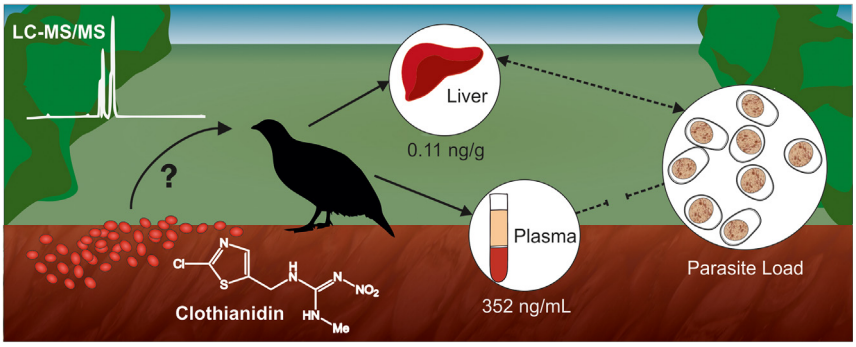
HIGHLIGHTS

- Gamebird carcasses analysed for clothianidin in plasma/liver and health parameters
- Detectable residues of clothianidin rose from 6% pre-sowing, to 89% post-sowing
- Detection frequency decreased over 30 days for plasma but not for liver.
- Faecal parasite load positively associated with clothianidin residue in livers only.
- Implications for future biomonitoring studies and agrochemical risk assessments

GRAPHICAL ABSTRACT



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ABSTRACT

Since neonicotinoid insecticides were introduced to the agricultural market, evidence of the negative impacts of these systemic compounds on non-target species has accumulated. Birds are one of the largest groups of species to inhabit farmland, but the extent of neonicotinoid exposure in avian communities is poorly understood and very little is known about how any exposure may affect wild birds. Here, free-living gamebirds were used as a model group to measure the extent of avian exposure to the neonicotinoid clothianidin via seed treatment. During a typical sowing period of winter cereals treated with clothianidin, blood and liver samples were collected simultaneously from individual hunted gamebird carcasses, both pre- ($n = 18$) and post-sowing ($n = 57$) and were analysed for clothianidin via LC/MS-MS. Body weight, fat score and faecal parasite load were also quantified in the birds to ascertain whether any of these health parameters were associated with clothianidin exposure under field conditions. Clothianidin was detected in 6% of individuals sampled pre-sowing and 89% of individuals sampled post-sowing. The frequency of clothianidin detection in plasma samples and the concentration of clothianidin in liver and plasma samples decreased significantly between the first week and 2–4 weeks post-sowing. Faecal parasite load was positively associated with concentrations of clothianidin in the liver (but not plasma) of partridge species, but there was no association between clothianidin concentration and fat score or body weight, for either sample type. This study provides clear evidence that treated seed is a source of pesticide exposure for gamebirds following autumn sowing. These findings have implications for gamebirds worldwide where seed treatments are in use, and will aid the design of any future avian biomonitoring studies for agrochemical compounds.

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1. Introduction

Neonicotinoids (NNs) are insecticides with a specific neurotoxic mode of action via nicotinic acetylcholine receptors (Tomizawa and Casida, 2005), and are the most widely used group of systemic insecticides on the global agricultural market (Simon-Delso et al., 2015). Seed treatments are one of the most common forms of NN application (Simon-Delso et al., 2015), for which the three main compounds are imidacloprid, clothianidin (CLO) and thiamethoxam. Following the use of NNs for nearly two decades, concerns were raised regarding the safety of non-target invertebrates (Pisa et al., 2017); as a result these three compounds were subject to a moratorium from 2013, preventing use on flowering crops within the European Union (EU), and subsequently banned in 2018 from being applied outdoors within the EU on the basis of a review of risks to bee health (Bass and Field, 2018). Despite these restrictions, NNs continue to be used in large quantities worldwide and are still applied to a large number of agricultural crops. The EU ban has highlighted the importance of biomonitoring for agrochemicals in non-target organisms (Mancini et al., 2019). In particular, the effect of NNs on wild birds has increasingly gained research attention as data suggest that this taxa may also be vulnerable to NN exposure and subsequent sub-lethal effects (Pisa et al., 2017). However, the extent of either of these parameters under field conditions remains unclear and there is a paucity of NN exposure data for species of farmland bird.

Gamebirds (Galliformes) are a group of avian species that may be susceptible to high levels of NN exposure via seed treatments due to the large proportion of agricultural seed present in their diets (Prosser and Hart, 2005), and the extent to which they frequent arable fields during the sowing season (Prosser and Hart, 2005; Bonneris et al., 2019; Bro et al., 2015; Lopez-Antia et al., 2016). Exposure of wild Galliformes to NNs has been confirmed, but there has only been a handful of studies across three continents (Millot et al., 2017; MacDonald et al., 2018; Botha et al., 2018; Turaga et al., 2016). Between 1995 and 2014, 105 NN poisoning incidents were reported across France, 47 of which were for species of gamebird (red-legged partridge *Alectoris rufa*, grey partridge *Perdix perdix* and ring-necked pheasant *Phasianus colchicus*) (Millot et al., 2017). The majority (73.3%) of these incidents occurred during the autumn sowing season and 36.7% of dead or dying birds were found in or adjacent to newly sown fields; as part of the same study, the NN compound imidacloprid was detected in the gizzards and livers of grey partridge (Millot et al., 2017). Imidacloprid residues have also been detected in the livers of Northern bobwhite quail *Colinus virginianus* (Ertl et al., 2018) and scaled quail *Callipepla squamata* in the USA (Turaga et al., 2016), wild turkeys *Meleagris gallopavo silvestris* in Canada (MacDonald et al., 2018), Cape spurfowl *Pternistis capensis* in South Africa (Botha et al., 2018), and in the crop and gizzard contents of red-legged partridge in Spain (Lopez-Antia et al., 2016). In addition, thiamethoxam has been detected in the eggs of grey partridge in France (Bro et al., 2016). Thus far there have been no such studies in the UK, despite the annual release during the autumn sowing season of millions of gamebirds into the environment for the shooting industry (Aebischer, 2019). A large proportion of autumn-sown cereals in the UK were treated with NNs prior to the ban in 2018, with approximately 90% of applications in the form of seed treatments (Garthwaite et al., 2013). Therefore, both managed and native populations of Galliformes may have been exposed to NNs during this time.

Multiple techniques have been used to measure NN exposure in wild birds to date, and as such residue data are available for a range of avian samples (e.g., organs, eggs, blood, feathers). The type of sample obtained from birds is often dictated by the size and/or status of the species. For example, blood or feathers are the only samples that have been obtained for small passerines and/or protected species using non-lethal sampling (Hao et al., 2018; Byholm et al., 2018; Taliansky-Chamudis et al., 2017; Humann-Guilleminot et al., 2019), whereas tissue samples are more commonly analysed for species of hunted Columbid or Galliforme (Millot et al., 2017; MacDonald et al., 2018; Botha et al., 2018; Turaga et al., 2016; Ertl et al., 2018). Existing data suggest that the concentration of NN compounds in birds may differ depending on the type of sample analysed, study species and the time of sampling (Bean et al., 2019; Zeid et al., 2019; European Food Safety Authority, 2006). For example, imidacloprid plasma concentrations were highest 6 h and 1 h post-exposure for white-crowned sparrow *Zonotrichia leucophrys* and Japanese quail *Coturnix japonica*, respectively (Hao et al., 2018; Bean et al., 2019), and there is conflicting evidence with regards to the accumulation of imidacloprid in liver tissue over multiple exposure events for Columbid and Galliforme species (Bean et al., 2019; Zeid et al., 2019; Lopez-Antia et al., 2015). These differences are attributable to the toxicokinetic properties of NNs, which generally remain poorly understood in avian physiology, particularly for species of wild bird. In a field-based context, very little is known about how patterns of NN exposure may be expressed via different types of biological sample, or how these measures of exposure may be used to assess the potential for sub-lethal effects associated with NN compounds.

Exposure to NNs has been reported to cause physiological sub-lethal effects in avian species in the laboratory (Gibbons et al., 2015), with some NN compounds (e.g., imidacloprid) being more toxic to birds than others (Tomizawa and Casida, 2003). For example, the dietary no-observed-effect level in bobwhite quail is reported to be 120, 300 and 525 ppm for imidacloprid, thiamethoxam and CLO, respectively (Mineau and Palmer, 2013). In particular, adverse changes to weight, fat stores and the immune system have been reported among species of Galliforme and Columbid dosed with imidacloprid (Lopez-Antia et al., 2015; Addy-Orduna et al., 2018). With regards to the immune system, aviary studies have reported that imidacloprid can negatively affect cell-mediated and humoral immunity (Lopez-Antia et al., 2015; Lopez-Antia et al., 2013; Kammon et al., 2012; Balani et al., 2011; Siddiqui et al., 2007), both of which are important for regulating parasite burdens in birds (e.g., in the gut and blood). As yet however, the effects of NN exposure on avian parasite load have not been investigated. To date, only one study has investigated NN-associated sub-lethal effects in free-living birds, and this reported weight loss and a reduction of fat stores in a passerine after individuals were dosed with imidacloprid at a migratory stopover site (Eng et al., 2019). Overall, there is a paucity of data to assess impacts of NNs on health parameters in free-living birds, and thus far the effects of any exposure arising from standard agricultural practice have not been investigated.

Managed populations of gamebird present an ideal test system to investigate NN exposure and associated sub-lethal effects in the field because it is possible to obtain several types of sample simultaneously from a large number of birds belonging to the same species or taxonomic group. In this study, the exposure of Galliformes to the NN CLO via ingestion of treated cereal seed was measured in situ using both blood plasma and liver samples collected during the autumn sowing period from the carcasses of hunted gamebirds. Specifically, the objectives of the study were to: 1) assess the extent and level of exposure of gamebirds to CLO via treated cereal seed during the autumn 2017 sowing period; 2) determine how patterns of NN exposure may be expressed via different types of biological sample by measuring the difference in the detection and concentration of CLO recorded in liver and plasma samples collected simultaneously from individual birds; and 3) investigate whether there were any changes to health parameters (weight, fat or parasite load) associated with the concentration of CLO in liver and/or plasma.

2. Methods

2.1. Study sites

Data collection took place at six sites in North Lincolnshire (UK) that were sown with Redigo-deter®-dressed wheat or barley (Bayer Crop Science Ltd., UK). Each site contained distinct populations of managed

gamebirds and was separated from the others by an average of 16 km (range: ~1.5–38 km) to ensure spatial independence.

2.2. Sample collection

Bird carcasses were collected between September and November in 2017 as part of standard shooting practices. These months are within the official shooting season in the UK, which opens on 1st September and closes on 1st February each year. Samples were collected from managed shoots (on independently scheduled shoot dates) once prior to the sowing of CLO-treated cereals (visit 1), again within 1 week post-sowing (visit 2), and a further one or two times 2–4 weeks post sowing (visit 3; Table 1). Between one and eight Galliforme carcasses were collected on each site visit depending on the relative success of the shoot (Table 1). Where possible, red-legged partridge *Alectoris rufa* were collected as the main study species; however, when red-legged partridge were not available, grey partridge *Perdix perdix*, pheasant *Phasianus colchicus* or woodpigeon *Columba palumbus* were taken in lieu. A total of 42 bird carcasses were collected within 10 min of time of death with the remainder (33 carcasses) collected at intervals up to a maximum of 3 h after time of death in order to ensure safe working within the constraints of an ongoing shoot. In all cases, death was established prior to sampling by use of a stethoscope. Carcasses were labelled, bagged and stored on ice for transportation. All carcasses were frozen at −20 °C within 6 h of the time of death. Blood samples were obtained post-mortem via heart puncture (using an 18 G needle and 2.5 mL syringe) immediately upon collection in the field. Up to 2 mL of whole blood was taken, stored in a heparinised microtainer, and then spun down at 1000 rpm for 5 min within 6 h of collection. Plasma was separated out from the sample and stored at −20 °C until analysis. Whole livers were excised at necropsy under laboratory conditions after all carcasses had been collected, and stored separately at −20 °C until analysis. It was not possible to visually confirm the presence or absence of red dye (indicating CLO treatment) on seeds that had been ingested into the gizzard at necropsy.

2.3. Health parameter data collection

Sex, age, weight, and fat score (measured as per standard British Trust for Ornithology protocol (Redfern and Clark, 2001)) were recorded for each carcass at necropsy. Faecal samples were extracted from the lower intestine of birds (where possible) to measure faecal parasite load. Faecal samples were weighed, dissolved in 100 mL sodium nitrate flotation fluid (1.20 SG; Vetlab Supplies Ltd., UK) and left to stand for 15 min to allow all parasite eggs to be suspended. Then approximately 2 mL of each sample was extracted from the surface of the flotation beaker. Samples were individually transferred to a McMaster worm egg counting slide (Vetlab Supplies Ltd., UK) and analysed under 10 × 10 magnification (Nikon Eclipse 80i, Nikon UK). The number of *Coccidia* eggs (a protozoan parasite belonging to the *Eimeria* genus) in the prescribed grid of the slide was counted, along with any other common parasitic eggs (e.g., nematodes belonging to the *Capillaria* genus). Total egg count (all parasite species) was transformed using the weight of the faecal sample to obtain a measure of parasite load per unit mass for each individual.

2.4. Residue analysis

In total, fresh livers from 75 birds (18 collected pre-sowing and 57 post-sowing) and plasma samples from 42 birds (9 collected pre-sowing and 33 post-sowing) were analysed for CLO using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.4.1. Extraction

For livers, 0.3 g of wet sample was weighed and spiked with a labelled internal standard (Clothianidin D3; QMX, Essex. UK). Extraction was carried out in 2 mL of 50:50 methanol:water (v:v) containing 0.2% formic acid and briefly vortexed. Samples were centrifuged at 3000 rpm for 5 min; HPLC grade water was added to the supernatant (4:1 v:v) and the samples were briefly vortexed again. The extracts were cleaned using Oasis HLB cartridges (60 mg, 3 cc size; Waters, Hertfordshire, UK). Solid phase extraction columns were pre-conditioned with methanol and deionised water, and eluted with acetonitrile. The extracts were evaporated using a Turbovap (Biotage, Uppsala, Sweden), re-dissolved in 1 mL of mobile phase (95% phase A, 5% phase B) and transferred into LC vials.

For plasma, each sample (20–50 μL) was spiked with a labelled internal standard (CLO D3; QMX, Essex, UK). The extraction was carried out in 20–50 μL (equivalent to sample volume) 95:5 water: methanol (v:v, containing 0.2% formic acid) and vortexed for 10 s, after which the solution was evaporated using a Turbovap. The residue was reconstituted with 200–500 μL of water:acetonitrile (95% phase A, 5% phase B) mobile phase, briefly vortexed and subsequently centrifuged at 3000 rpm for 5 min prior to being filtered (PES syringe filter with a pore size of 0.2 μm; Thermo Fisher Scientific; Hemel Hempstead, UK) and transferred to HPLC vials (Waters, Hertfordshire, UK).

2.4.2. Analysis

The analysis of liver samples was performed using a LC coupled to a triple quadrupole Quantum Ultra TSQ mass spectrometer (Thermo

Table 1
Overview of samples obtained from all sites and species composition of samples collected. Liver samples were obtained from all birds collected. The sub-set of birds from which plasma samples were obtained is indicated by numbers in superscript.

Site/species	Drive	Sowing date	Number of birds collected			Number of faecal samples collected		
			Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
			Pre-sowing	Post-sowing	Post-sowing	Pre-sowing	Post-sowing	Post-sowing
Site 1[a]	A,B	11/10/2017	6^{4}	–	10^{5}	0	0	4
Site 2	A	07/10/2017	6^{5}	8^{6}	6^{5}	0	8	6
Site 3	A	26/09/2017	–	4^{3}	5^{4}	0	4	4
Site 4[a]	A,B C	18/10/2017 01/11/2017	–	8^{3}	7^{3}	0	7	1
Site 5	A,B C	17/10/2017 25/10/2017	6	1^{1}	5^{1}	6	1	4
Site 6	A,B	08/10/2017	0	3^{2}	–	0	3	0
Red-legged partridge	*na*		17^{8}	16^{13}	27^{17}	6	16	18
Grey partridge	*na*		1^{1}	5^{2}	1^{1}	0	5	0
Pheasant	*na*		0	2	5	0	2	1
Woodpigeon	*na*		0	1	0	0	0	0
Total	12		18^{9}	24^{15}	33^{18}	6	23	19

On average, birds were collected three and 23 days post-sowing for visit 2 and visit 3, respectively; *na*: not applicable.
[a] Sites where two shoots were attended in the visit 3 timeframe.

Fisher Scientific; Hemel Hemsptead, UK), interfaced with ion max electrospray ionisation (ESI) and operated with Xcalibur™ (V.2.0.7; Thermo Fisher Scientific; Hemel Hemsptead, UK). Analyte separation was performed on a Phenomenex Synergi Fusion column (2.5 μm particle size, 50 mm × 2 mm ID; Phenomenex, Macclesfield, UK) using a water:methanol mobile phase gradient. For plasma samples, the analysis was performed using a LC coupled to a triple quadrupole Xevo TQ-S mass spectrometer (Waters, Hertfordshire, UK), interfaced with a Waters UniSpray source and operated with Masslynx software. Analyte separation was performed on a Waters Acquity BEH C18 column (1.7 μm particle size, 50 mm × 2.1 mm ID; Waters, Hertfordshire, UK) using a water:acetonitrile mobile phase gradient.

The LC programme for the two sample types was as follows. Liver: mobile phase A was 0.1% acetic acid in water and mobile phase B was 0.1% acetic acid in methanol (rate: 0.3 mL min^{-1}). Gradient elution for liver samples started from 95% A and 5% B, increased to 50% B in 15 min and to 100% B in a further 5 min, then decreased to 5% B in 0.1 min, held for 5 min, and returned to initial conditions. Plasma: mobile phase A was 0.1% formic acid in water and mobile phase B was 0.2% formic acid in acetonitrile (rate: 0.5 mL min^{-1}). Gradient elution for plasma samples started from 95% A and 5% B, increased to 70% B in 3 min, then returned to initial conditions. MS/MS was performed in single (using ESI in the positive mode) and multiple (using UniSpray in positive mode) action mode for livers and plasma, respectively. Two characteristic fragments (*m/z* 168.72 and *m/z* 131.56) were monitored for the compound CLO. Argon was used as collision gas.

2.4.3. Quality control

Three protocols were used during each batch run for quality control and assurance purposes: 1) a deuterated internal standard was added and analysed in all samples; 2) all batches contained a matrix-matched blank, which was analysed for CLO and the deuterated internal standard (Clothianidin D3; QMX, Essex, UK); and 3) during analytical runs a traceable National Institute of Standards and Technology certified standard (Clothianidin; SPEX, Certiprep, Stanmore, UK) was also analysed. The performance of the method was assessed for accuracy (recovery of the internal standards from all samples) and consistency (between-batch analyte linearity). Recovery for the total procedure was calculated using the labelled standards and all residue data were recovery- and blank-corrected. Sample recoveries ranged between 60 and 120%. Mean (±SE) recoveries were 78.0 ± 1.1% and 88.3 ± 2.4% for liver and plasma, respectively. The limit of detection (LOD) and limit of quantification (LOQ) for CLO were 0.004 ng/g wet weight (ww) and 0.006 ng/g ww, respectively, for liver samples and 0.15 ng/mL and 0.21 ng/mL, respectively, for plasma samples. The LOD was determined using the signal to noise ratio multiplied by three and the LOQ was calculated as the LOD plus the calculated expanded uncertainty of the method. The expanded uncertainty for CLO was calculated using the Nordtet TR537 handbook (Magnusson et al., 2012). Concentrations of CLO in plasma and liver samples were compared to one another in the units relevant to either sample type (liver: ng/g ww, plasma: ng/mL).

2.5. Statistical analysis

A combination of non-parametric analyses, generalised linear models (GLMs) and generalised linear mixed models (GLMMs) were used to analyse the data. Model fit for all GLMs and GLMMs was assessed by testing for over-dispersion (using the 'overdisp' function, (Bolker, 2018)) and by comparing modelled residuals to simulated residuals (using the 'simres' function in the 'Dharma' package (Hartig, 2016)). Final models were selected on the basis that the residuals did not significantly deviate from predicted residuals and that there was no over-dispersion. CLO concentrations in plasma (only) were log-transformed to improve model fit and all models were run using a negative binomial distribution to account for over-dispersion. For samples where the concentration of CLO for liver and plasma was measured as less than the LOD, the respective LOD value (i.e. 0.004 ng/g ww for liver and 0.15 ng/mL for plasma) was substituted prior to statistical analysis. All analyses were conducted in R (R Core Team, 2013) and models were run using the glmmTMB package (Brooks et al., 2017).

Patterns of exposure were analysed using Wilcoxon rank sum test (differences in CLO concentrations between groups), Fishers exact test (number of detects vs. non detects) and the Kruskal-Wallis test (between site differences). Data points included in analyses were restricted to those collected post-sowing (treatment group) unless stated otherwise. GLMMs were used to assess the association between CLO concentrations in samples (liver and plasma) and the number of days post-sowing; site was entered as a random effect to account for differences in CLO concentration between sites. The relationship between liver and plasma concentrations over time was analysed by dividing plasma CLO concentrations by liver CLO concentrations and modelling the resultant figures as a function of the number of days post-sowing. A simple linear model was used owing to the small number of samples available for which CLO was detected in both the liver and plasma ($n = 16$ in total; $n = 15$ after one outlier was removed). An additional GLMM was conducted using the same subset of data ($n = 16$) for liver and plasma CLO concentrations alone, with site added as a random effect. All health parameters were analysed using GLMs, with body fat, individual bird weight and faecal parasite load modelled as a function of

Table 2
Summary of clothianidin (CLO) detection in individuals collected pre- and post-sowing and the concentration of CLO recorded in liver and plasma samples obtained. The frequency of exposure is inclusive of both liver and plasma samples. Concentration data for CLO are presented for the subset of samples with concentrations above the limit of detection. The proportion of individuals that tested positive for CLO is calculated to the nearest 1%.

Species		Number of individuals		CLO detection (%)	Liver concentration (ng/g ww)			Plasma concentration (ng/mL)		
		Sampled	CLO detected		Range	Median	IQR	Range	Median	IQR
Pre-sowing (visit 1)										
All		18	1	6	*na*	0.13	*na*	*na*	*na*	*na*
Red-legged partridge	*Alectoris rufa*	17	1	6	*na*	0.13	*na*	*na*	*na*	*na*
Grey partridge	*Perdix perdix*	1	0	0	*na*	*na*	*na*	*na*	*na*	*na*
Pheasant	*Phasianus colchicus*	0	0	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Woodpigeon	*Columba palumbus*	0	0	*na*	*na*	*na*	*na*	*na*	*na*	*na*
Post-sowing (visits 2 & 3)										
All		57	51	89	0.01–37.0	0.07	0.51	0.40–3200	27.7	352
Red-legged partridge	*Alectoris rufa*	43	41	95	0.01–37.0	0.10	0.54	0.40–3200	47.1	382
Grey partridge	*Perdix perdix*	6	5	83	0.03–0.24	0.06	0.10	0.60–3.00	1.80	1.20
Pheasant	*Phasianus colchicus*	7	4	57	0.02–1.44	0.48	0.98	*na*	*na*	*na*
Woodpigeon	*Columba palumbus*	1	1	100	*na*	0.03	*na*	*na*	*na*	*na*

ww: wet weight; IQR: inter-quartile range; *na*: not applicable.

CLO concentration in liver and plasma samples. Individual bird weight was only analysed for red-legged partridge due to inter-species variation. One outlier with a very high number of faecal parasites (1050 eggs/g) was removed from all analyses that included parasite load (range for remaining values: 0–330 eggs/g; median and inter-quartile range: 6.1 and 64.5 eggs/g, respectively; Table S1).

3. Results

3.1. Prevalence and levels of exposure

CLO was detected in 6% (1/18) of birds collected pre-sowing and 89% (51/57) of birds collected post-sowing (inclusive of all species), with a significant difference in detection frequency between the two groups (Fishers exact test: OR = 94.3, $p < 0.001$; Table 2). CLO was detected in 86% (49/57) of liver and 54% (18/33) of plasma samples collected post-sowing, compared to only one liver sample and no plasma samples collected pre-sowing. The median CLO concentration in those samples which contained CLO residue was 0.11 ng/g ww (IQR = 0.5, $n = 51$) in liver and 352 ng/mL (IQR = 27.7, $n = 18$) in plasma. The largest recorded concentrations of CLO in liver and plasma were 37.0 ng/g ww and 3200 ng/mL, respectively. Residue concentrations in samples collected post-sowing differed significantly between sites for both liver (Kruskal-Wallis test: $\chi^2_5 = 16.2$, $p = 0.006$) and plasma samples (Kruskal-Wallis test: $\chi^2_5 = 11.5$, $p = 0.042$). The median concentration of CLO in the two sample types collected from each site, ranged between 0.02 and 1.30 ng/g ww (liver), and <LOD and 246.7 ng/mL (plasma), with sites 2 and 6 having the largest concentrations for both sample types (Table S2). Overall, partridges collectively had the highest frequency of detection, with 94% of individuals testing positive for CLO in plasma and/or liver. However, the concentration of CLO in liver samples was at least four-fold larger in pheasants compared to the other three species tested (Table 2). The concentration of CLO did not differ between male (33 liver and 22 plasma samples) and female (24 liver and 11 plasma samples) birds in either plasma or liver samples across all species (Wilcoxon rank sum test – liver: W = 581.5, p-value = 0.402; plasma: W = 198.5, p-value = 0.621).

3.2. Patterns of exposure in liver and plasma samples

Both liver and plasma samples were available for 42 out of the 75 birds included in the study (9 pre-sowing, 33 post-sowing). CLO was not detected in either the liver or plasma samples of those birds with both sample types that were collected pre-sowing, but 31 out of the 33 birds collected post-sowing tested positive for CLO: 13 (42%) birds tested positive for CLO in the liver only, two birds (6%) tested positive in the plasma only and 16 (52%) birds tested positive in both the plasma and liver. Overall, CLO was detected in 88% of liver samples and 55% of plasma samples post-sowing and there was a significant difference in detection between these two sample types (Fishers exact test: OR = 0.17, $p = 0.005$). When the LOD for liver was artificially set at 0.15 ng/g ww to match the LOD for plasma (0.15 ng/mL) to give a like-for-like comparison, CLO detection decreased from 31 to 20 birds out of the 33: two birds (10%) tested positive for CLO in the liver only, five (25%) in plasma only and 13 (65%) in both the liver and the plasma. Birds within each of these categories were collected on average at 24.0, 4.2 and 7.8 days post-sowing, respectively. When analysing only the data for red-legged partridge, the proportion of samples where CLO was detected in the liver remained similar between the two post-sowing visit groups (Fishers exact test: OR = 0.00, $p = 0.522$; Table 3). In contrast, CLO was detected in significantly more plasma samples during visit 2, compared to visit 3 across all sites (Fishers exact test: OR = 0.08, $p = 0.004$; Table 3). These trends remained the same when all species data were analysed (Table S3).

Where CLO was detected in both the liver and the plasma, the concentration of CLO was found to be on average 98% greater in plasma samples compared to liver. The concentration of CLO in red-legged partridges was significantly smaller during visit 3, compared with visit 2 for liver samples (Wilcoxon-rank sum test: W = 334.5, $p = 0.003$), and for plasma samples (Wilcoxon-rank sum test: W = 175.0, $p = 0.005$; Table 3; Fig. S1). These trends remained the same when all species data were analysed, except there was no significant difference in CLO liver concentrations between visit 2 and visit 3 (Table S3). The concentration of CLO in liver and plasma samples (inclusive of detect and non-detect samples collected post-sowing) was not found to decrease significantly with the number of days post-sowing in either red-legged partridges (Table 4; Fig. 1), or when all species were analysed (Table S4). There were 16 birds where CLO was detected in both sample types to allow a direct comparison of patterns of CLO concentrations in plasma and liver; the ratio of concentration in plasma to concentration in liver decreased with time from sowing (Fig. S2), but this relationship was not statistically significant. There was a significant positive relationship between CLO plasma and liver concentrations when the time from sowing was removed from the model and site was added as a random effect (Fig. S3).

3.3. Health parameters and CLO concentrations

Associations between health parameters and concentrations of CLO in liver and plasma samples were tested for red-legged partridges, both partridge species combined, and all species combined. There was a positive association between faecal parasite load and the concentration of CLO in the liver, which was statistically significant for analyses based on data for all species (Table S4; Fig. S4) and for both partridges combined (Table 4; Fig. 2), but not significant when data were analysed for red-legged partridge alone (Table 4). No association was found between plasma concentrations of CLO and faecal parasite load for red-legged partridge only, grey and

Table 3
Summary of clothianidin (CLO) detection and concentrations in plasma and liver samples throughout the study for red-legged partridge only. Data are presented for visit 1 (pre-sowing), visit 2 (1–7 days post-sowing) and visit 3 (8–30 days post-sowing). The proportion of samples for which CLO is detected is calculated to the nearest 1%. Data for liver are given with and without the equivalent LOD for plasma applied (0.004 and 0.015 ng/g ww, respectively).

Sample	Visit (group)	Number of samples		CLO detection (%)		CLO concentration[a]	
		Total	CLO detected	Visit	Group	Median	IQR
Plasma (ng/mL)	1 (pre-sowing)	8	0	0	0	*na*	*na*
LOD: 0.15	2 (post-sowing)	13	11	85	57	112	416
	3 (post-sowing)	17	5	29		17.2	23.0
Liver (ng/g ww)	1 (pre-sowing)	17	0	0	0	*na*	*na*
LOD set to: 0.15	2 (post-sowing)	16	11	69	50	2.42	11.6
	3 (post-sowing)	27	8	30		0.50	0.70
Liver (ng/g ww)	1 (pre-sowing)	17	1	6	6	0.13	*na*
LOD: 0.004	2 (post-sowing)	16	16	100	97	0.75	5.0
	3 (post-sowing)	27	25	93		0.06	0.3

IQR: inter-quartile range; LOD: level of detection; *na*: not applicable; ww: wet weight.
[a] Positive samples only.

Table 4
Summary of generalised linear models and generalised linear mixed model outputs for partridge species. Models were used to investigate CLO concentration in relation to the number of days post-sowing and health parameters, for plasma and liver samples.

Sample	Model + (random effects)	N obs	Disp	Estimate	SE	p-val
Red-legged partridge only						
Liver	CLO conc ~ days post-sowing + (site)	60	1.55	0.018	0.025	0.467
	parasite load ~ CLO conc	33	0.77	0.034	0.019	0.077
	weight ~ CLO conc	43	1.07	−0.001	0.002	0.731
Plasma	CLO conc ~ days post-sowing + (site)	38	1.43	−0.007	0.021	0.725
	parasite load ~ CLO conc	24	0.79	0.068	0.066	0.301
	weight ~ CLO conc	30	1.10	0.005	0.006	0.468
All	parasite load ~ weight	43	1.25	0.003	0.002	0.270
Red-legged partridge and grey partridge						
Liver	CLO conc ~ days post-sowing + (site)	67	1.70	0.023	0.023	0.304
	parasite load ~ CLO conc	39	0.77	0.042	0.018	0.024
Plasma	CLO conc ~ days post-sowing + (site)	42	1.57	−0.007	0.018	0.670
	parasite load ~ CLO conc	26	0.80	0.090	0.069	0.192

Models for fat score as a function of CLO concentration did not converge for liver or plasma samples.
CLO: clothianidin; conc: concentration (either in ng/g ww for liver or ng/mL for plasma samples); Disp: measure of model dispersion; N obs: number of observations used in each model; SE: standard error.

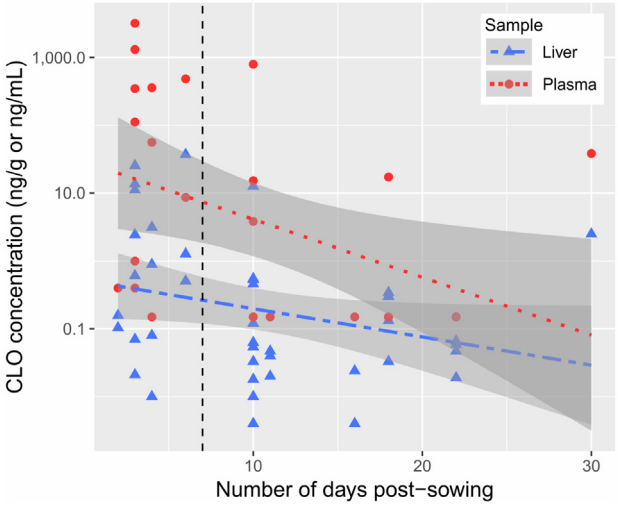

Fig. 1. Concentration of clothianidin (CLO) in liver and plasma samples collected from red-legged partridges post-sowing. Non-detects are represented as the LOD for both sample types (liver: 0.004 ng/g wet weight; plasma: 0.15 ng/mL). Each point represents one sample type obtained from one bird per site visit. The black dashed line represents the distinction between samples collected at visit 2 (1–7 days post-sowing) versus visit 3 (8–30 days post-sowing). A linear best-fit for each set of data points is represented by coloured lines, with 95% confidence intervals represented by the grey shading. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

red-legged partridge (Table 4), or when all species were analysed (Table S4). Neither CLO concentration (in either sample type), or parasite load were significantly associated with body weight of red-legged partridge (Table 4; Fig. S5). No significant associations between fat score and plasma or liver concentrations of CLO were detected when all species were analysed (Table S4). There was weak (non-significant) association between fat score and parasite load (all species; Table S4). The models for fat score did not converge when only red-legged partridge or partridge species data were used. All other associations remained the same as those for red-legged partridge when all species data were analysed (Table S4).

4. Discussion

This study provides strong evidence that NN-treated seed is a source of exposure for gamebirds following autumn sowing. The number of birds in the current study that tested positive for CLO post-sowing (89%) was high compared to the prevalence of CLO in birds collected pre-sowing (6%), and NN prevalence reported previously for other species of Galliforme. For example, NNs have been detected in the livers of 12% of northern bobwhite quails (Texas, USA; imidacloprid, CLO, thiamethoxam) (Ertl et al., 2018); 17% of scaled quails (rolling plains eco-region, USA; imidacloprid, CLO, thiamethoxam) (Turaga et al., 2016); 22% of wild turkeys (Ontario, Canada; thiamethoxam and CLO) (MacDonald et al., 2018); and 8% of eggs collected from grey partridges (France, thiamethoxam) (Bro et al., 2016). Furthermore, a study that used radio tracking to quantify the use of treated fields by wild birds estimated that 13% of grey partridge coveys were exposed to pesticides, including NNs (Bro et al., 2015). When comparing these data, it is of note that the LOQ for liver samples in previous studies was markedly higher than the LOQ obtained here (0.006 ng/g ww, compared to 1–3 ng/g ww in previous studies). However, when an LOQ of 1 or 3 ng/g is applied to the current dataset, the prevalence of CLO detection among individuals remains high at 77 and 70%, respectively. Notably, the prevalence of imidacloprid in grey partridge gizzards and livers (93 and 36%, respectively) collected as part of a study on wildlife poisoning events in arable land, were more similar to those described here (Millot et al., 2017). In the same study, imidacloprid-related poisoning incidents were also reported more frequently during the autumn sowing season than at other times of the year (Millot et al., 2017), which tallies with differences in CLO prevalence pre- and post-sowing observed in the current study.

When comparing the amount of CLO recorded in liver and plasma samples here to existing data for NN insecticides in wild birds (noting that direct comparison is confounded by differences in toxicity between NN compounds), concentrations were relatively similar to those recorded previously in livers (when LOQ is taken into account), but larger than those reported previously for plasma. To date, concentrations of NN compounds (CLO, imidacloprid, thiamethoxam) in liver samples collected from comparable Galliforme species and excluding poisoning events had a maximum of 120 ng CLO/g ww, 62 ng imidacloprid/g ww, and 160 ng thiamethoxam/g ww (MacDonald et al., 2018; Turaga et al., 2016); whereas concentrations of CLO recorded in the present study had a maximum of 37.0 ng CLO/g ww. Conversely, concentrations of NNs previously recorded in plasma collected from wild birds showed a maximum for any single compound of 3.28 ng imidacloprid/mL (Hao et al., 2018; Taliansky-Chamudis et al., 2017), which is far lower than the median concentration of CLO in plasma recorded here (352 ng/mL). This disparity may be attributable to the fact that the only comparable avian plasma data for NN exposure were obtained from two bird of prey species (Byholm et al., 2018; Taliansky-Chamudis et al., 2017) and one migratory passerine (Hao et al., 2018). Birds of prey are inherently more likely to experience secondary exposure (e.g., ingestion of contaminated prey), rather than primary exposure such as that via the direct ingestion of treated seeds. Equally, migratory passerines are likely to encounter a wider range of food sources across more varied habitats compared to sedentary Galliformes in arable fields. Also of note is that the majority of birds sampled previously were wild and some (e.g., those native to North America) inhabit non-arable habitats (MacDonald et al., 2018; Turaga et al., 2016; Ertl et al., 2018), whereas birds sampled here were hand-reared and released into an intensively farmed landscape. It is therefore likely that the spatial and temporal proximity of managed birds to drilling of CLO-treated seed contributed to large concentrations of CLO observed in plasma samples, as well as the overall number of individuals that tested positive for the compound. In addition, gamebirds are known to forage more frequently at field headlands than in field centres (Green,

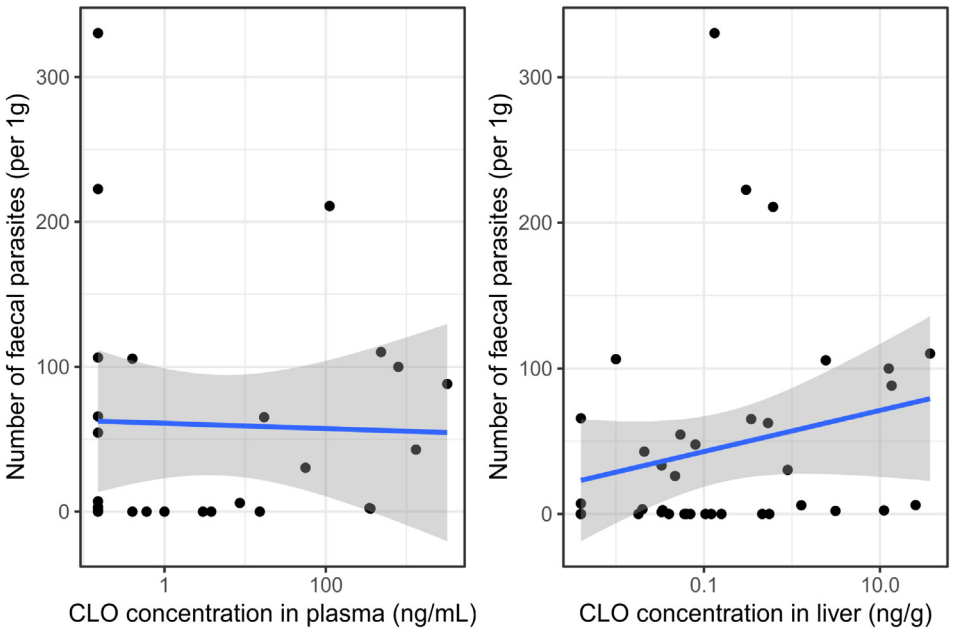

Fig. 2. Faecal parasite load plotted against the concentration of clothianidin (CLO) in plasma samples (left) and liver samples (right) for grey partridge ($n = 4$) and red-legged partridge ($n = 41$) combined. One outlier was removed from the data for liver (1050 faecal parasites per 1 g faeces and 0.5 ng/g wet weight CLO detected in the respective liver sample). Linear best-fits for the two data sets are represented by solid lines, with 95% confidence intervals represented by the grey shading. Data for all species are presented excluding woodpigeon (no faecal samples available). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1984), where there are relatively high densities of treated seed on the soil surface after drilling (Lopez-Antia et al., 2016; de Snoo and Luttik, 2004).

Concentrations of CLO differed between plasma and liver samples obtained from individual birds over the course of the study. These differences will reflect the time course of exposure, the toxicokinetics of CLO in the birds and the interval between exposure and measurement of residues. No firm conclusions can be drawn because there is a paucity of toxicokinetic information for CLO in birds, but the literature does contain information for imidacloprid. Controlled avian experiments have shown that imidacloprid concentrations in plasma can peak 1 h after exposure (Bean et al., 2019), with large individual variation in concentrations observed up to 6 h after exposure (Hao et al., 2018). Comparatively, concentrations of imidacloprid in liver have been reported to be 46–84% of those in plasma and appear to be more consistently dose-dependent across existing studies (Bean et al., 2019; Zeid et al., 2019), with reported cases of accumulation over multiple exposure events (Lopez-Antia et al., 2015). In a field setting, where treated seeds is a primary, concentrated source of exposure, the expectation is that the frequency and level of exposure would decrease with the number of days post-sowing as seed densities and the concentration of CLO on remaining seeds decline. Here, the frequency of CLO detection in plasma decreased significantly between one week (visit 2) and two-to-four weeks (visit 3) post-sowing, and the concentration of CLO in the liver and plasma also significantly decreased over this period. However, the frequency of CLO detection in livers was similar at visits 2 and 3. It seems likely that, as noted for other contaminants (Espín et al., 2016), plasma samples provide a shorter-term labile measure of recent exposure, whereas liver samples provide a more stable measure of exposure. However, further pharmacokinetic studies would be required to confirm that there are such differences in CLO toxicokinetics and to quantify the resultant relationships between dose and plasma/liver residues. Overall, liver samples provided a more sensitive detection method for CLO (Fig. S3), had a higher frequency of detection (CLO was detected in the liver and plasma of 88 and 55% of birds, respectively, when both samples were available from the same bird for analysis), and concentrations in liver tended to be maintained over a longer period of time than those in plasma (Fig. S2).

Of the three health parameters measured, faecal parasite load was the only one that was found to be associated with CLO concentrations in biological samples. However, the association was present only with concentrations in the liver and when both partridge species and all species were modelled collectively. This positive association may be explained by the potential effect of NNs on the avian immune system via nicotinic acetylcholine receptors, which are present on many cell membranes throughout the body, including white blood cells (Kalamida et al., 2007). Previously, NNs have been found to adversely affect both the humoral and cellular immune response of bird species, including the reduction of antibody titres and T-cell mediated immunity (Lopez-Antia et al., 2013; Siddiqui et al., 2007). Incidentally, T-cells have been well documented as an important response to coccidiosis, the parasitic disease caused by *Coccidia*, which accounted for the majority of parasites found in faecal samples (Table S1), and secretory immunoglobulin-A antibodies are thought to bind to the coccidial surface to inhibit the protozoan parasite (Yun et al., 2000). As such, there is a plausible pathway by which NN exposure could be associated with increased faecal parasite load, to the detriment of individual bird health. Alternatively, this association may be explained by birds transitioning from a managed-captive environment to being released into the field. It is common practice to feed captive-bred galliformes with grain treated with anthelmintics prior to release, and so any increase in faecal parasite load may have been exacerbated by a lack of anthelmintic-treated feed, post-release stress (Millán, 2009), and/or an increase in CLO-treated seed consumption. Faecal parasite loads in avian hosts can also vary greatly between individuals based on reproductive status, time of sampling, climate and the phase of the parasitic cycle (Villanúa et al., 2006). Further field-based studies would be required to establish the true cause of the association observed here, particularly as a significant effect was detected when managed and unmanaged partridge species were included in the analyses, rather than red-legged partridge alone. Interestingly, the association between faecal parasite load and concentrations of CLO only existed for liver samples, and not for plasma samples - presumably because plasma concentrations are more transient (Espín et al., 2016). This finding may be important to consider

when investigating sub-lethal effects of NNs in the field, as the sample type used to measure exposure may dictate whether or not an association is detected.

No association was detected for either of the remaining health parameters measured (fat score or body weight) in relation to CLO exposure, although both have been reported previously to be negatively affected in NN exposure studies with captive birds. For example, a significant reduction in body weight was recorded in CLO-dosed South American eared doves *Zenaida auriculata* (Addy-Orduna et al., 2018) and imidacloprid-dosed red-legged partridges (Lopez-Antia et al., 2015), whilst white-crowned sparrows dosed with imidacloprid lost between 17 and 26% of body mass during a 3-day period, with fat scores following a similar trend (Eng et al., 2017). Notably, avoidance of imidacloprid-treated seed in captive birds has been found to be a conditioned aversion mediated by sickness (Lopez-Antia et al., 2014), but the extent to which avoidance influences feeding behaviour and subsequent potential for effects in the field has been questioned (Millot et al., 2017). Differences in dose as well as differences in the inherent toxicity of imidacloprid and CLO may explain the lack of association between exposure and either fat score or body weight under our field-based design; it is also likely that migratory passerine species (such as white-crowned sparrows) are more sensitive to fat loss compared to reared Galliformes. As the birds used in this study were heavily managed for the shooting season, it is also possible that the provision of supplementary (untreated) cereal seeds by gamekeepers, resulted in more consistent weight and fat scores of birds throughout the duration of the study.

4.1. Conclusion

The present study provides clear evidence that, in the UK and likely elsewhere, treated cereal seeds are a significant source of pesticide exposure for gamebirds during autumn sowing. CLO was detected in both the liver and plasma, although the difference in concentrations between the two sample types indicates that they provide different measures of exposure in the field. These data demonstrated that a health parameter (faecal parasite load) can be associated with one measure of NN exposure, but not the other, which will be an important factor to consider when designing any future studies investigating the sub-lethal effects of NNs on free-living birds, and possibly other vertebrates. Furthermore, the positive association between faecal parasite load and liver CLO concentration indicates a need for further research on the interaction between NN compounds and avian parasites and/or disease in a field-based setting. Field data collected here significantly contribute to the growing body of evidence for NN exposure in Galliformes worldwide; they will be of value to the design of any future avian biomonitoring studies and to the development of risk assessments for insecticide seed treatments.

Ethics statement

All data collection and research was approved by Animal Welfare Ethical Review Bodies panel at the University of York. Fieldwork design was also approved by an independent panel at the Game and Wildlife Conservation Trust.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140493.

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