THE EFFECTS OF NEONICOTINOID SEED TREATMENTS ON FARMLAND BIRDS

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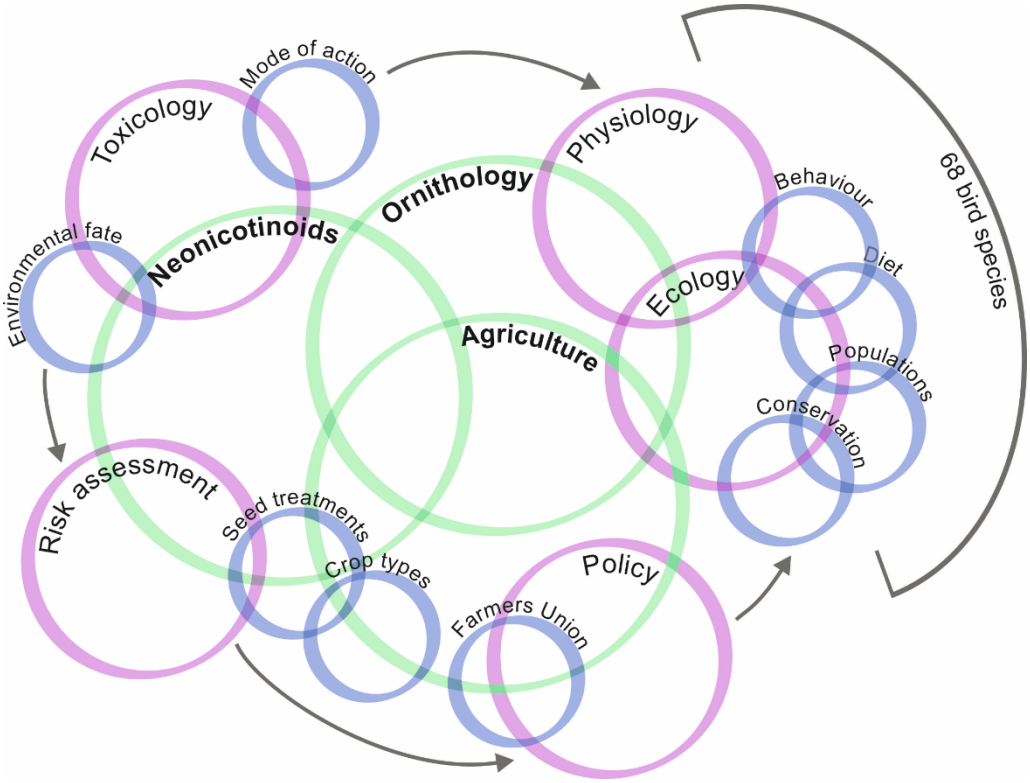

"A jack of all trades is a master of none,

but often times better than a master of one."

– English proverb dating from the 14th century

Abstract

Neonicotinoids are the most common group of agricultural insecticides used worldwide, with seed treatments accounting for a large proportion of applications. Since neonicotinoids were introduced to the agricultural market, concerns have been raised regarding their effect on non-target organisms, and in the last 5 years avian-related research has gained momentum. However, the extent and impact of neonicotinoid exposure in free-living birds, particularly farmland communities, remains poorly understood.

Here, data were collected for agricultural plant material and multiple species of farmland bird from neonicotinoid-treated fields, to assess the exposure pathway associated with seed treatments. Biological samples were obtained from 15 species of bird to measure levels of exposure and data were collected to investigate whether there were associated physiological sub-lethal effects. Long-term data sets for neonicotinoid use and bird populations were also modelled to assess the impact of seed treatments on farmland bird species over the last 21 years.

Seed treatments were found to be a significant source of exposure for farmland birds. Exposure was confirmed in 9% of individuals pre-sowing, compared to 68% of individuals post-sowing, and 30% of species overall. Exposure was found to be associated with one physiological parameter, which could be detrimental to bird health. There was no consistent evidence to suggest that dietary exposure to neonicotinoid seed treatments has impacted bird populations historically, however three bird species warrant further investigation in this regard.

These data suggest that current risk assessment and insecticide product safety protocols do not effectively safeguard farmland bird communities from neonicotinoid exposure during sowing, and imply that exposure may be widespread in bird communities where neonicotinoids are in use. Results obtained here highlight the need for field-based data in ecotoxicological risk assessments and should be considered in relation to any future systemic insecticide seed treatments.

(292/300 word limit)

List of contents

List of figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

List of tables

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

List of supplementary material

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX

Acknowledgements

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There are two people at CEH Wallingford, without which, the modelling chapter would not have been a possibility. I would like to thank Nick Isaac for agreeing to collaborate with me, and Stephen Freeman whose model we adapted for the purpose. Both Nick and Stephen valiantly volunteered to talk me through the model, despite my minimal mathematical background, and at that point in time, only a beginner's knowledge of R.

All of the chemical analyses presented in this thesis were made possible by the hugely helpful team at CEH Lancaster. A special thanks goes to: Darren Sleep and Nicola Thompson, who patiently taught me the required protocols, dealt with the mood swings of the analytical equipment and saw me through some mild laboratory-based panics when my 'one-shot' samples were at stake. I would also like to thank Elaine Potter, who gave me a crash course in avian necropsy, and Ann Kretzschmar who gave me a place to stay (often at short notice) during my time at Lancaster.

All of the avian field samples included in this thesis were collected in the autumn of 2017; 432 birds were caught and processed within 32 days. This would not have been possible without the team of ringers who volunteered to accompany me on my mist-netting excursions, and all of the shoot staff (and their four-legged companions) who helped me to collect gamebird samples. Alongside Jenny Dunn and Vivienne Hartwell, a special mention and thanks goes to Stuart Britton, who cheerfully offered much of his time, and always managed to catch whatever species I optimistically requested. A further thanks is due to all of the lab technicians at the University of York and University of Lincolnshire who helped facilitate the collection of these data.

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Declaration

Various co-authors contributed to data chapters two, three and four; contributions from co-authors are detailed on the title page of these chapters, as would appear in submission of a journal publication.

In chapter two, data are presented for sites in East Anglia and Lincolnshire. All data collection that took place in Lincolnshire was done so by me as part of this PhD research. I prepared Lincolnshire samples for chemical analysis (data/sample management, methodology development, sample preparation, sample extraction and initial processing of concentration data), whilst CEH operated the analytical equipment, assisted in methodology development and provided the finalised concentration data. All data collection in East Anglia was done by the Royal Society for the Protection of Birds (RSPB) as part of the project 'The impact of neonicotinoids on farmland birds'. All aspects of the chemical analyses related to samples obtained from East Anglia were undertaken by the Centre for Ecology and Hydrology (CEH; Lancaster), except for sample preparation that was undertaken by the RSPB under my supervision. All subsequent data management, statistical analysis, data presentation and interpretation for both the East Anglia and Lincolnshire datasets was undertaken by me, as well as all written work and the assimilation of these data for chapter two.

I undertook all data collection and analyses included in chapter three. My roles during the chemical analyses of avian samples presented in this chapter were as follows: data/sample management, methodology development, sample preparation, sample extraction and processing initial concentration data. CEH operated the analytical equipment, assisted in methodology development and provided the finalised concentration data. All data management, statistical analyses, data presentation and interpretation, and written work was undertaken by me.

In chapter four, several long-term data sets were used to create the final data frame that was entered into the model. These data were sourced and used with permission from the Breeding Bird Survey (British Trust for Ornithology), the Pesticide Usage Survey (Fera Science Ltd.) and the Agcensus database (hosted by EDiNA). The Freeman and Newson model (2008) was adapted for use in chapter four by me and my co-author Nick JB Isaac (CEH, Wallingford). All data management, statistical analyses, data presentation and interpretation and written work was undertaken by me.

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

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Publication status

The current publication status of each chapter is detailed below.

Chapter	Title	Status	Details
1	[Working title] The state of play: neonicotinoids and farmland birds	*TBC*	*TBC*
2	From seeds to plasma: confirmed exposure of multiple farmland bird species to a neonicotinoid seed treatment	Published (Mar 2020)	Sci Total Environ. 19(723):138056
3	High prevalence of neonicotinoid residue in liver and plasma samples collected from gamebirds during autumn sowing	Submitted (Feb 2020)	Sci Total Environ.
4	Using long-term datasets to assess the impacts of dietary exposure to neonicotinoids on farmland bird populations in England	Published (Oct 2019)	PLoS One. 14(10):e0223093.

If it is bad for the bees, is it bad for the birds?

Introduction

Neonicotinoids (NNs) are a class of systemic insecticide that were introduced to the agricultural market in the early 1990s, and have subsequently caused many debates within academic, regulatory and agricultural sectors, regarding the effectiveness and ecotoxicological safety of these compounds. The release of NNs into the environment over the last three decades has triggered a complex set of adverse effects on pollinators [1], and has raised numerous concerns within the scientific community about the potential effects on other wildlife taxa [2]. In response to an extensive review of the risk of NNs to pollinators conducted by the European Food Safety Authority (EFSA), the European Union (EU) banned the outdoor use of the compounds imidacloprid, thiamethoxam and clothianidin, in 2018. However, few other countries have followed suit, and the use of NNs worldwide continues in parallel with the debate regarding their environmental safety.

Worldwide integrated assessments have attempted to understand the full extent of the environmental impacts of NNs [1, 3-5]. These assessments have compiled data from multiple research groups on the behaviour of NN compounds in the wider environment, NN alternatives within agricultural production, and the impact of NNs on non-target organisms. In ecotoxicology specifically, the majority of research to date has focused on pollinators, but many knowledge gaps pertaining to other non-target organisms remain. Over the last 5 years, the number of studies on NNs in avian species has risen and momentum has grown within this research area. Birds are one of the major species groups to inhabit farmland and are often used as indicator species for ecosystem health [6]. As such, birds are not only at risk from the impacts of NNs, but could also be an important species group for understanding the wider effects of NNs throughout the environment. To date, a handful of studies have confirmed NN exposure in wild birds [7-13], and there is a mounting body of evidence from laboratory- and aviary-based studies that has confirmed that NN compounds are toxic to birds at relatively small doses [14], and that sub-lethal effects on avian physiology and behaviour can occur [15]. As yet, translating evidence of toxicity and/or sub-lethal effects to a field-based setting or population scale has rarely been attempted, and the gap between laboratory research and information to inform NN usage policies in an avian context remains large.

Due to the paucity of data regarding NNs and farmland birds, and the relative infancy of this research area, there are many research gaps that could have been investigated as part of this thesis. Currently, one of the largest is understanding the extent to which wild birds are exposed during standard agricultural practices, and the impact that this exposure may have. To gain a full

understanding of the interaction between NNs and wild birds, it is important to put toxicity data obtained from aviary studies with strict dosing regimens into an environmentally-relevant context by measuring rates of exposure and/or sub-lethal effects under standard agricultural conditions. It is equally important to assess the potential impacts of exposure in the field at a population scale, by examining plausible pathways in the context of historical NN use. Using a combination of these approaches, this thesis investigates how exposure affects individual wild birds, avian communities and subsequently populations. To this end, the main aim of this thesis is to collect and use field-based evidence to understand the interaction between agricultural applications of NNs and farmland birds *in situ*. The purpose of these data and subsequent analyses, is to bridge the gap from individuals in aviaries to communities in the field, to determine whether NNs are significantly contributing to continued farmland bird declines, and if so, how. To provide a cohesive body of work, data collection was designed around graduating themes of exposure to effect, and individuals to populations, which necessitated the use of several approaches, and a focus on a specific exposure pathway.

There are multiple pathways by which birds can be exposed to NNs in the wild. Compounds can be applied to crops in the form of foliar sprays, soil drenching and prophylactic seed coatings, which have the potential to cause exposure via dermal, inhalation and ingestion routes. To measure the effect of NN use on birds in the field, it was important to select an exposure pathway that would pose a large risk to farmland birds, both in terms of the frequency and level of exposure that birds may be subject to. Thus, exposure via the ingestion of NN seed treatments was chosen as the focal exposure pathway. Dermal- or inhalation-based pathways were discounted due to the lack of evidence to suggest these are a significant risk to farmland birds, and the relatively small concentrations of NNs that birds may be exposed to via these routes. Seed treatments specifically were chosen as a refined dietary pathway, as agricultural seed makes up a significant portion of multiple farmland bird diets [16], and seed treatments accounted for >90% of NN applications in the UK [17], and ~60% of NN applications worldwide [18]. Furthermore, NNs in this instance may also be considered as a model for other pesticides applied as seed coatings.

Research objectives

In order to assess the effect of neonicotinoid seed treatments on wild birds, the main research objectives of this thesis were:

1. To investigate the frequency and level of NN exposure among farmland bird communities in response to the use of NN seed treatments according to standard agricultural practice;

2. To assess whether wild birds experience sub-lethal effects as a result of NN exposure from treated seed, sown according to standard agricultural practice; and
3. To investigate whether historic, long-term NN usage has had any impact on farmland bird populations in the UK in the context of dietary exposure to NN seed treatments.

To achieve objectives one and two, field data were collected during the autumn sowing seasons of 2015-2017. This was prior to the ban on NNs in 2018, but after the moratorium on imidacloprid, clothianidin and thiamethoxam use on flowering crops that began in 2013. The moratorium had a noticeable impact on the type of NN compound used for seed treatments in the UK, with a switch from imidacloprid to clothianidin taking place during this period. Therefore, all field data included in this thesis was collected from fields sown with clothianidin-treated seed. To achieve objective three, historic data for pesticide use was obtained for the years 1994-2014. As such, the use of imidacloprid, clothianidin and thiamethoxam seed treatments are included in these analyses, which is representative of the popularity of each compound relative to the agricultural policies in place during the timeframe in which these data were collected.

Chapter overview

Chapter 1

A review of the literature to date. Part one introduces NNs with an overview of the chemistry, usage and policies associated with this group of insecticides. Part two assimilates data from studies pertinent to NNs and birds, providing an overview of toxicity, known sub-lethal effects, potential exposure pathways and examples of NN exposure in wild birds. Collectively this chapter aims to bring together knowledge from multiple strands of evidence to present our current understanding of the effect (and potential effects) of NNs on farmland birds, and identifies the challenges associated with this field of research.

Chapter 2

This chapter investigates whether seed treatments are a source of exposure to farmland bird communities in the UK, and the factors that influence patterns of exposure observed via this pathway. Data were collected on all aspects of the exposure pathway: from treated seed to avian blood samples. The availability of treated seed and bird abundance in treated fields were recorded to understand the potential exposure farmland birds could be subject to via NN seed treatments. Concentrations of NN were measured in agricultural seed and seedling samples to assess the level of NNs available to birds in the field. Avian blood samples from multiple passerine species were

analysed for NN compounds to confirm the frequency and level of exposure among a typical agricultural avian community. And finally, patterns of exposure in wild birds were analysed in conjunction with physiological parameters to investigate the possibility of sub-lethal effects in the field.

Chapter 3

In chapter two, biological samples obtained from passerine species are analysed for the NN compound clothianidin as a measure of exposure during a typical autumn sowing season. In this chapter, similar data are collected for three species of gamebird. By sampling from a smaller species pool, with species that are managed as part of the shooting industry, this chapter allowed for further analyses of the sub-lethal effects of NN exposure in the field. Furthermore, blood plasma and liver samples (the two biological samples most commonly analysed for NN compounds) were simultaneously obtained from individual birds to examine how patterns of NN exposure are expressed in differing biological samples. In this instance, data for physiological health parameters (body weight, body condition, fat score and parasite load) were recorded to assess whether there were sub-lethal effects associated with NN exposure, expressed in either sample type.

Chapter 4

After investigating whether exposure occurs on an individual level in the field, the final chapter focused on whether this exposure is translated to population-scale effects for species of farmland bird in England. This chapter used long-term datasets collected as part of the pesticide usage survey (Fera Science Ltd.), the Breeding Bird Survey (British Trust for Ornithology) and annual cropping data (Defra) to model the change in bird abundance in relation to NN seed treatments over a period of 21 years. Model outputs were obtained for 21 species of farmland bird that were put into dietary exposure groups, based on the proportion of agricultural seed and seedling material in each species diet. These groups allowed model outputs to be interpreted in the context of dietary exposure to NN-treated seed. The effectiveness of the modelling approach and the use of historic data, which was not originally collected for this purpose, are also discussed in a biomonitoring context.

Conclusions drawn from each chapter feed into multiple lines of evidence concerning NN exposure and the potential for effects of this on wild birds. These data span multiple avian species at an individual and population level, which may help to discern the impact of NNs on farmland birds in the UK, as well as being relevant in the context of NN usage and bird species worldwide. It is hoped that results from this research will contribute to the body of evidence necessary to make informed

choices regarding NN-related policies, and will provide useful information relevant to any future insecticide seed treatments.

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Literature Review

1.1 NEONICOTINOIDS: AN OVERVIEW

1.1.1 Origin

Neonicotinoid (NN) insecticides are a relatively new group of systemic compounds that are designed to provide long-term protection to crops from invertebrate pests [1]. The development of NNs was propelled by the necessity for a new suite of compounds after target invertebrates gained resistance to existing pesticides, including pyrethroids, carbamates and organophosphates [2, 3]. Imidacloprid (IMI) was the first NN to be made commercially available for agricultural use in the early 1990s (year of patent: 1985) and was followed by a number of other similar compounds, such as clothianidin (CTD) (year of patent: 1989) and thiamethoxam (THX) (year of patent: 1992) [1, 4]. Overall there are eight available compounds that are currently in use worldwide: IMI, CTD and THX, primarily used for crop seed coatings; acetamiprid (ACE) and thiacloprid (THC), which are more often used as foliar or ground sprays for agricultural purposes (FERA, 2017); followed by nitenpyram (NPM) and dinotefuran (DFN), which both have uses outside of agriculture (e.g., residential/veterinary pest management). The eighth NN-type compound to be released on the agricultural market was sulfoxaflor, which was approved for use in the European Union (EU) in 2015 and is used as a foliar spray [5]. Furthermore, over 600 *cis*-NN compounds (isomers of NNs) have also been synthesised, a small number of which are being developed for the Chinese insecticide market [2].

1.1.2 Structure & mode of action

NNs consist of substituted aromatic heterocyclic rings, typically adjoining to a methylene bridge ending in an electron-withdrawing terminal group, which differs depending on the specific compound (**Figure 1.1**) [6]. NNs have a small molecular size (between 250 and 300 g/mol) and are therefore moderately-to-highly water-soluble (184 to 590 mg/L) [7]. Solubility is inherent to the systemic function, and allows compounds to be taken up into the plant xylem by diffusion and distributed internally. NNs are also photostable, non-ionised at environmental pH values and not readily hydrolysed [8].

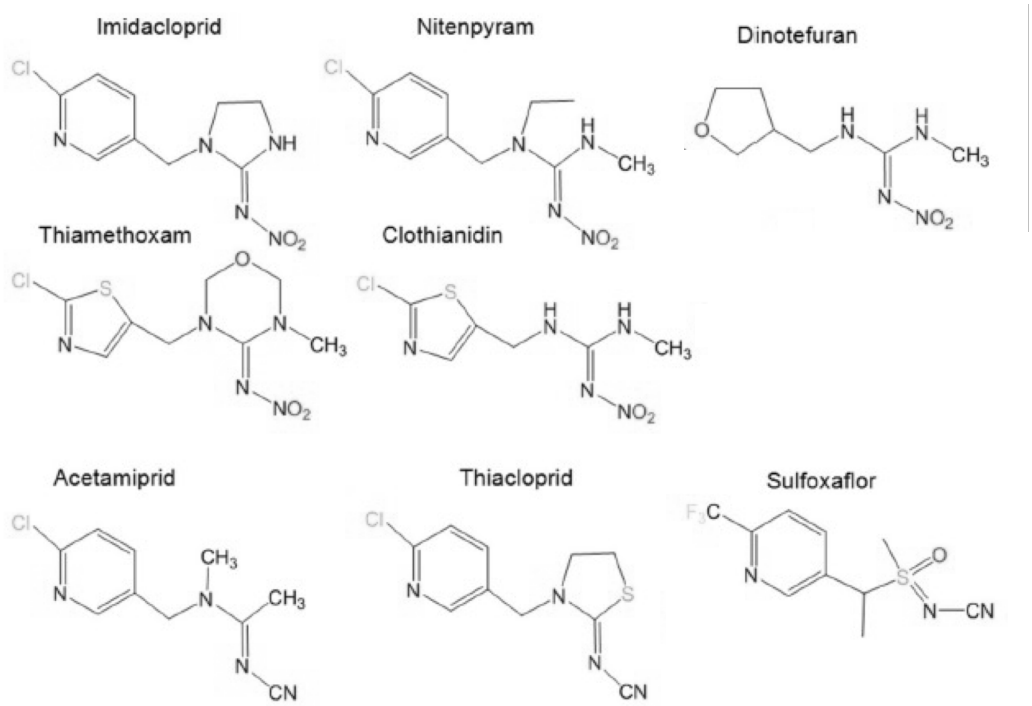

Figure 1.1 Chemical structure of eight NN compounds commercially available on the agricultural market. Reproduced from Simon-Delso (2015) [9].

NNs act as agonists (e.g., blocking agents) to nicotinic acetylcholine receptors (nAChRs), which are pentameric transmembrane proteins that respond to the neurotransmitter acetylcholine [10]. There are many variations of these proteins, which are expressed at various locations within an organism's central and peripheral nervous system, as well as in other non-neural and non-muscle cell types [10, 11]. nAChRs are made up of five sub-units, which can include any one of 17 polypeptides; broadly speaking, these sub-units can be configured to have either five or two binding sites as part of a homogenic or heterogenic structure, and different binding sites can exist among the many nAChR subtypes [11]. nAChRs are known to be responsible for controlling and regulating multiple aspects of an organism's biology (**Table 1.1**), as well as muscular or neuronal disease initiation [10]. However, the function of many subtypes of nAChRs have not yet been identified.

Table 1.1 Known functions of nAChRs (summarised from Kalamida *et al.,* 2007) and physiological processes associated with each.

Function	Systems affected (physiological manifestation)	Associated nAChR subtype
Expressed in the muscular system		
Neuromuscular transmission	Muscles (movement)	$(\alpha1)2\beta1\gamma\delta$ (foetal) $(\alpha1)2\beta1\varepsilon\delta$ (adult)
Autoantigen	Neuromuscular disease (myasthenia gravis)	
Gene expression	Neuromuscular synapses (movement)	
Expressed in central nervous system		
Regulating the release of neurotransmitters (DA, glutamate, 5-HT, ACh, norepinephrine, 4-aminobutyate)	Central nervous system (movement) Hypothalamus-pituitary (hormone regulation)	Various (DA: α4β2 and α6β2β3 subtypes)
Gene expression	Embryogenesis (early neuronal development)	Various
Cognition	Hippocampus (memory, plasticity) Ventral tegmental area (reward/addition)	α7, α4β2, α3β4
Expressed in non-neural/muscular cells		
Regulation of anti-inflammatory pathway(s)	Immune system (macrophage-mediated cytokine, IL and TNF production)	α7
Other cholinergic mechanisms	(Angiogenesis and endothelial cell growth)	Mainly α7

DA: dopamine; ACh: acetylcholine; 5-HT: serotonin; IL: interleukin; TNF: tumour-necrosis-factor.

NNs were designed to illicit a mode of action whereby nAChRs found in the central nervous system of invertebrates are blocked, leading to death and paralysis of the target pest species (such as aphids, beetles and leafhopper species) [8]. In the majority of insects, acute toxicity (LD_{50}) occurs at concentrations between 4 and 5 ng of NN per insect, dependant on the compound applied [12]. However, it has also become apparent that NNs (IMI, THC, ACE) have multiple binding sites across many classes of insect, resulting in non-uniform responses to exposure of differing levels [2].

The negatively charged tip of NN compounds is designed to be more specific to the invertebrate nAChR sub-binding sites, compared to mammalian counterparts (**Figure 1.2**) [13]. The difference between the invertebrate and vertebrate nAChR structure is thought to result in a higher binding affinity of NN compounds to insect nAChRs than mammalian nAChRs [8, 13], therefore increasing the specificity of NNs to target pest species. Although the binding affinity for NNs in mammals is relatively low, the α4β2 sub-site of nAChRs is the most commonly affected (**Table 1.1**), the effects of which are thought to be centrally mediated, with poisoning symptoms similar to that of nicotine [1].

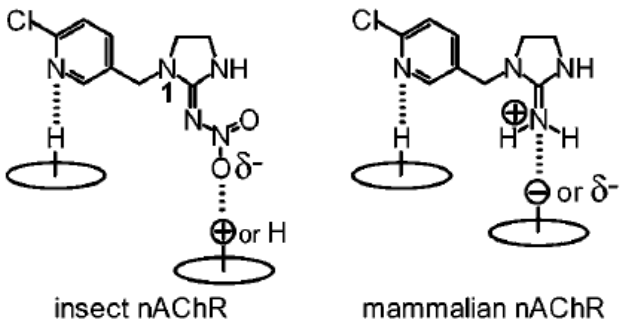

Figure 1.2. The structure of mammalian and insect nAChRs. Reproduced from Tomizawa & Casida (2003) [8].

1.1.3 Usage

NNs are the most predominant class of insecticide to be developed in the past 30 years [1] and have a variety of uses. NN products have been developed for urban pest control, external veterinary parasitic treatments and to regulate aquatic pests in the fish farming industry; however, the largest market for these compounds by far are plant protection products (horticulture, ornamentals and agricultural crops) [9]. Since their introduction in the early 1990s, global agricultural NN usage has increased continuously (**Figure 1.3**), and in 2017 NN treatments accounted for just under 30% of the agricultural insecticide market [14]. In the UK specifically, there was a ~26-fold increase in the weight of NNs applied annually to crops between 1994 and 2011 [12], and in 2012 93% (by weight) of all insecticide seed treatments were NN-based [2]. Outside of the UK, only three other countries have data on NN usage: California (use), Sweden (sales trends) and Japan (domestic shipment), and all except Sweden have documented a steady rise in NN use between 1990 and 2012 [2]. Overall, the reliance on NN compounds for agricultural pest control has grown substantially; however, there is a propensity for these compounds to be used as prophylactic treatments (rather than combative) and therefore it is debated whether cropping yields are significantly benefited as a result of increased NN applications [15-17].

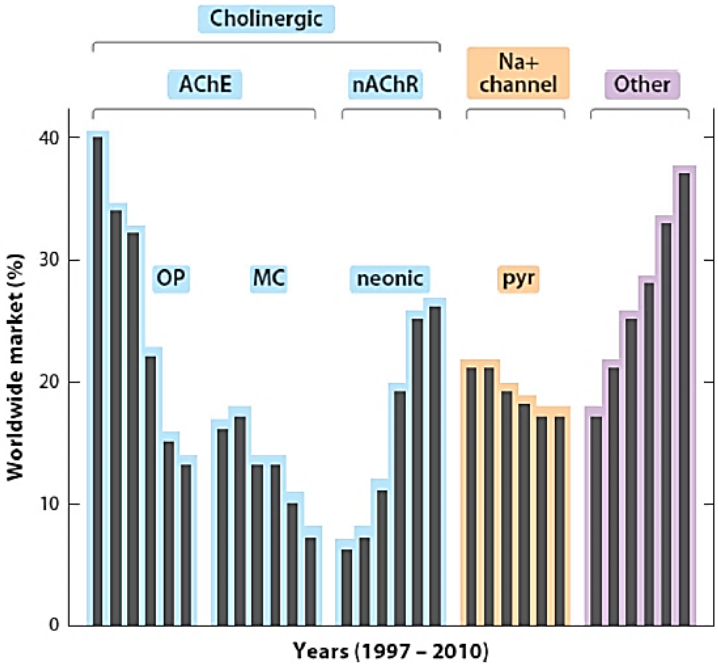

Figure 1.3. Worldwide insecticide market between 1997 and 2010. Reproduced from Casida & Durkin (2013) [18]. Data is shown for the years 1997, 2000, 2002, 2005, 2008 and 2010. OPs: organophosphates; MCs: methylcarbamates; pyr: pyrethroids; AChE: acetylcholinesterase; nAChR: nicotinic acetylcholine receptor.

NNs are registered for use on more than 120 different crops worldwide [9]. In the UK, NNs have been applied to oilseed rape (OSR), wheat, barley, linseed, sugar beet, potatoes, rye, oats and grain maize [19], with wheat (1994-2018) and OSR (1994-2012) making up the majority [19]. Comparatively, maize is the main crop type that is treated with NNs in the USA; in 2011 over 79% of maize was sown with NN-treated seeds [20]. Across all crop types, prophylactic seed coatings are the most common application of NNs [9]. In 2011 it was estimated that globally, approximately 60% of all applications were as seed treatments or soil drenching [21], and between 1994 and 2014 more than 90% of annual UK NN applications were in the form of seed coatings [19], with the amount of OSR treated with NNs rising from 37.4 to 83.0% between 2002 and 2011 [22]. Ground sprays, foliar and granular applications account for the remaining proportion of NNs applied, although these are generally used within a smaller pool of crops, and are combative rather than prophylactic treatments.

Due to changes in agricultural policies during the period of NN use, there has been a shift in the most common NN compounds applied in agriculture. In the EU, IMI was the most popular compound for the first 20 years that NNs were available on the commercial market [9]. However, in 2013 the EU brought about a moratorium that meant that CTD took precedence over IMI, with regards to seed treatment applications (**Figure 1.4**) [19]. Outside of the EU, Canada is the only other

country to follow a similar path, whereas IMI continues to be widely used in all remaining countries where NNs are registered for use [9].

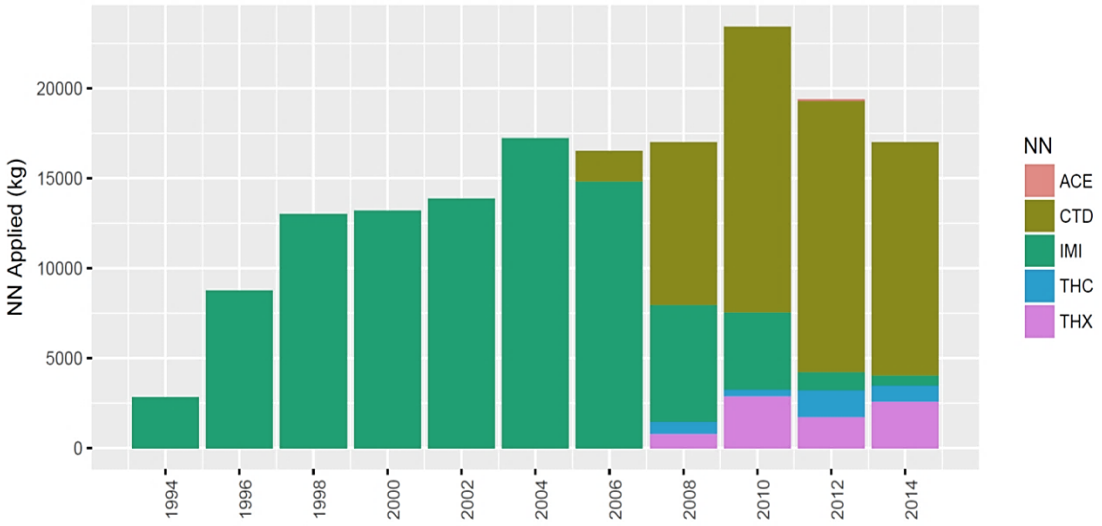

Figure 1.4. Change in neonicotinoid usage in the UK between 1994 and 2014. Data obtained from Pesticide Usage Scheme (Fera Science Ltd.) [19]. NN: neonicotinoid; ACE: acetamiprid, CTD: clothianidin, IMI: imidacloprid, THC: thiacloprid, THX: thiamethoxam.

1.1.4 Ecological risk assessments

There are multiple agencies worldwide that are responsible for conducting regulatory risk assessments for active substances, prior to their release onto the agrochemical market. Each agency has their own specific ecological risk assessment procedure, but overall the protocols used are broadly similar to one another [23]. Generally, a multi-tiered approach is used with varying levels of refinement to account for the complexity of assessing different exposure pathways within an ecosystem. Essentially, there are two key aspects of assessing the potential effect of a substance on non-target organisms: a) estimating the toxicity (or hazard) of the substance; and b) identifying and quantifying potential exposure pathways. These are collectively referred to as the toxicity exposure ratio (TER) approach, whereby the hazard of a substance is directly compared to the estimated amount of the substance in the environment, to produce a TER value that is judged against safety thresholds [24]. Unique risk assessment protocols are usually employed for different species groups and pesticide application methods, due to the variation in the residue unit dose (RUD; mg of toxicant per fresh weight of material) associated with different application techniques and the vulnerability of different species to potential exposure pathways. Therefore, to provide more detail on the principles of a typical ecological risk assessment procedure in relation to NNs, the European Food Safety Authority (EFSA) risk assessment protocol for birds and treated seed is used from here on as a relevant example [24].

The ecological risk assessment process for plant protection products is broadly divided into three main tiers: a screening step, first tier and higher tier assessments (**Figure 1.5**) [24]. The screening step uses predicted environmental concentrations (PEC) and worst-case scenario data to identify those substances (and associated methods of application) that pose an ecotoxicological threat and therefore require further assessment [25]. This step is mandatory for the majority of pesticide application methods; however, there is no screening step for seed treatments in relation to birds, and therefore the risk assessment method begins at tier 1 [24]. Tier 1 requires data for acute toxicity (LD_{50}; dose that causes 50% mortality in the test population) and chronic toxicity (encompassing reproductive endpoints such as, body weight, egg quality, fertility and chick survival) to be collected under laboratory conditions. With regards to birds specifically, the mallard *Anas platyrhynchos* and bobwhite quail *Colinus virginianus* or Japanese quail *Coturnix coturnix japonica* are the standard test species used [24]. Toxicity data for these species are subsequently converted to a daily dose unit (usually mg of active substance per kg body weight per day) so that they may be used in conjunction with total daily intake (TDI) data to calculate the acute and long-term TERs. The TDI is calculated using the RUD relevant to the dietary component in the exposure pathway (in this example, seed) and homogenous dietary data for 'generic' focal species. In this example, a generic species is assumed to be 100% granivorous and therefore provides a worst-case scenario for toxicant ingestion as part of the TDI. If the TERs are below the required safety thresholds, further assessment is required as part of the higher tier.

The main objective of using higher tier assessments is to provide a more realistic exposure estimate, so several refinement options are available and are usually selected on a case-by-case basis (**Figure 1.5**) [24]. Common refinement steps for seed treatments include the use of radio-tracking data for farmland bird species to assess the time spent in treated fields during periods of pesticide application, and the use of focal species to recalculate TDI values and subsequent TERs. The difference between 'generic focal' (tier 1) and 'focal' species (higher tier), is that the former refers to fictitious species with generalised traits, whilst the latter refers to real data for bird species known to inhabit crop types where the substance can be applied. For birds specifically, up to 21 avian focal species are available as part of the EFSA risk assessment. This particular refinement allows dietary data to be heterogeneous and based on the proportion of time each species spends in each crop type (e.g., a more realistic estimate of TDI). The aim of using field data, specific species data and/or refined dietary data in this manner is to improve the quality of the assessment and overall likelihood that the risk has been adequately assessed.

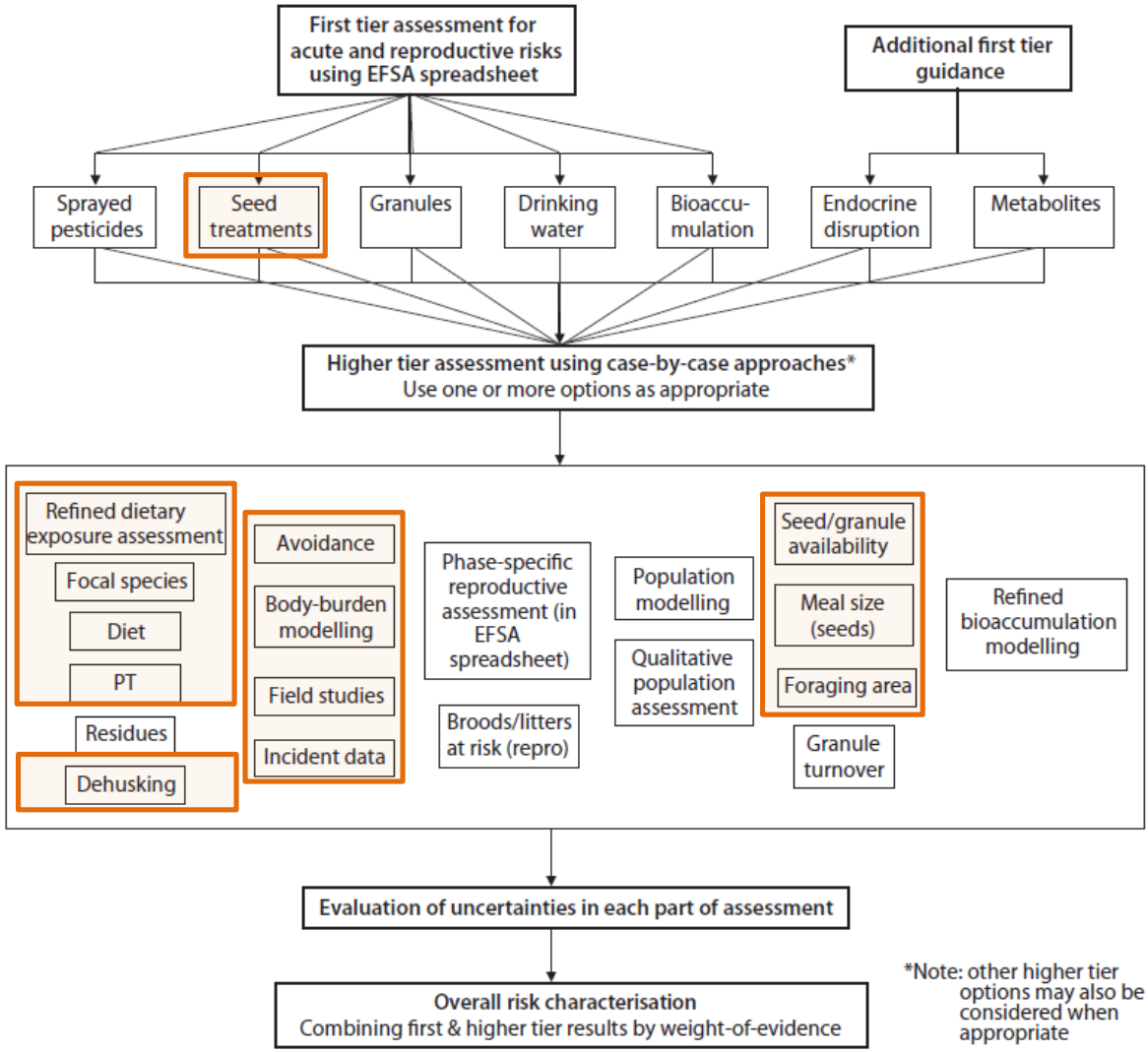

Figure 1.5. Overview of the EFSA risk assessment procedure for plant protection products for birds and mammals. First and higher tier assessment occur after the initial screening step, which is not shown here. Highlighted boxes refer to refinement options applicable to seed treatments. Reproduced from the EFSA (2009) [24].

Overall, the risk assessment procedure and associated data required for each new substance are respectively, lengthy and extensive. Current protocols are thorough across many aspects of ecotoxicological risk and must cover a wide range of scenarios for multiple pesticide application methods and numerous non-target species. However, an accurate estimation of risk is naturally difficult to obtain when it is impractical to collect data for every species potentially exposed, and as a result the majority of the process relies on extrapolations or assumptions, rather than species-specific or field-based data [26]. NNs in particular have come under scrutiny with regards to the effectiveness of risk assessments and their ability to protect wildlife associated with agricultural landscapes. Over the last few years, a large number of research-led NN-related studies have discovered a range of adverse impacts on non-target organisms, which were beyond the scope of

EFSA risk assessment protocols [14, 27]. Similarly, reviews of the effect of NNs on pollinators and invertebrates have called for regulatory procedures to be tightened, given the range of risks associated with systemic compounds in the wider ecosystem [28]. Furthermore, an extensive report in the USA suggested that the Environmental Protection Agency (EPA) significantly underestimated the impact of NN use on aquatic ecosystems and avian species in the USA [4]. Specifically, the authors proposed that toxicity to birds had been underestimated by a factor of 1.5-10.0, and that concentrations of NNs in water bodies across North America are above safety thresholds for aquatic food chains [4]. In a broader sense, concerns have also been raised that risk assessment protocols are not sufficiently spatially explicit (e.g., exposure is assessed on a field rather than landscape-scale) [23], and that the use of focal and test species to estimate the risk for all potential species affected is not effective [29]. In addition, sub-lethal effects of toxicants are known to increase in treatment groups where multiple agrochemicals are applied, highlighting the risk of pesticide mixtures in the environment [30, 31]; however, this is not formally accounted for in assessment protocols at present [32].

1.1.5 Neonicotinoids & non-target organisms

Serious concerns were raised by the scientific community regarding the effect of NNs on non-target organisms after only five years of commercial use [7]. In particular, pollinators were identified as a vulnerable species group because of the broad-spectrum mode of action and the rate of decline experienced by pollinators over the last decade [12]. Since doubts were first raised, a large research effort has been undertaken worldwide to understand the potential effects of NNs in the environment.

Research pertaining to pollinators has been the main focus over the last decade and many adverse effects of NN use on species from this group have since been identified. It has now been confirmed that first generation NNs are highly toxic to bees [2]. For example, the LD_{50} for honeybees in laboratory studies was found to be 3.7-490 ng/bee (IMI) varying greatly, within and between colonies, dependant on environmental factors, subspecies tested, specimen condition and method of exposure (contact or ingestion) [28]. Within controlled experiments, sub-lethal effects of NNs (multiple compounds) on pollinators and other non-target invertebrates have been found to be extensive [28, 33, 34]. NNs have been shown to affect several stages of ontogenetic development, reduce mobility [28], alter olfactory-based and learnt behaviours [35], as well as impair immune responses [36]. Equally, field experiments, predictive and real-world modelling approaches have also produced an overwhelming body of evidence to suggest that NN usage is detrimental to pollinators at an individual, colony and population scale [33, 34]. And finally, exposure and the

associated sub-lethal effects by which pollinators are affected have been confirmed in the field [14, 37].

Outside of insect taxa, evidence of the effects of NNs on other non-target species is sparse, although avian research is one of the largest areas to gain momentum over the last 5 years. Generally, the vertebrate research field is lagging compared to pollinators, with a limited number of studies advancing beyond the laboratory. The majority of mammalian studies are based on mouse or rat models, most fish studies are limited to the laboratory, and aviary studies are confined to fewer than 15 test species [27]. Sub-lethal effects on vertebrate species described in the literature include adverse effects on reproduction, biometrics, development, behaviour, oxidative stress, hormone receptors, navigational ability, gene transcription and immune responses across multiple aquatic and terrestrial species [27, 34, 38]. Many of these sub-lethal effects are seen to occur with low-level chronic exposure, as well as acute exposure events [27]. One study has also proposed that the number and prevalence of emerging wildlife diseases has increased in line with patterns of NN usage due to immune suppression across non-target organisms, both invertebrate and vertebrate species (including white-nose virus in bats, trichomonosis in birds and chytridiomycosis in amphibians) [39]; however, this theory is yet to be substantiated.

1.1.6 Agricultural policy

As a result of an increasing number of studies that provided evidence for negative impacts of NNs on pollinators and intensive lobbying, EFSA actioned a 2-year moratorium as of 2013 on the use of CTD, THX and IMI across all countries in the EU [40]. It was agreed that the effect of NNs on pollinators specifically would be reassessed, with a view to collect and review evidence by 2017. Specifically, the moratorium prohibited the use of NNs on spring-sown flowering crops (e.g., those that may cause an exposure pathway to pollinators). In the UK, this meant that the application of NNs was largely restricted to CTD and THX seed treatments applied to winter sown cereals. However, in 2015 following lobbying from the National Farmers Union, the UK government made an allowance to apply the banned compounds for up to 120 days on OSR crops in areas that were particularly susceptible to yield loss as a result of cabbage stem flea beetle *Psylliodes chrysocephalus* [16]. As a result, approximately 5% of spring-sown OSR was still treated annually with NNs in the UK during the moratorium. In the subsequent review (2017-2018), the impact on pollinators was found to be significant and a decision was made to ban the use of CTD, THX and IMI in all outdoor environments (e.g., restricted to use in greenhouses) as of April 2018, with a 6-month buffer period. Therefore, the use of IMI, CTD and THX ceased in the EU as of 2019.

Worldwide, CTD, THX and IMI continue to be used as seed treatments and/or foliar sprays. In the USA, the EPA highlighted the risks to invertebrates and took steps to improve labelling of NN products following the 2013 EU NN moratorium [41]; however, specific restrictions were not imposed and NNs continue to be used on a large scale across most states. In Canada, the government actioned a phase-out period (of 3-5 years from 2016) for the use of IMI [42]; a current decision process regarding a similar action is ongoing for CTD and THX. China and Japan are two of the biggest users of NNs in Asia [9]. China has six NN compounds registered and is one of the largest producers and exporters of IMI globally [43]. China has also invested in the development and use of new *cis*-NN compounds and shows no sign of halting the use of NNs in any capacity [9]. The Australian government has taken a similar stance to the US EPA and has not restricted the use of any NN compound, but has similarly highlighted the risks associated with use on product labels [44]. Data on the usage of NNs in Africa and South America remain sparse, however a worldwide study of NN in honey samples revealed that NNs are prevalent in samples from all continents (excluding the poles) [45] and in terms of policy, there are currently no known restrictions on NN usage within these continents.

1.1.7 Summary (I)

The mode of action for NNs is selective for nAChRs, however the full extent of the nAChR function within invertebrate and vertebrate physiology remains unknown. As binding affinity is not completely exclusive to insect species, the potential impact of these compounds on non-target vertebrates should be considered. It has now been confirmed that initial risk assessments underestimated the effect of NNs on non-target pollinator species, and this has since been reflected in changes to EU agricultural policies relating to NN use. Despite the growing body of research-led evidence of the adverse impacts of NNs on non-target organisms and doubts regarding the effectiveness of NN seed treatments, the global use of NN compounds continues, and the prospect of future insecticidal seed treatments remains. Consequently, it is important to gather and assess further evidence on the effect of NN seed treatments on other farmland taxa, including birds.

1.2 NEONICOTINOIDS & FARMLAND BIRDS

1.2.1 Farmland birds & pesticides

Worldwide, agricultural intensification is thought to be the largest threat to avian fauna [46]. Significant declines in farmland birds, specifically, have been well documented over the past 30 years and have been attributed to many aspects of agricultural intensification, including habitat loss, time-shifts of seasonal practices and the increased use of agro-chemicals [47, 48]. A recent review of farmland bird declines in North America found that pesticide use was the most commonly reported negative driver of population decline in farmland birds (42% of all studies, 93% of which reported negative impacts), followed by habitat loss and alterations [47]. Similarly, insecticide application was found to be one of the higher ranking variables to explain farmland bird declines in the UK during agricultural intensification between 1962 and 1995 [48], and has been cited in multiple reports as one of the key land use changes that has contributed to avian population change [49-51].

According to Donald *et al.* (2001) approximately 120 bird species of European conservation concern are supported in some way by farmland as a habitat type [52]. In the UK, farmland bird populations dropped substantially between 1970 and 2013: of the 19 farmland indicator species (those deemed dependent on farmland habitat), 12 experienced population declines of between 23 and 97% [53]. The steepest declines occurred between the mid-1970s and the early-1990s, when the number of commercial pesticides in use rose from 137 to 344 [54]. After NNs were introduced in the 1990s, farmland bird populations have continued to decline, but at a slower rate (**Figure 1.6**).

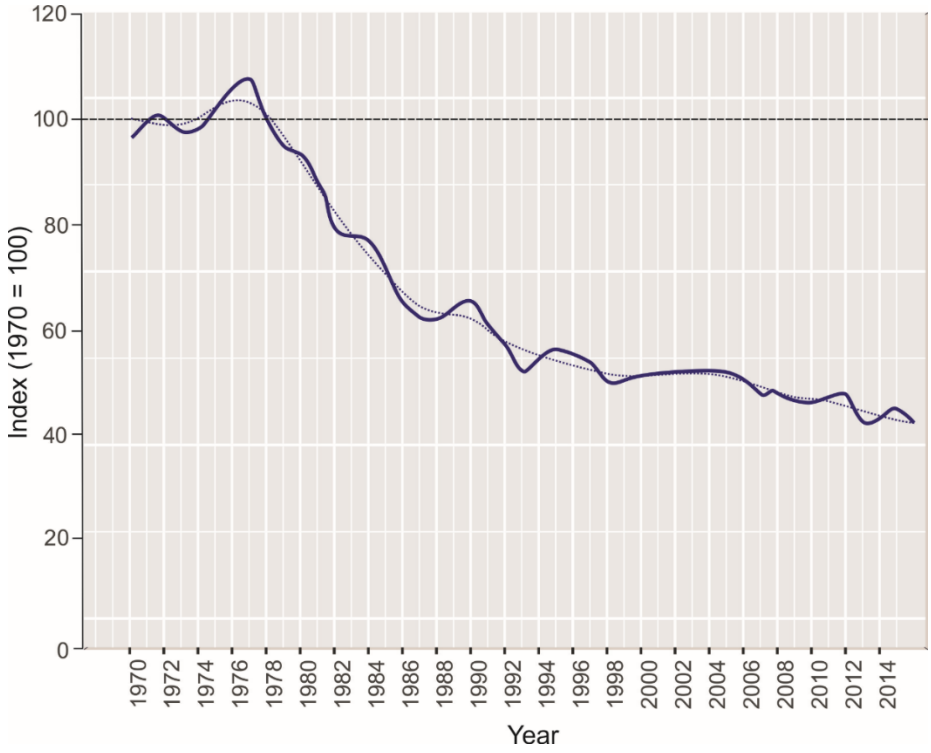

Figure 1.6. Population trend for the 19 farmland bird indicator species between 1970 and 2014 in England. Dotted line: smoothed trend; solid line: unsmoothed trend; dashed line: index baseline. Data sourced from the British Trust for Ornithology, Royal Society for the Protection of Birds, Defra and Joint Nature Conservation Committee. Reproduced from Defra (2015) [55].

As yet, it is unclear whether NN applications specifically are contributing to farmland bird population declines in the wider context of agricultural intensification, and there is a paucity of data with regards to the frequency and level at which birds are exposed to NNs in their natural environment. Currently, the literature on NN exposure and any associated effects (either at individual or population level) is largely restricted to aviary studies, and a complete picture of the effect of NN usage on farmland birds has not been achieved. This part of the review aims to assess what is currently known about NNs and birds, including toxicity data, known effects of NNs on avian physiology and the likelihood of farmland birds being exposed to NNs during standard agricultural practice.

1.2.2 Toxicity & toxicokinetics in birds

ACE and IMI are the most acutely toxic NN compounds to birds among all of those available on the commercial market [1], but toxicity differs significantly between all NN compounds available. For example, when considering the two compounds most commonly used as seed treatments, IMI is over 13-times more toxic to birds than CTD [4]. Overall, the range of LD_{50} values across multiple NN compounds and avian species is large (15-2716 mg/kg bw; **Table 1.2**), highlighting the variation in

toxicity within this class of insecticide and the sensitivity of different species (and sizes) of bird to NNs.

Table 1.2. Toxicity thresholds for neonicotinoid compounds provided by commercially prescribed studies. Reproduced from Mineau & Palmer, 2013 (Table 2.1 and 3.1) [4].

Compound	Species	Acute toxicity	Reproductive endpoints*		
		LD_{50}	NOEL	LOEL	Dose range
		mg/kg/bw	*ppm*	*ppm*	*ppm*
IMI	Bobwhite	152	120	240	0-240
	Mallard	283	120	240	0-240
	Canary	25-50	n/a	n/a	n/a
	Grey partridge	15	n/a	n/a	n/a
	Japanese quail	31	n/a	n/a	n/a
	Rock Dove	25-50	n/a	n/a	n/a
	House sparrow	41	n/a	n/a	n/a
CTD	Bobwhite	>2000	500	n/a	0-500
	Mallard	>752	250	525	0-500
	Japanese quail	430			
ACE	Bobwhite	180	250-400	500-800	100-800
	Mallard	98	125	250	62.5-500
	Zebra finch	5.7	n/a	n/a	n/a
THX	Bobwhite	1552	300	900	100-900
	Mallard	576	300	900	100-900
THC	Bobwhite	2716	466	n/a	53-466
	Mallard		28	48-55	14-418

*Collected as part of the standard 'avian reproductive test' for chronic toxicity endpoints, conducted as part of regulatory risk assessments [56].
LD50: lethal dose in 50% of test population: NOEL: no-observed-effect level; LOEL: lowest-observed-effect level.

Several studies have addressed the toxicokinetic properties of NN compounds in avian physiology, although data are mainly limited to IMI. Data presented are for the most part consistent; NN compounds are thought to be eliminated rapidly from birds, as is observed in small mammals [57], with the compound being detectable in some parts of the body for longer than others [58, 59]. For example, IMI has been found to remain in the blood and liver for 6-8 and up to 16 hrs post-dosage, respectively [58, 60]. Furthermore, the largest concentrations of IMI post-dosing have been consistently reported in muscle tissue, compared to organs or plasma [58, 61]. Conversely, data between studies is somewhat contradictory with regards to the potential bioaccumulation of IMI within avian anatomy. For example, the accumulation of IMI has been observed in the liver of red-legged partridge over a 28-day dosing period [62], as well as dose-dependently in the brain, kidney, liver and muscle of rock pigeon *Columba livia domestica* [61]. However, in a study of Japanese quail *Coturnix japonica* no observable accumulation of IMI was detected in the liver over a 3-day dosing

regimen [58], nor was accumulation observed in data obtained from domestic chickens, presented as part of the EFSA risk assessment for IMI [59]. Further toxicokinetic research over a larger number of avian species would be required to fully understand behaviour of each NN compound within avian anatomy, and any differences thus far observed. These data would be useful for determining the likely effects of NNs in relation to patterns of exposure in wild birds, but is likely to be outweighed by the large welfare expense that this would incur.

1.2.3 Potential exposure pathways

In general, exposure may occur via three main routes (inhalation, dermal contact and ingestion), all of which are relevant to NNs and birds. Inhalation of, or dermal contact with NNs by birds is possible via the use of seed treatments (inhalation of dust released during the sowing process), foliar sprays (overspray of NNs onto birds) or ground drenching applications (contact with soil or plants treated). However, the RUD a bird is subject to as a result of these exposure routes is likely to be much smaller than the amount that could be ingested. The ingestion of NNs may take place via the same three application methods (seed treatments, foliar and ground sprays), but the ingestion of treated seed specifically is likely to pose the greatest risk, as the majority of NNs are applied as seed coatings [19, 21] and a single seed can contain up to 1.34 mg of NNs [4]. NNs originating from seed treatments can also be dispersed throughout other components of avian habitat at differing concentrations due to several factors, including high compound solubility, systemic plant uptake, and long half-lives [7], resulting in many potential pathways of dietary exposure to wild birds (**Figure 1.7**). Consequently, dietary exposure arising from NN seed treatments will be the focus for the remainder of this section.

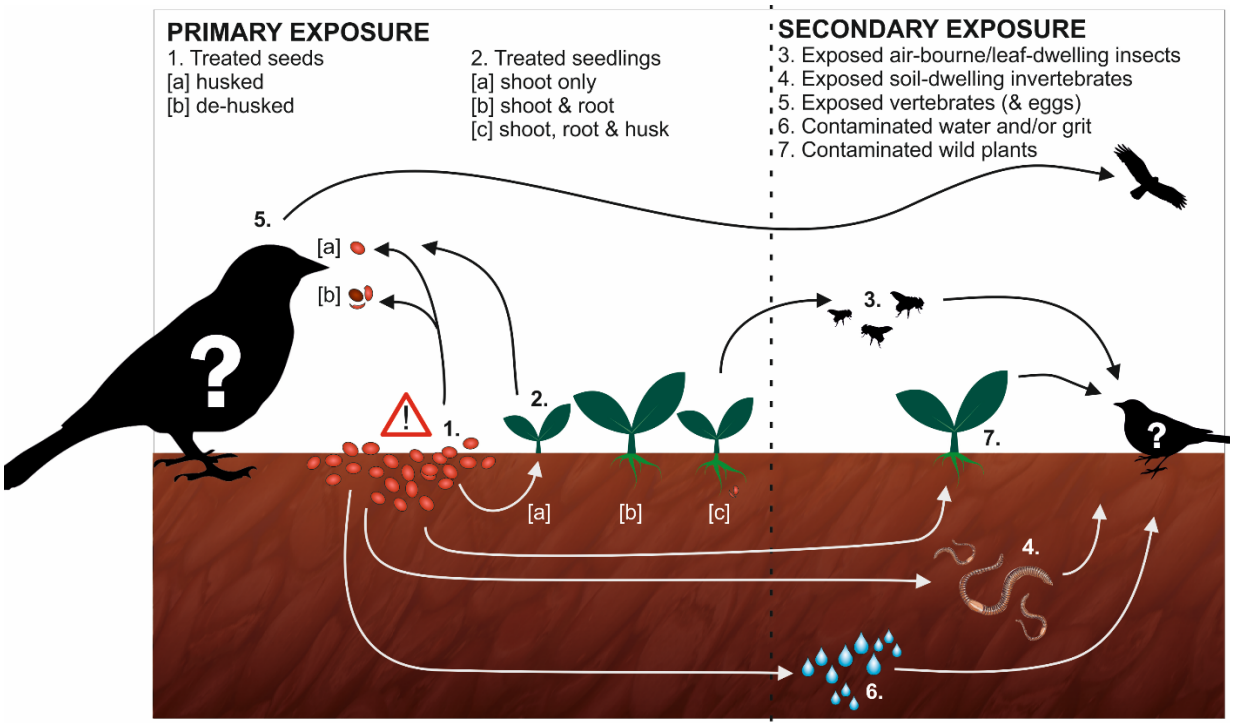

Figure 1.7. Dietary exposure pathways for avian species to NNs via seed treatments. Primary exposure relates to the ingestion of treated crop material (seeds and seedlings), whereas secondary exposure refers to pathways by which NNs may reach birds via the ingestion of contaminated food items.

Primary exposure: ingestion of treated seed

As seeds constitute a major component of many farmland bird diets [63], the ingestion of NN-treated seed is a major pathway by which wild birds may come into contact with NN compounds. To date, a handful of studies have measured the number of NN-treated seeds on the soil surface after sowing; average seed densities of 43.4 (± 5.5 SE; Spain), 0.22 (± 0.16 SE; Canada) and 0.06-31.26 (± 0.05-34.69 SE; USA) seeds per m^2 have been recorded [29, 64, 65]. Although there is some disparity between these data, one common feature between studies is that more seeds are found at headlands compared to field centres [64-66], which is attributable to the effectiveness of drilling techniques [66]. Spillage of treated seed has also been reported in various crops, with the number of spills equating to 1.0-2.8 per field (sugar beet and pea/spring wheat, respectively) and 5-12,500 seeds per spillage (sugar beet/maize/pea and flax, respectively) [66].

According to industry guidelines, one seed can contain between 0.012 and 1.34 mg of NN, depending on the compound used and the crop type to which it is applied [4]; however, thus far there are few data for the variability of the amount of NN compound found on seeds *in situ*. When translated to the potential level of exposure a bird may be subject to as a consequence of consuming treated seed, it has been estimated that acute toxicity would be reached if 0.1-202.5 seeds were consumed by a 15 g bird (at 5% tail of acute sensitivity), and chronic toxicity would

occur if 0.03-15.8 seeds were consumed [4]. In one study, the number of seeds on the soil surface within an area of 6-50 m^2 was enough to provide dose equivalent to the LD_{50} for sensitive avian species or a chronic dose for non-sensitive bird species [64]. There is also evidence to suggest that rainfall after sowing can reduce the concentration of NNs on treated seeds, therefore altering the potential level of exposure a bird may be subject to [67].

Interestingly, two separate studies found that three species of bird (red-winged blackbird *Agelaius phoeniceus*, brown-headed cowbird *Molothrus ater* and red-legged partridge *Alectoris rufa*) avoid NN-treated seed; however, this only occurred as a learnt behaviour as a result of post-ingestion distress, suggesting that there is no difference in palatability between treated and non-treated seeds and, that there is a lack of olfactory cues associated with NN compounds [68, 69]. Although there is evidence for avoidance of treated seed where choice is presented, the ingestion of treated seeds has also been reported to be more likely in an aviary setting when more food sources are present, where it is more difficult for birds to distinguish untreated seeds from treated seeds [69]. This is of note when considering the availability of treated seeds to wild birds, whose habitats often have unpredictable, limited and/or diverse natural food resources.

Primary exposure: ingestion of treated seedlings

Existing data for multiple compounds and crops have found that between 1 and 15% of the original NN application to seeds is taken up by crop plants after germination [70, 71], and that it is possible to measure the parent compound in crop seedlings between 20 and 134 days post-sowing [6]. For example, sugar beet leaves have been found to contain 5.3% of IMI applied to the seed 64 days after sowing [72]. Many bird species, such as skylark *Alauda arvensis* and those from the *Columbidae* family will feed on crop seedlings [63, 73], therefore making this a relevant exposure pathway to consider in the context of NN seed treatments. As the percentage of active compound found in seedlings is relatively low compared to that in seeds, this exposure pathway is likely to be less of a risk to birds than the ingestion of treated seed. However, it is worth noting that some species will pull up and consume entire seedlings [74], which may also include the original seed coating.

Secondary exposure: neonicotinoids in the wider environment

Due to the soluble properties of NN compounds, the potential for significant levels of NNs to leach in to the surrounding substrate and water table from seed coatings is high [75]. A number of studies have measured NN residue in soils, surface water and non-target plant materials, the majority of which found evidence of both recent (applied in that year) and historic (applied before year of

study) NN residue [7, 76, 77]. A UK-based study found that the minimum level of NNs in wild plants at the boundaries of NN-treated fields were higher than those in crop samples, and contained NN compounds that were applied 3 years previously [77]. And most recently, a large-sale study in Switzerland detected NNs in 93% of organic soils and crops, and 80% of soils and plants, sampled from organic and 'ecological focus areas', respectively [78].

Newly available data suggest that there can be both lateral and vertical transportation of NNs within soils from treated seeds, which is exacerbated by precipitation [79]. The time during which NNs can transported from seed coatings is limited by each compound's DT_{50} value [67], which ranges considerably between soil types and can be relatively large [7, 14]; across all NN compounds, DT_{50} values in soil are reported to be between 3.4 and 1,230 days [7]. It is therefore possible that some NNs may move throughout the environment and accumulate over time, particularly with repeated applications to the same areas of agricultural land. In one study, IMI was recorded in 91% of sites when only 15% of the sites had been planted with treated seed in that year [7], whilst another study recorded larger IMI concentrations at sites that had had two consecutive years of IMI treatment (coated seeds), compared to sites that had only been planted with treated seed in the previous year [80].

Potential exposure pathways relevant to bird species as a result of NN leachate and contaminated soil include the ingestion of exposed soil-dwelling invertebrates and contaminated water (**Figure 1.7**). Indeed, NNs have been detected in a large proportion of surface waters sampled worldwide, throughout numerous habitat types [81]. The potential accumulation of NNs over time in the wider landscape may also predispose birds to chronic low level exposure, as well as shorter periods of high-level exposure during the sowing season. Compared to the direct ingestion of treated crop material, these pathways are likely to result in extremely low levels of exposure, but nevertheless should be considered in the wider context of NNs and wild birds.

Secondary exposure: consumption of contaminated prey items

Recent research has reported that above-ground invertebrates may be subject to sub-lethal concentrations of NNs in treated fields under a field-realistic scenario [78]. Therefore theoretically, a bird may ingest NNs in small quantities contained within invertebrate prey items. The extent or possibility of exposure would depend on whether the ingested insects had: i) come into contact with NN-contaminated plants (whether these be wild or crop species); ii) the level of NN in said plant; and iii) the ratio of exposed:non-exposed invertebrates consumed by the bird within a given amount of time [82]. As yet, very few data are available to inform this exposure pathway.

Comparatively, there is evidence to suggest that vertebrate prey items (such as eggs and small birds) can be contaminated with NNs [60, 83], and NNs have been recorded in some top-level predators, including bird of prey species [84, 85].

1.2.4 Measuring exposure in wild birds

Measuring NN exposure in wild birds is not straight forward. Data for this subject area are limited to only a few species and multiple approaches have been used. A small number of studies employed radio-tracking and observational data to estimate the likelihood of exposure (as is similar to regulatory risk assessments), or measured the consumption of treated-seeds to confirm exposure. Results from these studies evidenced that multiple species of farmland birds will frequent areas that have been sown with NN-treated seeds [86], feed on seeds to which NNs may be applied [70], and consume NN-treated seed under field conditions [64, 65]. Whilst this information is extremely useful in identifying vulnerable species groups, or behaviours of specific species during the sowing season, it does not quantify the level of NN compound within an individual's system, therefore making it difficult to estimate the impact that this exposure will have. Moreover, radio-tracking techniques are only able to provide an estimate for the level of exposure, rather than confirmation, which is perhaps one of the main drawbacks of this technique being used for regulatory risk assessments.

Arguably the best quality data for measuring NN exposure in avian species are obtained by directly testing samples collected from wild birds for the compounds themselves. By using this approach, ingestion of NN compounds can be unequivocally confirmed and some measure of the level of exposure is possible. To date, NN residues have been measured in wild birds by analysing various samples including blood plasma, liver, feathers and eggs (**Table 1.3**), while some studies have also recorded concentrations of NNs in the crop or gizzards of avian species to confirm (rather than measure) exposure [87, 88]. The disadvantage of using biological samples is the associated cost to animal welfare, particularly when sample collection is invasive (e.g., blood); however, when considering the potential impact of a toxicant on multiple species and avian populations, the benefit of these data may outweigh the welfare implications. This trade off highlights the need for effective, non-invasive biomonitoring techniques, which if in existence and employed effectively, may be of significant benefit to farmland birds with regards to the use of agrochemicals [89]. A candidate sample type in this instance is feathers, which have been successfully utilised in one study to quantify NN exposure over a large area [90]. However, one disadvantage to this technique at present is the need to pool samples, which may lead to overestimation of exposure within a population, and there are uncertainties surrounding external contamination and the use of feathers

to measure internal concentrations of NNs [91]. Notably, there are many species of gamebird that are commonly hunted in farmland habitat as standard practice that could potentially be used as part of biomonitoring efforts, therefore negating the need for additional captures and/or sampling efforts, although monitoring would be limited to one species group. Overall, a consistent method for quantifying agrochemical exposure in wild birds remains one of the larger research gaps in NN- and pesticide-related research, which if identified could greatly improve the effectiveness of the protection afforded by regulatory risk assessment procedures.

Table 1.3. Summary of neonicotinoid concentrations recorded in avian samples collected from the field. Samples ordered by sample type and year of study.

Sample type	Reference	Species	Scientific name	Samples *N*	Positive samples *%*	Compounds detected	Minimum residue reported	Maximum residue reported	Unit
Plasma	Byholm *et al.* (2018) [85]	Honey buzzard	*Pernis apivorus*	10	60	IMI, THC	0.0089	0.31	ng/mL
Plasma	Hao *et al.* (2018) [60]	White-crowned sparrow	*Zonotrichia leucophrys*	36	80	ACE, IMI, THX, THC	0.0025	0.18	ng/mL
Plasma	Taliansky-Chamudis *et al.* (2017) [84]	Eurasian eagle owl	*Bubo bubo*	30	3	IMI	n/a	3.28	ng/mL
Liver	Ertl *et al.* (2018) [92]	Northern bobwhite quail	*Colinus virginianus*	57	12	n/a	<LOQ	<LOQ	ng/g
Liver	Botha *et al.* (2018) [93]	Cape spurfowl	*Pternistis capensis*	3*	100	IMI	16.0	29.0	ng/g
Liver	MacDonald *et al.* (2018) [94]	Wild turkey	*Meleagris gallopavo silvestris*	40	22	CTD, THX	8.6	160	ng/g
Liver	Millot *et al.* (2017) [87]	Grey partridge; pigeon	*Perdix perdix; Columbid*	57	28	IMI	0.3	43.5	mcg/g
Liver	Turaga *et al.* (2016) [88]	Northern bobwhite quail; scaled quail	*Colinus virginianus; Callipepla squamata*	98	17	CTD, IMI, THX	3.65	62.29	ng/g
Feather	Humann-Guilleminot *et al.* (2018) [90]	House sparrow	*Passer domesticus*	146*	99	ACE, CTD, IMI, THX, THC	n/a	140.48	ng/g
Cloacal fluid	Bishop *et al.* (2018) [95]	Rufous hummingbird; Anna's hummingbird	*Selasphorus rufus; Calypte anna*	8*	75	IMI	0.068	1.96	ng/mL
Eggs	Bro *et al.* (2016) [83]	Grey partridge	*Perdix perdix*	52**	8	CTD, THX	<LOQ	67	ng/g

*Pooled samples.

**Clutches of eggs sampled, rather than number of eggs sampled.

ACE: acetamiprid; CTD: clothianidin; IMI: imidacloprid; LOQ: level of quantification; THC: thiacloprid; THX: thiamethoxam.

1.2.5 Effects of NNs on birds

Direct effects

The mode of action for NNs in birds is poorly understood. However, agonists of the nAChR in vertebrates can result in disruption to neurotransmitters, gene expression, cognition, immune function and other cholinergic pathways (such as angiogenesis; **Table 1.1**), and it is therefore possible that exposure to NNs may produce multiple symptoms in avian species. Tests performed by industry during compound development provide a measure of lethality (LD_{50}) and reproductive endpoints as standard, but the protocols used are restrictive and are often unrepresentative of natural conditions. Outside of regulatory ecotoxicological studies, a growing number of research-led experiments have investigated other sub-lethal effects in addition to those associated with reproduction (**Table 1.4**). Overall the number of study species is small, and due to the welfare costs and time requirements, there are a paucity of large data sets. Despite this, a wide range of adverse physiological and behavioural effects have been observed among both domestic and wild bird species (**Table 1.4**), indicating that NN exposure has the potential to disrupt many aspects of avian physiology and/or behaviour, which in turn could affect survivorship and breeding success in the wild. Thus far, using 'environmentally-relevant' doses in aviary experiments is the main method to gain perspective on how a measured sub-lethal effect may present in the field. However, as these doses are based either on estimates for NNs in species' diets or represent of 0.5% the relevant LD_{50} [30, 31, 62], it remains difficult to reconcile these values with actual levels of exposure, for which there is little data. To date, only one study has successfully investigated a sub-lethal effect of NNs on wild birds in the field by employing an experimental design whereby migratory birds were captured, dosed with IMI, then re-released and tracked [96]. As yet, no studies have attempted to investigate sub-lethal effects in avian species that may be associated with levels of NN exposure caused by NN applications as part of standard agricultural practice. Understanding how sub-lethal endpoints measured in aviary studies manifest in a field-based setting and wild birds *in situ*, remains a large challenge within this area of research.

Table 1.4. Overview of observed sub-lethal effects of neonicotinoids on bird physiology and behaviour. All data were collected as part of aviary studies with experimental doses of NN compounds at varying concentrations.

Reference	Year	Compound	Sub-lethal effect	Details	Species	Scientific name
Humann-Guilleminot [97]	2019	ACE	Reproductive	Decline in sperm density and SOD activity	Zebra finch	*Taeniopygia guttata*
Rawi [98]	2019	IMI	Neurological	Decreased serum AChE; increased norepinephrine, serotonin; neuronal degeneration, pyknosis, neurophalgia, gliosis, eosinophilic neuron degeneration, demyelination, focal minue haemorrhage	Japanese quail	*Coturnix coturnix*
Ravikanth [99]	2018	IMI	Biometric	Decrease in GSH and serum total protein; increase in serum ALP	Chicken	*Gallus gallus domesticus*
Salvaggio [100]	2018	THC	Embryonic	Increase in teratogenic effects	Chicken	*Gallus gallus domesticus*
Zeid [61]	2018	IMI	Oxidative stress Morphological	Decrease in serum levels of GSH, SOD activity; increases in malondialdehyde levels, alanine aminotransferase, LDH, uric acid, plasma TNF-α, plasma AChE; alterations in brain and liver structural morphology	Rock pigeon	*Columba livia domestica*
Addy-Orduna [101]	2018	IMI, CTD, THX	Biometric Behavioural	Weight loss and behavioural symptoms of intoxication (diminished response, fluffed-up appearance, uncoordinated)	South American eared doves	*Zenaida auriculata*
Gobeli [102]	2017	IMI	Reproductive	Embryonic deformities and altered organ mass	Bobwhite quail	*Colinus virginianus*
Eng [96, 103]	2017; 2019	IMI	Biometric Behavioural	17-25% loss in body weight and decrease in fat stores; disruption to orientation capabilities	White-crowned sparrow	*Zonotrichia leucophrys*

Compounds: ACE: acetamiprid; CTD: clothianidin; IMI: imidacloprid; THX: thiamethoxam.
Enzymes: AChE: acetylcholinesterase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; SOD: superoxide dismutase; GPX; glutathione peroxidase.
Other: GSH: glutathione; TSH: thyroid stimulating hormone; TNF: tumour-necrosis factor.

Table 4 (cont.). Overview of observed sub-lethal effects of neonicotinoids on bird physiology and behaviour. All data were collected as part of aviary studies with experimental doses of NN compounds at varying concentrations.

Reference	Year	Compound	Sub-lethal effect	Details	Species	Scientific name
Pandey & Mohanty [30, 31, 104]	2015; 2017	IMI	Reproductive	Changes to the pituitary-thyroid axis (hypothalamic and testicular) including: T4, T3, TSH, GSH; reduction in testicle weight and volume, as well as testicular regression; changes to thyroid weight, volume and follicles and presence of lesions	Red munia	*Amandava amandava*
Lopez-Antia [62]	2015	IMI	Immunosuppression Reproductive Oxidative stress Biochemical	Dose-dependent reduction in cell-mediated immune response, magnesium, LDH, glucose, carotenoid based coloration (eye ring), as well as smaller clutch size and delayed lay date; dose-dependent increase in SOD activity in red blood cells, coloration in the beak yolk vitamins and carotenoids	Red-legged partridge	*Alectoris rufa*
Hoshi [105]	2014	CTD	Reproductive Oxidative stress	Increase in vacuolization, DNA fragmentation in seminiferous tubules, number and size of vacuoles in hepatocytes; abnormal histology in the granulosa cells of ovaries; significant differences in egg-laying rates and embryo weights; decrease in GPX-4 and manganese SOD	Japanese quail	*Coturnix coturnix*
Tokumoto [106]	2013	CTD	Reproductive	Decrease in embryonic length, fragmentation of germ cells and delayed embryonic development	Japanese crested ibis	*Nipponia nippon*
Lopez-Antia [107]	2013	IMI	Immunosuppression Reproductive Oxidative stress Biochemical	Reduction in cellular immune response, egg size, fertility, activity of GPX and total GSH levels in erythrocytes, eye ring colour, total proteins, albumin, cholesterol, calcium and magnesium and haematocrit	Red-legged Partridge	*Alectoris rufa*

Compounds ACE: acetamiprid; CTD: clothianidin; IMI: imidacloprid; THX: thiamethoxam.

Enzymes ALP: alkaline phosphatase; LDH: lactate dehydrogenase; SOD: superoxide dismutase; GPX; glutathione peroxidase.

Other GSH: glutathione; TSH: thyroid stimulating hormone; TNF: tumour-necrosis factor.

Table 4 (cont.). Overview of observed sub-lethal effects of neonicotinoids on bird physiology and behaviour. All data were collected as part of aviary studies with experimental doses of NN compounds at varying concentrations.

Reference	Year	Compound	Sub-lethal effect	Details	Species	Scientific name
Goyal & Sandhu [108]	2012	THX	None	No significant effect seen	Chicken	*Gallus gallus domesticus*
Kammon [109]	2012	IMI	Immunosuppression	Decrease in humoral responses	Chicken	*Gallus gallus domesticus*
Balani [110]	2011	IMI	Immunosuppression	Decrease in the total number of leukocytes	Chicken	*Gallus gallus domesticus*
Siddiqui [111]	2007	IMI	Immunosuppression Biochemical	Decrease in haemagglutination inhibition antibody titre and total albumin	Chicken	*Gallus gallus domesticus*
Cox [112]	2001	IMI	Behavioural	Inability to fly, uncoordinated	House Sparrow	*Passer domesticus*

Compounds ACE: acetamiprid; CTD: clothianidin; IMI: imidacloprid; THX: thiamethoxam.
Enzymes ALP: alkaline phosphatase; LDH: lactate dehydrogenase; SOD: superoxide dismutase; GPX; glutathione peroxidase.
Other GSH: glutathione; TSH: thyroid stimulating hormone; TNF: tumour-necrosis factor.

Population-scale & indirect effects

Very few papers consider how the sub-lethal effects of NNs on avian physiology and behaviour translate to population-scale effects, although the use of agricultural pesticides has previously affected avian species at this scale [113], and the sub-lethal mechanisms by which toxicants may affect populations have been identified [114]. This area of ecotoxicology remains challenging and approaches available to tackle this issue are limited [89]. To date, only two studies have analysed historic NN usage data in conjunction with avian population data, both of which looked at very different aspects of NN exposure and exemplified the advantages and disadvantages of using modelling approaches for this purpose.

The first study looked at changes in the population of Northern bobwhite quail in conjunction with climatic and land-use variables, including the application (kg) of NNs. This study found a negative association between the population of Northern bobwhite quail and NN use across multiple habitat types (Texas, USA), during periods of low and high NN application [115]. Although this study was thorough and took multiple environmental factors into consideration, the specific mechanism or exposure pathway for this association was not identified, and only one species of farmland bird was investigated. In a broader review, changes in bird populations (93% negative) were attributable to pesticides in 42% of 122 studies included [47], which suggests that any association between NNs and avian populations is unlikely to be isolated to one species. This highlights the need for further studies of this ilk, and for a greater number of study species, which may be able to put any observed trends in to a relative context.

The only study thus far to investigate the effect of NNs on populations of multiple bird species, focused on indirect, rather than direct effects of NNs. Potentially, one of the major indirect effects of NN usage is a decline in the availability of insectivorous food items. In 2014, Holland *et al.* found a significant association between surface water concentrations of NNs and the decline in free-living insectivorous bird species in the Netherlands, postulating that this group of species had declined in response to a lack of insect prey items as a consequence of NN use [76]. The use of long-term data in this manner provided a correlative statistic for the association between the presence of NNs in the environment and avian population change, but was unable to provide data for the hypothesised exposure pathway to explain this association. Several studies have indeed found that insect populations have been negatively impacted by long-term NN use [22], but to date no study has unequivocally linked NN-related changes in insect populations to avian population trends. With regards to NNs specifically, the case for this particular indirect effect remains strong as evidence for the negative impacts of these compounds on non-target invertebrates continues to grow [34].

Developing a means to advance population studies beyond correlative statistics to mechanistic associations is required to fully understand and evidence the impact of NNs on birds at a population scale.

1.2.6 Factors that may influence susceptibility to exposure & sub-lethal effects

Some bird species may be more susceptible to NN exposure than others, depending on the heterogeneity of the diet and the composition of NN-related food items within it. In addition, foraging behaviours and time spent handling food items may also influence the level of exposure a bird is subject to. For example, up to 85% of NN applied to seeds can be lost when the husk is removed [70]. With regards to seed treatments specifically, there are a wide range of species among several avian taxonomic groups for which grain or seed is a major dietary component [63], and up to 30 bird species have been observed consuming crop seed in pesticide-related studies [64, 70]. Corn bunting *Emberiza calandra*, in particular, has previously been identified as a good focal species, as their habitat and granivorous dietary preferences make them particularly vulnerable to NN exposure via seed treatments [64]. Conversely, insectivorous species, or those that rely on insect prey items when raising young, should also be considered. Although these species are not vulnerable to NN exposure via the ingestion of treated seed, they may be predisposed to the indirect effects of NNs via the loss of insect prey items. Overall, it is unlikely that any particular avian species ecology is fully 'safeguarded' against the effects of NNs, due to their chemical properties and widespread use; however, the impacts of each associated pathway are yet to be quantified.

The timing of exposure may also be an important factor to consider when evaluating the potential impacts of NNs on bird populations and individual health. For example, exposure via winter-sown crops may not be pertinent to potential reproductive endpoints, unless the species in question breeds all year round. Comparatively, any adverse effects on the immune system remain a consistent threat throughout a bird's life history, but may be of more importance during energy deficits caused by the breeding season, when birds generally are at lower levels of condition and/or are undergoing post-breeding moult, or over winter when there are a lack of food resources [116]. In these instances, both spring- and winter-sown crops could cause exposure during vulnerable periods of time for many species. Additionally, the effects of toxins/contaminants on the avian endocrine system are reported to be more significant in migrant bird species, due to additional energy expenditures associated with long-distance travel [117]. NNs specifically have already been shown to affect navigational ability and body weight in a migrant species of bird [103], but the potential for other adverse physiological effects must also be considered, especially as sowing

seasons often overlap with the departure of individuals to wintering grounds, when it is necessary for birds to be at peak condition. Indeed, small songbirds exposed to IMI at a migratory stop-over site as part of a field-based dosing experiment were seen to delay their migration by a median of 3.5 days, which in turn can adversely affect subsequent survival and reproductive success [96].

1.2.7 Summary (II)

In order for a toxicant to have a significant adverse impact on an organism's population, a chain of events must take place on a consistent basis. Firstly, the organism must be susceptible to the mode of action of the compound; secondly exposure must occur in that organism's natural environment, at a level that induces adverse health effects; and finally, these adverse effects must translate from an individual to a population-level change. In the case of NNs and birds, we know that avian physiology can be impacted by the NN mode of action. We also know that NNs cause both acute and chronic toxicity, manifesting in various physiological and behavioural changes in a laboratory setting. Furthermore, there is evidence that exposure to NNs occurs in populations of multiple species of bird, worldwide. However despite this line of evidence, the extent to which NNs impact wild birds during standard agricultural practices remains unclear. Current laboratory-based studies are essential for precisely measuring the effects of NNs on bird anatomy, but they cannot provide an accurate estimate of how NNs impact birds in the wild where the level of exposure is unknown. This paucity of field-based studies needs to be addressed to better understand the relationship between NNs and farmland birds, with an aim to quantify the frequency and level of exposure, as well as any associated effects among bird communities *in situ*. In doing so, conclusions related to the impacts of NNs on wild birds would move away from a hypothetical baseline, towards unequivocal evidence.

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From seeds to plasma: confirmed exposure of multiple farmland bird species to clothianidin during sowing of winter cereals

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Investigation	●		●				●			
Methodology	●	●	●	●						●
Resources		●	●		●				●	
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ABSTRACT

Neonicotinoids are the largest group of systemic insecticides worldwide and are most commonly applied as agricultural seed treatments. However, little is known about the extent to which farmland birds are exposed to these compounds as a result of standard agricultural practices. This study uses winter cereal, treated with the neonicotinoid clothianidin, as a test system to examine patterns of exposure in farmland birds during a typical sowing period.

Surface seed densities and bird abundance were recorded at 25 farms post-sowing to assess the availability of clothianidin-treated seed to birds and potential species at risk. The concentration of clothianidin in treated seeds and crop seedlings collected from a subset of farms was measured via liquid chromatography-tandem mass spectrometry, and camera traps were used to monitor seed consumption by wild birds *in situ*. Avian blood samples were collected from 11 species of farmland bird from a further six sites to quantify the prevalence and level of clothianidin exposure associated with seed treatments. The weight, body condition and haematocrit score of individual birds were recorded to investigate the potential for sub-lethal effects associated with clothianidin exposure.

Clothianidin-treated seeds were found on the soil surface at all farms surveyed at an average density of 2.8 seeds/m^2, and with seed spillages recorded at 17 out of 25 farms. The initial concentration of clothianidin in seeds (median: 254.5 µg/g) varied around the target application rate, whilst crop seedlings contained on average 5.9% of the clothianidin measured in seeds. Exposure was confirmed in 32% of bird species observed in treated fields (n = 66). Clothianidin was detected in 50% of individual blood samples collected post-sowing; the median concentration recorded in positive samples was 12 ng/mL. Neither weight, body condition nor haematocrit were found to be associated with NN exposure in birds sampled.

Results here provide clear evidence that a variety of farmland birds are subject to clothianidin exposure following normal agricultural sowing of clothianidin-treated cereal seed. Furthermore, the widespread availability of seeds at the soil surface was identified as a primary source of exposure. Overall, these data are likely to have global implications for bird species and current agricultural policies where neonicotinoids are in use, and may be pertinent to any future risk assessments for systemic insecticide seed treatments.

2.1 INTRODUCTION

Since their introduction in the early 1990s, neonicotinoid (NN) insecticides have grown in use and by 2014 accounted for approximately one-third of the insecticide market worldwide [1]. NNs are the most widely used class of systemic insecticide in agricultural practice, consisting of seven commercially available compounds that are applied in more than 100 countries [1]. The most commonly used (in descending order) are imidacloprid (IMI), thiamethoxam (THX) and clothianidin (CTD), which are predominantly applied as seed treatments and have been registered for use on more than 140 crops worldwide [1]. NNs act as agonists for the nicotinic acetylcholine receptors in the central nervous system of invertebrates, causing paralysis and death [2], and are designed to be taken up into xylem and distributed throughout the plant to provide long-term protection. It was assumed that these factors would predispose NNs to being less of a risk to many non-target species compared to older insecticides [1]. However, in 2018 the EU banned the outdoor use of three widely used NN compounds, due to their impact on key pollinator species [3]. Subsequently, concerns have also been raised regarding their potential effect on other non-target species, particularly farmland birds [4-6].

Over the last 50 years, farmland birds have undergone substantial population declines across Europe and North America that have been attributed to agricultural intensification, of which increased pesticide application is part [7, 8]. Historically there has been evidence of insecticides adversely affecting birds both directly (e.g., the effect of the organochlorine DDT on birds of prey [9]), as well as indirectly (e.g., decreased food availability during the breeding season as a result of broad spectrum insecticides [10]). However, the effect of systemic insecticides (such as NNs) on individual birds in the field is largely unknown. NNs specifically are toxic to birds at relatively small concentrations, but the level of toxicity differs markedly between compounds [11]; for example the LD_{50} for bobwhite quail *Colinus virginianusis* is 152 mg/kg/body weight for IMI, but >2000 mg/kg/body weight for CTD [12]. In aviary conditions, NNs are also known to cause sub-lethal effects in birds, such as adverse impacts on the reproductive system [13-16], alterations to the immune system [13], neurotoxic symptoms [17, 18], oxidative stress [19] and changes to behaviour [20, 21]; many of these sub-lethal effects have been reported at environmentally-relevant doses in laboratory studies using wild bird species. Furthermore, Mineau and Palmer (2013) estimated that the ingestion of 1.3 imidacloprid- or 4.4 clothianidin-coated wheat seeds would be sufficient to breach adverse reproductive end points in a small (15 g) songbird [22].

Many farmland birds have a high proportion of agricultural seeds and plant material in their diet [23], and so have potential for exposure to NNs applied as seed treatments through ingestion of either treated seed or seedlings [22]. The risk from dietary exposure is assessed within regulatory assessment procedures, by combining information on toxicity of the respective compound and estimates for levels of exposure that wild birds may be subject to via seed treatments [24]. As part of this procedure in the EU, NN seed treatments required 'higher tier' assessment options, such as the use of radio-tracking data and focal species dietary data, to estimate the level of exposure in farmland bird species more accurately [12]. Product application instructions also play an important part in safeguarding wildlife from pesticide use. With regards to NN seed treatments specifically, product labels clearly state that seeds should be buried at a minimum depth of 4 cm, and that no seed should be left on the soil surface after drilling [25]. Nevertheless, little is known about the effectiveness of these safeguards, and patterns of exposure to seed treatments in wild birds remain poorly understood.

To date, only a handful of studies have collected field data to investigate exposure of wild birds to agricultural seeds treated with pesticides. With regards to seed availability, studies in Spain, Canada and the USA found between 0.04 ± 0.03 (SE) and 43.4 ± 5.5 NN-treated seeds per m^2 at the soil surface after drilling [26-28]. Only one study has measured NN concentrations in treated seed collected from the field and reported highly variable concentrations among three seed types and three NN compounds [28]. Furthermore, numerous papers report that NN uptake in crops is highly variable [29, 30], such that residue taken up by seedlings can vary between 1 and 15% of that in treated seeds [31, 32]. In terms of exposure of birds to seed treatments, 30 species of wild bird were observed consuming pesticide-treated seed in newly sown fields as part of a study in Spain [26], a US study documented 10 confirmed bird species and various unidentified sparrow species feeding at experimentally-placed NN-treated seed piles [28], and a UK-based report observed 18 bird species feeding on the types of crop seed that could be treated with NNs [33].

NN residues have been measured in a range of avian samples, but only from a limited number of farmland species. Thus far, NNs have been detected in the liver, crop or eggs of four gamebird and three columbid species [34-37]. NN poisonings have also been documented in grey partridge *Perdix perdix* and columbid species, of which more than 70% (of 101 incidences and 734 mortalities) occurred during the autumn sowing period [35]. Only three studies have measured concentrations of NNs in avian plasma: two in raptor species [38, 39], and one in a songbird species [40], with 38 out of 76 individuals testing positive for NNs across the three separate datasets. Most recently, a

large study in Switzerland measured NN concentrations in the feathers of house sparrows *Passer domesticus* and found 100% prevalence of NNs (consisting of five compounds) in 146 pooled samples collected from 62 farms across the Swiss plateau [41].

Thus, there is evidence that a range of farmland birds have the potential to be exposed to NN treated seeds and that residues of NNs can be detected in biological samples taken from birds. To link these lines of evidence together and form an understanding of the entire exposure pathway for NN treated seeds, we conducted a field-based study that investigated patterns of NN (specifically CTD) exposure within a typical farmland bird community, via treated seeds sown according to standard agricultural practice. The objectives of the study were to: 1) measure availability of treated seeds on the soil surface after sowing; 2) quantify CTD concentrations in treated seeds and seedlings collected from the field; 3) identify avian species that may be exposed to CTD in recently sown cereal fields; 4) monitor avian blood plasma for CTD contamination in samples collected from multiple bird species pre- and post-sowing; and 5) measure physiological parameters in individual birds to investigate the possibility of sub-lethal effects associated with CTD ingestion.

2.2 METHODS

Data were collected from 31 sites located in the regions of East Anglia (UK) and North Lincolnshire (UK) during the autumn sowing seasons of 2015 (21 farms), 2016 (19 farms) and 2017 (6 sites). These were the two regions in the UK that annually received the greatest mass of NN applied as seed treatments [42]. Avian blood plasma samples were obtained from sites in Lincolnshire (**Table 2.S1)**, whilst seed, seedling and bird survey data were obtained from farms in East Anglia (**Table 2.1**). All sites were sown with Redigo-deter®-dressed wheat (Bayer Crop Science Ltd., UK); this seed treatment contained only CTD.

Table 2.1. **Sampling numbers for surface seed density surveys, seed samples, seedling samples, bird abundance surveys and camera traps at sites in East Anglia during 2015 and 2016.**

Sampling details	**Farm Number**																									**Total**	
Number of:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*Sampling effort*	*Farms sampled*
Fields sampled at each farm*	1	1	1	2	2	2	2	1	1	2	1	1	1	1	1	2	2	2	2	2	2	2	1	2	2	**39**	n/a
Seed density surveys	5	5	5	8	8	3	8	5	3	8	5	3	3	3	5	7	8	8	7	8	8	7	5	8	8	**151**	**25**
Seed samples																										**110**	**15**
Pooled				6	7		6	5		4					3	1	5	7	7	3	4	3		5	7	73	15
Individual					8			10										9						10		37	4
Seedling samples																										**88**	**13**
Pooled				1	4		3									2	2	1	3	2	4	1		4	1	28	12
Individual				10			9	20										21								60	4
Bird surveys	5	5	5	10	8	5	9	5	4	10	4	6	5	5	5	8	10	10	8	9	8	9	5	10	9	**177**	**25**
Camera traps	1	2	2	8	1	1					2	2	2			1	4	1	1	1		3	4	1	3	**40**	**18**

*Farms with two fields were sampled in 2015 and 2016.

2.2.1 Density of seeds on the soil surface

Seed density surveys were conducted on 21 fields (autumn 2015) and 18 fields (autumn 2016), across 25 farms in East Anglia sown with CTD-dressed wheat seeds (either Redigo Deter® or Deter®; Bayer PLC, UK). Surveys took place at each site for up to two weeks post-sowing (on or as close as possible to days 1, 3, 6, 9 and 12, where day 0 was the day of sowing). At each visit, the number of treated wheat seeds visible on the soil surface was recorded in 60 quadrats (0.25 m^2), comprising 20 quadrats in the field centre and 20 quadrats at each of two field headlands; quadrats were evenly distributed along transects diagonally bisecting the headland and field centre.

2.2.2 Treated seed & seedling sample collection

NN-treated wheat seeds present on the soil surface were collected during seed density surveys (1-14 days post-sowing) from 24 fields that were distributed across a subset of 15 farms in East Anglia. CTD-treated wheat seedlings were collected from 20 fields across a subset of 13 farms. Whole seedlings inclusive of roots and shoots (as extracted from soil) were collected from each site in weeks 2-4 and 5-13 post-sowing, covering two stages of wheat growth (small seedlings, growth stages 10-16 and early tillering plants, growth stages 20-26).

Pooled samples, generally comprising of up to 10 individual seeds or seedlings were collected from the field centres and headlands of each field on each sampling occasion. In addition, individual seeds and seedlings were collected and analysed separately at four of the 15 farms per sample type (**Table 2.1**). Seed and seedling samples were stored at -20°C until analysis.

2.2.3 Bird abundance surveys & camera trap data

Bird surveys took place at the same 25 farms where surface seed densities were recorded. Surveys were undertaken in the months of September to December 2015 and 2016, at the same sampling time points used to assess surface seed densities (on or as close as possible to day 1, 6, 9, and 12), and at a further two time points (2-4 weeks and 5-13 weeks post-sowing) to coincide with seedling collection. Birds utilising treated fields were recorded by: 1) scanning the entire field on arrival and counting the number of birds present; 2) walking along, and counting birds in field boundaries; and 3) flush counts whilst walking field transects (maximum of three transects per field separated by at least 100 m). The location (field boundary, centre or headland) and number of each species observed were recorded, excluding birds flying over the site. The locations of any seed piles (defined as >10 seeds at one location) that were spilt during standard agricultural practice were also noted as part of these surveys. Motion-sensitive infrared camera traps (Bushnell, USA) were

placed at 40 such seed piles across 18 farms and remained active until seeds were depleted. Cameras recorded bird feeding activity by recording 10 sec of continuous video footage when triggered by movement. On average, each camera recorded data for 8.5 days (range: 1-26 days). Camera footage was processed to obtain the following for each bird observed: species, time and date of observation, time at seed pile and the number of CTD-treated seeds (visually identifiable by red dye) consumed.

CTD exposure estimated from the mean and maximum number of seeds consumed by each species (per visit) and the mean concentration of CTD measured on treated seed, was compared to known avian sub-lethal (no-observed-effect-level; NOAEL) and lethal (LD_{50}) toxicity thresholds for CTD-treated seed consumption. These thresholds were modified for each species from the value given by Mineau & Palmer (2013) for a 15 g bird at the 5% tail of sensitivity [22], based on the average weight of each species included in the present study [43].

2.2.4 Collection of avian plasma samples

Plasma samples were obtained from birds captured at five farm sites and one garden site in North Lincolnshire. All capture sites were immediately adjacent to fields drilled with CTD-dressed wheat seeds (treated with Redigo-deter®), except the garden site which was within 50 m of the treated field. Sites were separated by an average of 22 km (range: 5-40 km) to ensure spatial independence. Birds were sampled across the six sites at two time points between September and November 2017: pre-sowing, before any treated seed was drilled at each farm (temporal control group), and within 2 weeks after fields were drilled (temporal treatment group). Birds were caught between sunrise and midday, using up to 66 m of mist-nets per visit situated along field/site boundaries and cover crops. Birds were extracted from mist-nets and processed following standard British Trust for Ornithology procedures (species, age, sex and bird weight recorded where possible [44]). Blood was taken from designated species via brachial venepuncture under Home Office licence (**Table 2.S1**). The maximum amount of blood taken from any bird was equal to 1% of its body mass. A health check took place prior to blood being taken, and again prior to release. Blood was collected and stored on ice in heparinised haematocrit tubes and centrifuged (1000 rpm, 5 min) within 3 hrs of collection. Haematocrit scores (proportion of red blood cells in blood volume) were obtained for each sample of more than 40 µL and plasma was decanted into individual Eppendorf tubes. Samples were stored at -20°C until they were analysed.

2.2.5 Residue analyses

In total, 111 seeds (73 pooled, 38 individual samples), 93 seedlings (32 pooled, 61 individual samples), and 96 plasma samples from individual birds were analysed for CTD using liquid chromatography-tandem mass spectrometry (LC-MS/MS; see **Supplementary Note 2.S1** for extraction and LC-MS/MS method details). Three protocols were used during each LC-MS/MS 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 CTD and the deuterated internal standard; and 3) during analytical runs a traceable National Institute of Standards and Technology certificated standard (Clothianidin; SPEX, London, 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 corrected. Ten samples (2 seed, 5 seedling, 3 avian plasma samples) with recoveries <60% and >120% were excluded from subsequent data analyses. The mean (± SE) recoveries for the remaining samples were 99.9 ± 0.9% for seeds, 103.9 ± 1.2% for seedlings and 82.7 ± 1.6% for avian plasma. The limit of detection (LOD) and limit of quantification (LOQ) for clothianidin were 0.4 ng/g and 0.6 ng/g, respectively for seeds and seedlings, and 0.15 ng/mL and 0.21 ng/mL, respectively for plasma samples. The LOD was determined using three-times the signal-to-noise ratio, and the LOQ was calculated as the LOD plus the calculated expanded uncertainty of the method. The expanded uncertainty for CTD was calculated using the Nordtet TR537 handbook [45]. With regards to avian plasma samples specifically, there was no significant difference in the recoveries between samples of differing volumes (20-50 µL; Kruskall-Wallis: $\chi^2_3 = 1.15$, $p = 0.763$).

2.2.6 Statistical analysis

Due to the heterogeneity of the data, spatial and temporal patterns of CTD exposure were initially analysed using non-parametric tests, followed by generalised linear mixed models (GLMMs), where possible (**Table 2.2**). In these analyses, all 'pooled' residue data for seed and seedling samples included the concentration of CTD obtained from samples analysed as a pool of items (one data point per pool), and data for samples analysed as individual items (one mean data point per group of individual samples collected at the same farm and on the same date). For statistical analyses relating to the burden of CTD in any sample item (seed or seedling), the total mass of CTD in any pooled sample was divided by the number of sample items in that pool. The fit of all GLMMs was assessed by measuring over-dispersion and the visual and statistical assessment of modelled versus simulated residuals. All models were also tested for zero-inflation and inter-correlation between

fixed effects. All GLMMs except for those modelling surface seed density were run using a negative-binomial distribution to account for over-dispersion (surface seed density was run using a Gaussian distribution). All analyses were conducted using R [46].

Table 2.2. Summary of models used for data analyses.

Independent variable		Fixed effects	Random effect
Seed densities			
log(mean SD per site, per survey +1)	~	location(headland) + N days post-sowing + year	(1|field)
Seed and seedling residue data			
CTD residue in seeds	~	cumulative rainfall (between sowing and sample collection) + N days post-sowing	(1|farm)
Bird abundance data			
bird abundance (per survey, per species guild)	~	mean SD (per site, per survey)	(1|field)
Sub-lethal effects			
weight	~	log(CTD concentration in plasma collected post-sowing)	(1|capture time)
body condition	~	log(CTD concentration in plasma collected post-sowing)	n/a
haematocrit	~	log(CTD concentration in plasma collected post-sowing)	n/a

Mean surface seed density (SD) was calculated for each field, at each farm, in each year and at each field location (headland and field centre).
CTD: clothianidin; N: number of.

Two GLMMs were used to analyse parameters related to surface seed densities, and seed and seedling residue data (**Table 2.2**). The variable 'number of days post sowing' was structured such that all data points were categorised into the following five groups: 0-1, 2-4, 5-7, 8-10 and 11-14 days post-sowing. The variable 'cumulative rainfall' referred to the amount of rain that fell at each field, in each year between the date of sowing and date of sample collection. Rainfall data were collected from weather stations in the Met Office MIDAS network [47], and matched to fields based on the geographical proximity (usually within 1.6 km). With regards to random effects, data for a 'farm' included data from all years, and 'field' referred to individual fields across all farms, across both years.

To analyse bird abundance as a function of surface seed density (**Table 2.2**), the mean surface seed density was calculated per field per survey event and assigned to each bird abundance record (total number of each species observed in each field at each survey event). Published dietary data [48] were then used to determine whether agricultural seed is present or absent in the diet of species observed in this study, as a means of refining the species groups included in each avian GLMM

model. Specifically, models were run for those species where agricultural seed was deemed as 'present' in the diet (defined as those species where the term 'crop grain' – or a specific seed to which NNs are known to be applied, such as plants of the genus: *Beta, Triticum, Hordeum, Linum, Secale, Brassica, Avena* were included in the list of known food items; **Table 2.S2**) and for those species where agricultural seed was 'absent' from the diet (those species where the previously mentioned terms were not included in the available dietary data; **Table 2.S2**). A number of additional models (each representing a specific taxonomic species guild; **Table 2.S2**) were then run using a subset of species where agricultural seed was deemed to be present in the diet. For each multi-species analysis, the dependent variable was the aggregate count summed across species.

Wilcoxon sum rank tests were used as a preliminary analysis to ascertain whether bird weight, body condition or haematocrit score differed between those individuals where CTD was detected in plasma samples, compared to those where it was not. To standardise bird weight as a parameter across multiple species, the percentage difference between the recorded weight of birds at capture and the average species weight (obtained from the British Trust for Ornithology 2005 Ringing Scheme data [43]) was used in these analyses. The residuals of from general linear models, where log body weight was modelled as a function of log wing length, were used as a measure of body condition for each individual. Residuals were obtained for each species from separate linear models to account for significant differences between species biometrics (this was not necessary for age or sex). Robin *Erithacus rubecula* and greenfinch *Chloris chloris* were excluded from the analyses because only one sample was available for either species post-sowing. Subsequently, GLMMs or generalised linear models were used to assess whether there was an association between the level of CTD exposure and haematocrit, body condition or bird weight (**Table 2.2**). To ensure good model fit, health parameters were modelled as a function of the log of CTD concentrations in plasma on a negative-binomial distribution to account for over dispersion (except for body condition which was under dispersed and modelled using a Gaussian distribution). The LOD (0.15 ng/mL) was used as the CTD concentration value for individuals where the concentration was recorded as 'non-detect'. Time of capture was entered as a random effect for the model concerning bird weight, to account for diurnal changes across all species. It was not possible to include 'species' as a random effect in the haematocrit model due to issues with model convergence, therefore a generalised linear model was used instead (**Table 2.2**).

The first-order dissipation half-life (DT_{50}, days) for CTD on seed samples was calculated from the amount of residue on all seed samples collected at each day post-sowing using **Equations 1 and 2**, where C_0 and C_t are the concentrations of CTD in each sample at time 0 and time (t), respectively. t is the number of days post-sowing at which the sample was collected, and k is the first-order rate constant.

$$C_t = C_0 \, . \, e^{-kt} \qquad \textbf{[Eqn 1]}$$

$$DT_{50} = \frac{\ln(2)}{k} \qquad \textbf{[Eqn 2]}$$

2.3 RESULTS

2.3.1 Surface seed densities

Seeds were present on the soil surface in 38 out of 39 fields, and at all farms in at least one year (21/21 farms in 2015; 18/19 farms in 2016). Seeds were present in 20% (1804/8930) of quadrats; the number of seeds recorded at the soil surface across all quadrats ranged between 0 and 364 seeds/m^2, with a mean of 2.8 ± 12 (SE) seeds/m^2. In addition, the presence of seed piles (>10 seeds at one location) was confirmed at 17 out of 25 farms across both years.

There was a significant difference in surface seed densities between farms across both years (Kruskall-Wallis: $\chi^2_{24} = 862.3$, $p < 0.001$); the mean (± SE) number of seeds at each farm ranged from 0.11 ± 0.07 to 12 ± 2.5 seeds/m^2. The mean density of seeds on the soil surface after drilling was found to be higher at field headlands (3.7 ± 0.36 seeds/m^2), compared to field centres (0.9 ± 0.06 seeds/m^2; paired Wilcoxon signed-rank test: $V = 89.5$, $p < 0.001$; **Figure 2.1**). When all parameters were entered into a GLMM, mean surface seed density was found to decrease significantly with the number of days post-sowing and was positively associated with seed location (headland) (**Table 2.3**).

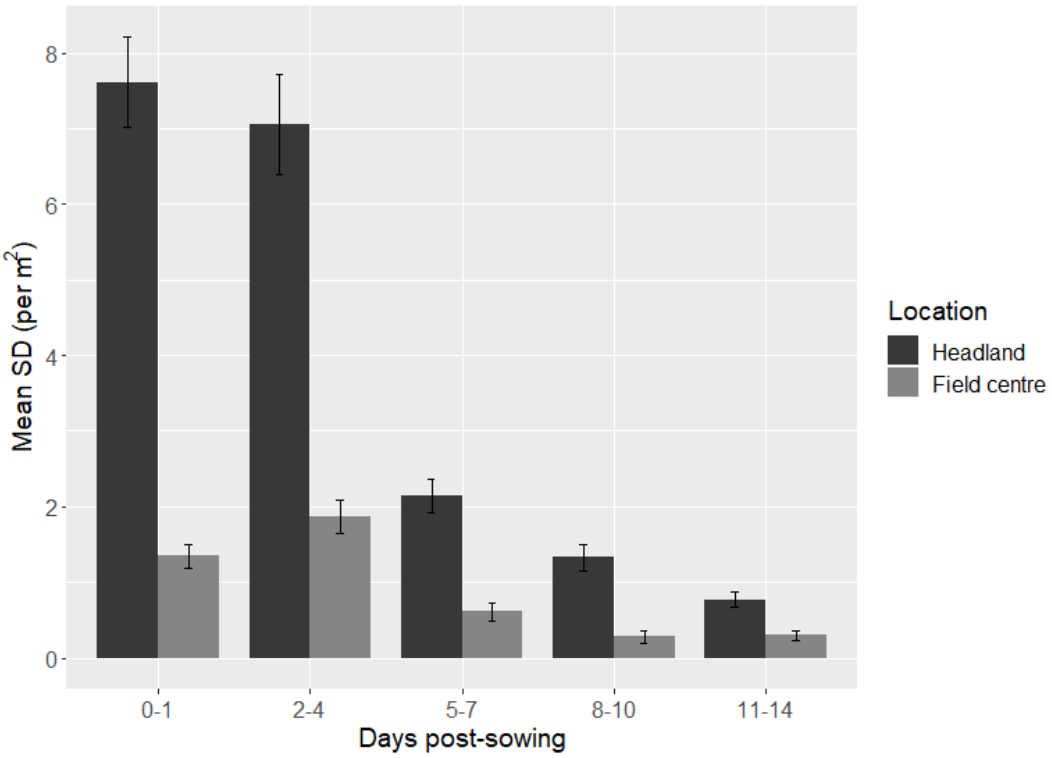

Figure 2.1. Surface seed densities between day 0 (sowing date) and 14-days post-sowing. Mean surface seed density (SD) per m^2 was calculated across all farms (n) for days 0-1 (n=24), 3 ±1 (n = 20), 6 ±1 (n = 25), 9 ±1 (n = 20) and 12 ±1 (and data from one farm collected on day 14; n = 24) post-sowing, with standard error bars. Data are shown separately for headland and field centre.

Table 2.3. Summary of generalised linear mixed model outputs for seed density and seed residue data collected from East Anglia.

Model	**Model output**			
	Disp	*Est*	*SE*	*p-value*
SD ~ field location(headland) + number of days post sowing + year				
Field location(headland)		0.33	0.03	**<0.001**
Number of days post-sowing	0.09	-0.14	0.01	**<0.001**
Year		-0.12	0.07	0.076
CTD residue in seeds ~ number of days post sowing + cumulative rainfall				
Number of days post sowing	0.59	-0.10	0.06	0.088
Cumulative rainfall		-0.14	0.01	**<0.001**

Disp: model dispersion; Est: model estimate; SE: standard error; SD: surface seed; CTD: clothianidin; density.

2.3.2 Clothianidin residue: seed & seedling samples

Seeds

The concentration of CTD recorded in pooled seed samples collected within 2 weeks post-sowing varied between 0.01 and 550.9 µg/g. However, the CTD residue recorded in pooled seeds did not significantly differ between farms within the first 24 hrs post-sowing (Kruskall-Wallis: $\chi^2_{13} = 20.6$, p = 0.080), or during the entire study period (Kruskall-Wallis $\chi^2_{14} = 18.2$, p = 0.197). CTD residue in pooled seeds decreased with the number of days post-sowing (**Table 2.3**) with a dissipation half-

life of 4.2 days (**Figure 2.2A**). The median CTD concentration in pooled seeds collected within 24 hrs post-sowing was 254.5 µg/g (IQR = 173.6; n = 27), compared to 90.3 µg/g (IQR = 154.7; n = 33) in seeds collected 2-7 days post-sowing and 48.2 µg/g (IQR = 83.6; n = 16) in those collected 7-14 days post-sowing. There was also a significant negative association between CTD residue in seed samples and cumulative rainfall at each farm (**Table 2.3**). The loss of CTD from seeds sampled at the earliest compared to the latest day post-sowing (at any one site, in either year; n=20) yielded an average loss of 13% of remaining residue per mm of rain.

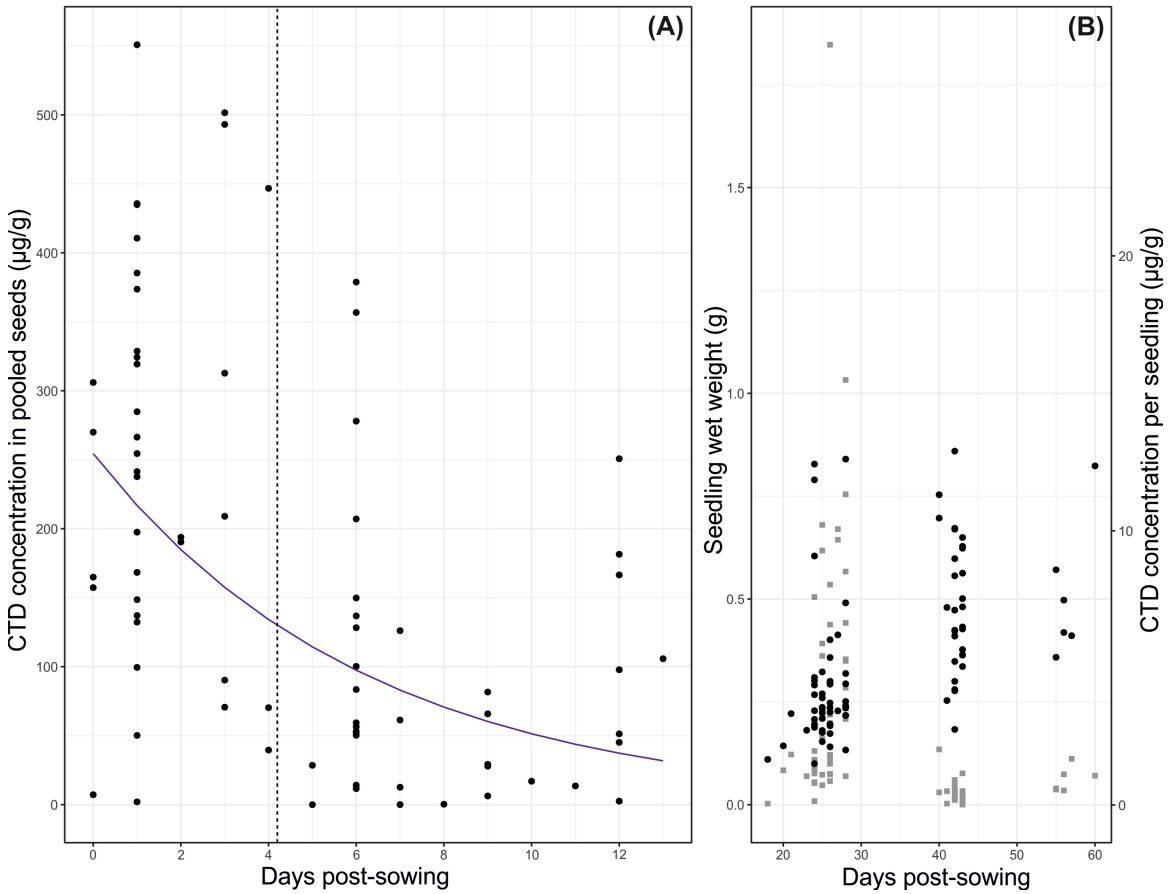

Figure 2.2. A) Concentration of clothianidin (CTD) in pooled seed samples collected between 0 and 14 days post-sowing. Each data point represents a pooled sample, or the mean taken from a group of individual samples from the same site and on the same day. Blue line: curve describing dissipation of residues on seeds over time. Dashed line: dissipation half-life (4.2 days) calculated using all sample values. **B) Wet weight of single seedlings and concentration of CTD in seedling samples between days 18 and 60 post-sowing.** Grey squares represent the concentration of CTD in each seedling sample (individual and pooled). Black circles represent the weight of each seedling; data points are either the weight of an individual seedling or an estimate of individual seedling weight calculated by dividing the weight of a pooled sample by the number of seedlings in that pool. All samples with a concentration >15 µg/g are seedlings that were analysed individually; one outlier was removed from these data (seedling weight = 0.17 g, CTD concentration = 104.5 µg/g, collected 28 days post-sowing).

CTD residue measured in individual seeds collected within 24 hrs of sowing varied around the target application rate of 500 µg/g (calculated from the Redigo Deter® product label, which states that 200 mL (containing 50 g of CTD) should be applied to 100 kg of seed [25]), with CTD concentrations ranging between 104.6 and 606.9 µg/g per seed (**Figure 2.3**). The mean (±SE) residue in individual seeds collected within 24 hrs of sowing was 278.3 ± 19.4 µg/g and the coefficients of variation for groups of individual seeds collected at each of the four farms within this time period ranged from 22 to 39%. Individual seeds collected 24 hrs post sowing contained on average 55.6% of the target application of CTD.

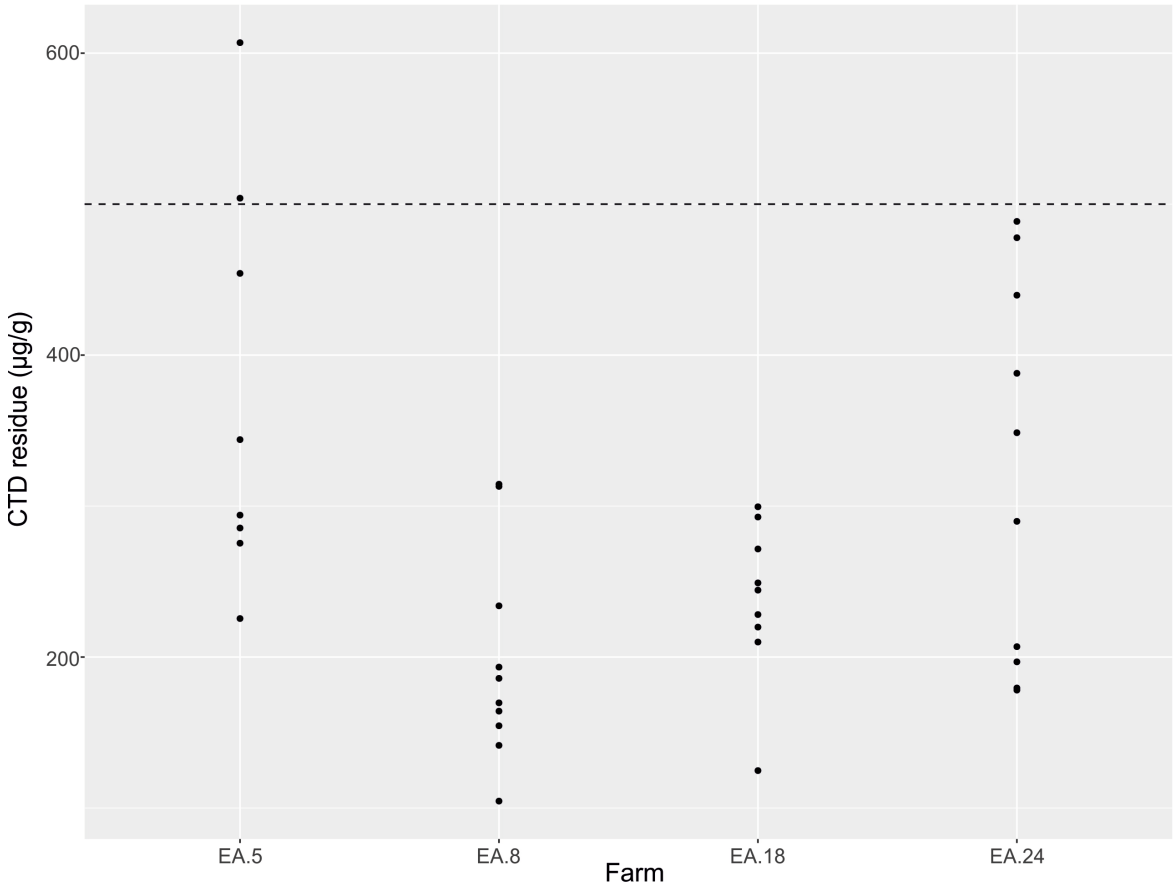

Figure 2.3. Variation in clothianidin (CTD) concentrations recorded in individual seeds collected within 24 hrs sowing. Individual seed samples were collected from four farms: EA5 and EA8 (sampled in 2015), EA18 and EA24 (sampled in 2016). Coefficients of variation for each farm were as follows: EA5 = 36%, EA8 = 35%, EA18 = 22%, EA24 = 39%. Dashed line: target application rate for CTD to winter cereal seeds in the UK, as per manufacturer's instructions (500 µg/g) [25].

Seedlings

The median residue in pooled seedling samples was 1.1 µg/g (IQR: 1.4; n = 34), which was 122-fold lower than the median residue measured in pooled seed samples. Residue also varied greatly in

pooled seedling samples across the study period (0.003 – 15.8 µg/g). The median residue in pooled seedlings collected 2-4 weeks post-sowing was 1.8 µg/g (n = 17), compared to 0.5 µg/g (n = 17) for those collected 5-13 weeks post-sowing (representative of the two seedling growth stages sampled). The concentration of CTD decreased significantly with increasing seedling mass (Spearman's rank correlation r_S = -0.305, p = 0.003; **Figure 2.2B**). As with seeds, residue in seedlings did not differ significantly between farms (Kruskall-Wallis χ^2_{12} = 9.8, p = 0.632).

The concentration of CTD measured in individual seedlings ranged between 0.1 and 104.5 µg/g (mean: 4.8 ± 1.8 µg/g), with coefficients of variation for groups of individual seedlings from each farm ranging between 124 and 198%. On average seedlings contained 5.9% of the CTD residue recorded in seed samples collected 0-2 days post-sowing (based on the mass of CTD per seed or seedling across pooled and individual samples).

2.3.3 Bird survey & camera trap data

A total of 65 bird species were recorded in fields sown with treated seed during the surveys undertaken in 2015 and 2016 (**Table 2.S2**). Songbirds made up the largest proportion of species observed in treated fields throughout the study period, whilst gulls accounted for several of the larger numbers of birds observed (**Figure 2.S1A**). Starlings *Sturnus vulgaris* were the most frequently observed songbird, accounting for 48% of all observations, followed by finch species (26%), comprised of large flocks of linnet *Linaria cannabina* (**Figure 2.S1B**).

A significant positive association was found between mean surface seed density (calculated for each site, at each survey visit) and bird abundance (recorded at the same site and survey visit) for those species where agricultural crop seed was recognised as being 'present' in the diet, but no association was found for those species where crop seed was deemed 'absent' from the diet (**Table 2.4**; **Figure 2.S2A**) [48]. When data were analysed for species guilds that are known to consume agricultural seed, surface seed densities were found to be positively associated with the number of 'other passerines' (starling observations accounted for 79% of data points in this guild) and buntings (comprising of yellowhammer *Emberiza citrinella* and reed bunting *Emberiza schoeniclus* observations; **Figure 2.S2**). Gamebirds exhibited a weaker positive association (p = 0.083; **Figure 2.S2**), but no significant association was detected for crows, finches, gulls or columbids (**Table 2.4**).

Table 2.4. Summary of generalised linear mixed model outputs for avian data. Bird abundance (up to 14 days post-sowing for specific species guilds) was modelled as a function of surface seed densities, whilst health parameters (weight and haematocrit) were modelled as a function of clothianidin (CTD) concentrations in avian plasma samples.

Model	**N species (N obs)**	**Model output**			
		Disp	*Est*	*SE*	*p-value*
Bird abundance ~ seed density					
Species with agricultural crop seed absent in diet	37	1.09	0.07	0.08	0.418
Species with agricultural crop seed present in diet	34	1.27	0.26	0.07	**<0.001**
Bird abundance (species with agricultural seed present in diet, split by taxonomic guild) ~ seed density					
Buntings (*Emberizidae*)	2	0.41	0.38	0.16	**0.018**
Crows (*Corvidae*)	5	0.68	0.20	0.14	0.148
Finches (*Fringillidae*)	3	0.45	0.17	0.22	0.447
Gamebirds (*Phasianidae*)	3	0.53	0.25	0.14	**0.083**
Gulls (*Laridae*)	5	0.38	0.16	0.20	0.408
Other passerines (*Alaudidae, Passeridae, Prunellidae, Sturnidae*)*	4	0.58	0.43	0.18	**0.015**
Pigeons & doves (*Columbidae*)	4	0.55	0.23	0.17	0.194
Thrushes (*Turdidae*)	1	0.59	0.18	0.14	0.193
Sub-lethal effect ~ concentration of CTD in plasma					
Weight	(70)	0.89	-0.006	0.01	0.451
Body condition	(70)	0.002	-0.002	0.01	0.208
Haematocrit	(70)	0.99	0.008	0.01	0.425

See **Table 2.S2** for a full list of species included in each 'taxonomic guild' used for bird abundance data.
*Shorelark *Eremophila alpestris* excluded from the model (only one individual recorded throughout survey period). Starlings *Sturnus vulgaris* made up 79% of observations in this group.
Est: model estimate; Disp: model dispersion; N: number of; obs: observations; SE: standard error for model estimate.

Fifteen bird species were observed consuming treated-seed at seed piles (**Table 2.5**)**.** The maximum time spent and number of seeds consumed at a seed pile during any singe visit was 11 min and 15 seeds (woodpigeon *Columba palumbus*; **Table 2.S3**). Individual birds at seed piles were found to consume 1.4–65.2% and <0.1–3.2% of the sub-lethal and lethal threshold for CTD, respectively, per visit (based on the mean and maximum number of seeds consumed and the mean concentration of CTD detected on seeds in the present study – 0.016 mg/seed; **Table 2.5**). In general, smaller species (<30 g body weight) were found to ingest a larger proportion of the amount of compound required to reach either toxicity threshold compared to larger species (>30 g body weight; **Table 2.5**).

Table 2.5. Summary of camera trap data for bird species observed consuming treated seed at seed piles. Data are ordered by the maximum proportion (%) of the toxicity thresholds (for CTD) that each species consumed.

English name	Latin	Average species weight	Total individuals	Seeds eaten per individual, per event			% of CTD toxicity threshold reached (mean*)		% of CTD toxicity threshold reached (max*)	
		(g)	*(n)*	*Mean*	*SE*	*Max*	*LD_{50}*	*NOAEL*	*LD_{50}*	*NOAEL*
Woodpigeon	*Columba palumbus*	507	115	9.37	1.63	152	0.2	4.0	3.2	65.2
Dunnock	*Prunella modularis*	21	4	2.25	0.48	3	1.1	23.3	1.5	31.1
Chaffinch	*Fringilla coelebs*	22	14	1.36	0.17	3	0.7	13.4	1.5	29.6
House Sparrow	*Passer domesticus*	27	16	1.81	0.16	3	0.7	14.6	1.2	24.2
Feral Pigeon	*Columba livia domestica*	360	4	19.25	6.88	37	0.6	11.6	1.1	22.3
Magpie	*Pica pica*	213	34	4.29	0.54	13	0.2	4.4	0.7	13.3
Red-legged Partridge	*Alectoris rufa*	530	48	6.42	0.78	28	0.1	2.6	0.6	11.5
Robin	*Erithacus rubecula*	19	4	1.00	0.00	1	0.6	11.4	0.6	11.4
Jay	*Garrulus glandarius*	167	1	7.00	n/a	7	0.4	9.1	0.4	9.1
Grey Partridge	*Perdix perdix*	490	31	5.55	0.88	20	0.1	2.5	0.4	8.9
Carrion Crow	*Corvus corone*	509	61	5.05	0.62	19	0.1	2.2	0.4	8.1
Rook	*Corvus frugilegus*	452	8	3.88	1.38	13	0.1	1.9	0.3	6.3
Pheasant	*Phasianus colchicus*	1200	21	6.95	1.42	22	0.1	1.3	0.2	4.0
Stock Dove	*Columba oenas*	326	1	5.00	n/a	5	0.2	3.3	0.2	3.3
Jackdaw	*Corvus monedula*	232	2	1.50	0.50	2	0.1	1.4	0.1	1.9

*Calculated using the mean or maximum number of seeds consumed per visit for each species, an estimated concentration of 0.016 mg of CTD per seed (equal to the average mass of CTD per individual seed in this study). Endpoint values for NOAEL and LD_{50} were obtained from Mineau & Palmer (2013), for a 15g bird at the 5% tail of sensitivity, which were moderated by the average weight for each species (obtained from the BTO [43]). NOAEL in this instance refers to reproductive effects only [22].

CTD: clothianidin; LD_{50}: median lethal dose; NOAEL: no-observed-adverse-effect level; SE: standard error of the mean.

2.3.4 Clothianidin residue: avian plasma samples

Significantly more avian plasma samples tested positive in the post-sowing group (36/71, ~51%), compared to the pre-sowing control group (4/36, ~11%; Fisher's exact: OR = 8.0, CI = 34.7, $p < 0.001$). Samples were available from ten species post-sowing and nine species pre-sowing, of which nine and two species tested positive for CTD, respectively. Greenfinch was the only species to test negative in the post-sowing group, whereas blackbird *Turdus merula* and starling were the only species to test positive in the pre-sowing group (3/5 and 1/1 birds tested, respectively; **Table 2.6**). All four birds that tested positive pre-sowing were female, whereas males and females were equally represented in samples collected post-sowing. Concentrations of clothianidin in all positive samples ranged between 0.5 and 69,300 ng/mL, with a median value of 12.0 ng/mL (n = 40; **Table 2.6**). The median CTD concentration in positive samples collected pre-sowing was 3.6 ng/mL (n = 4), whereas the median in post-sowing samples was 12.5 ng/mL (n = 36).

Table 2.6. Summary of the prevalence of clothianidin (CTD) in avian samples collected post sowing and the concentrations of the compound measured in individual plasma samples collected from each species. Data are ordered by maximum concentration measured in any one individual bird from one species (from highest to lowest). CTD prevalence post-sowing is calculated to the nearest 1%.

Species		**Number of samples post-sowing**			**CTD prevalence post-sowing**	**Residue in all positive samples (ng/mL)**			
		Total	*ND*	*POS*	*%*	*Minimum*	*Maximum*	*Median*	*IQR*
Yellowhammer	*Emberiza citrinella*	10	3	7	70	2.0	69300	29.4	4530
House sparrow	*Passer domesticus*	5	3	2	40	6740	7500	7120	380
Tree sparrow	*Passer montanus*	9	3	6	60	3.3	4880	22.5	37.2
Chaffinch	*Fringilla coelebs*	9	2	7	78	0.6	352	29.3	1000
Dunnock	*Prunella modularis*	15	10	5	30	0.5	444	3.7	54.3
Blackbird	*Turdus merula*	7	2	5^{3}	71	2.4	127	9.4	8.0
Reed bunting	*Emberiza schoeniclus*	6	5	1	15	3.0	3.0	3.0	0.0
Starling	*Sturnus vulgaris*	0	0	0^{1}	n/a	2.0	2.0	2.0	0.0
Robin	*Erithacus rubecula*	1	0	1	100	1.7	1.7	1.7	0.0
Goldfinch	*Carduelis carduelis*	8	6	2	25	0.8	1.4	1.1	0.3
Greenfinch	*Chloris chloris*	1	1	0	0	0.0	0.0	0.0	0.0

^{X}Superscript values give the number of positive samples obtained for each species that were collected pre-sowing.
ND: non-detect for CTD; POS: tested positive for CTD; IQR: inter-quartile range.

There was a significant difference between the concentration of CTD found in avian plasma samples collected from different farms post-sowing (Kruskall-Wallis $\chi^2_5 = 17.4$, $p = 0.003$). However, there was no significant difference in the concentration of CTD recorded post-sowing between species (with five or more positive samples; Kruskall-Wallis $\chi^2_4 = 2.4$, $p = 0.662$; **Table 2.6**). For species where measurements of CTD concentration in plasma were available, four were observed consuming treated seeds at seed piles (dunnock, robin, house sparrow *Passer domesticus* and chaffinch *Fringilla coelebs*) and five were observed in treated fields (yellowhammer, blackbird, reed bunting, goldfinch *Carduelis carduelis* and starling); all tested positive for CTD (**Table 2.S2**). Tree sparrow *Passer montanus* was the only species to test positive for CTD that was not observed in treated fields in East Anglia, whereas greenfinch was observed in treated fields in East Anglia, but did not test positive for CTD (only one sample was obtained for analysis).

Neither body weight, body condition nor haematocrit differed significantly between individuals where CTD was detected in the plasma, compared to those where it was not (Wilcoxon sum rank test – weight: $W = 557$, $p = 0.523$; body condition: $W = 665$, $p = 0.541$; haematocrit: $W = 712$, $p = 0.523$; **Figure 2.S3** ,**2.S4, 2.S5**). Equally, no association was found between the concentration of CTD in plasma samples and either body weight, body condition or haematocrit in birds sampled post-sowing (**Table 2.4**). There was weak evidence that bird weight varied with the hour of capture (Kruskall-Wallis $\chi^2_5 = 9.9$, $p = 0.076$) and evidence that haematocrit score differed between species (Kruskall-Wallis $\chi^2_9 = 42.2$, $p < 0.001$) in samples collected post-sowing. Two individuals with the highest CTD concentrations in plasma samples (yellowhammer and tree sparrow) exhibited intoxication symptoms at sampling (fluffed up appearance, sluggish movement) and had red dye around their bills. Both these individuals, in addition to a third (chaffinch) also had red faeces.

2.4 DISCUSSION

Results from this study collectively confirmed that 21 species of farmland bird were exposed to CTD, providing the first account of exposure in an avian community over a typical cereal sowing period. Exposure was identified via direct observations of CTD ingestion via treated seed (15 species) and/or the presence of CTD residue in plasma (10 species), in approximately one third of all species observed in CTD-treated fields. The median concentration of CTD residue recorded in plasma samples here was larger than any NN residue reported in an avian species to date [39], except for poisoning incidents [35]. This study provides evidence that seed treatments are a source of NN exposure in wild birds, and identifies multiple factors that may affect patterns of exposure observed in the field.

According to application instructions provided by the manufacturer, treated seeds are required to be buried at a depth of 4 cm and are to be reincorporated into the soil if left on the soil surface after drilling [25]. Here, CTD-treated seeds and seed piles were available on the soil surface at the majority of farms surveyed, which is in accordance with previous research that identified high prevalence of wheat seed at the soil surface compared to other crop types [28, 49]. The current study found that treated seed was present on the soil surface in almost all the fields surveyed across both years, whilst seed piles were found at 68% of farms sampled. However, the number of seeds on the soil surface differed significantly between farms, suggesting non-uniformity across drilling practices; this variability has previously been attributed to differences in soil type or farm machinery [28, 49]. Surface seed density was also higher at the headlands compared to field centres as found in previous studies [26-28, 49], and may be indicative of differences in localised efficiency of farm machinery at burying seeds at the prescribed depth [25]. Overall, treated seeds were found to be broadly available at the soil surface within the first two weeks post-sowing, with an average of 2.8 seeds per m^2 available to birds across all farms surveyed. Availability decreased over longer periods, presumably due to consumption of seed by wildlife.

The median concentration of CTD on pooled samples of treated seed collected from the soil surface within the first 24 hrs after sowing was 49% lower than the target application rate (as prescribed by product labels), and did not significantly differ between farms. However, there was high intra-farm variability in CTD concentrations on seeds collected and analysed individually across multiple farms, suggesting that the application of active compound is not standardised across individual seeds. These results tally with a similar study that also found variable concentrations of NNs that were below the application rate on soybean and corn seeds [28]. Comparatively, the amount of residue measured in seedlings was considerably smaller than that in seeds (on average seedlings contained only 5.9% of the CTD measured in treated seeds), which tallies with previous studies that have found between 1 and 15% of residue in treated seeds is taken up by the seedling [31, 32]. CTD concentrations were negatively associated with the wet weight of individual seedlings, presumably reflecting growth dilution of residues (see also [30]) as seedlings developed. Similar patterns of CTD concentrations were found in seedling samples compared to seeds, which also exhibited low inter-farm variability in pooled samples, but high intra-farm variability in individual samples across farms surveyed. These results highlight the potential for large variability in exposure arising from the consumption of either seeds or seedlings. However, data here suggest that treated seedlings are a smaller source of exposure to farmland birds when compared with treated seeds.

During the period 0-13 weeks post-sowing, 66 species of bird were recorded in fields in East Anglia and Lincolnshire that were sown with CTD-treated seeds. Of these, exposure was confirmed in ~32% of all species recorded (**Table 2.S2**). The species exposed were not restricted to any one taxonomic group: plasma samples tested positive in species of sparrow, bunting, finch and thrush, whilst species of columbid, galliforme, corvid and passerine were observed consuming treated seed at spilt seed piles. Observations relating to galliformes and columbids are consistent with previous observations of NN poisonings during autumn months and the detection of NN residues in samples of liver and eggs collected from quail, partridge and pigeon [34-37]. Furthermore, exposure was confirmed here for a similar species composition to that in Roy *et al.* [28], and 12 of the 30 species observed consuming pesticide-treated seeds in a previous study conducted in Spain [26]. This included multiple sparrow species, such as house sparrow, which have also been reported to be extensively exposed to NNs across the Swiss plateau [41]. Overall exposure was not limited to any specific species ecology or taxonomy, other than that the majority of species exposed are known to have cereal grain in their diet (**Table 2.S2**) [48].

The prevalence of CTD residues in plasma samples collected post-sowing (~50%) was broadly similar to that reported previously. Of the three other studies that have measured NN residue in plasma samples collected from wild birds, positive samples accounted for 3% (n=30 bird of prey samples), 60% (n=10 bird of prey samples) and 80% (n=36 passerine samples) of the total sample size [38-40]. Comparatively, Humann-Guilleminot *et al.* reported 100% prevalence of NN residue in 146 pooled house sparrow feather samples (each pool contained one feather from three individuals) [41]. When comparing these data, differences in sample type, time of sampling (in relation to exposure) and test species are all likely to explain the observed variation between studies. Firstly, pooled samples may inflate the overall exposure prevalence compared to samples analysed from single birds. Furthermore, NN in feathers may have been laid down over a period of several days or weeks during moult, whereas NN residue is known to exit the blood stream 6-8 hrs after the compound is ingested [40, 50]. Therefore blood residues are more likely to provide a snap-shot of exposure whilst feather residues may reflect aggregated exposure over a longer period. Secondly, birds of prey are likely to experience secondary exposure via consumption of contaminated prey, whereas passerines are more likely to experience primary exposure via the direct ingestion of treated seed. This will predispose passerines to higher levels and frequencies of exposure than predatory species. Notably, the median and maximum concentration of CTD in plasma recorded in the present study exceeded any previous records of NN residue in avian plasma. To date, 3.28 ng IMI/mL was the highest NN concentration reported for a bird of prey plasma sample (obtained from Eurasian eagle

owl *Bubo bubo* [39]) and 0.17 ng IMI/mL the highest NN concentration in a passerine plasma sample (obtained from a white-crowned house sparrow *Zonotrichia leucophrys* [40]). Here we recorded a median concentration across all positive samples of 12 ng CTD/mL, whilst the maximum concentration recorded (in one yellowhammer) was 69,300 ng CTD/mL. As this is the first study to measure NN residue in plasma samples collected directly post-sowing (compared to those conducted outside of the sowing season), it is possible that these data are not unusual during this time period and may be representative of a period of 'peak' exposure as a result of the increased availability of treated seed.

Surveys in East Anglia confirmed that surface seed densities were a significant predictor of bird abundance in treated fields for species groups such as buntings and passerines, as well as gamebirds. These findings tally with those species that were seen to have the highest concentrations of CTD in plasma samples (such as yellowhammer and tree sparrow), as well as multiple gamebird species that were observed consuming treated seeds at seed piles. Unfortunately, only one plasma sample was obtained for starling, which was the species that made up the largest proportion of birds observed in treated fields (and were associated with seed densities); however, this sample did test positive for CTD. Interestingly, starling and blackbird were the only two species to test positive for CTD pre-sowing, both of which are migratory and highly dispersive in autumn [48] and may therefore have had access to sites outside of those sampled where drilling had already taken place. Also of note is that species such as goldfinch, reed bunting and blackbird are not typically known to consume cereal seed [23], and therefore alternative exposure pathways aside from ingestion of treated seed should be considered. One plausible explanation is the contamination of soil, water and wild plants with NN compounds originating from seed treatments, as has been evidenced by previous research [51, 52]. This hypothesis is further supported by seed residue data presented here, as well as existing literature, which suggests that NNs leach from treated seeds over time and with increased rates of precipitation [53, 54].

Although this study confirms that birds are exposed to CTD, what remains less clear is the impact that this level of exposure is likely to have on avian fitness and health in the wild. Here, we used haematocrit and body weight (both of which have previously been found to be negatively associated with IMI exposure [17, 55]), as well as body condition, as indicators for adverse effects of CTD exposure in the field. We did not find any associations, but as we were not able to account for some confounding factors introduced by the field-based study design (such as species differences in haematocrit), it is possible that any association may have been masked. Furthermore,

seeds in this study were treated with CTD rather than IMI (the latter of which is known to be more toxic to birds [11]), and therefore any symptoms of toxicity are likely to be on the lower end of the known spectrum for NN compounds. When examining CTD toxicity thresholds in the context of the number of seeds consumed at seed clusters, one wood pigeon was found to ingest sufficient seed to reach 65% of the generic NOAEL threshold for reproductive effects, whilst smaller species (<30 g) were found to ingest 11 to 31% of the compound required to reach the reproductive NOAEL threshold per feeding event. These estimations are constructed based on single visits, so could be under-estimating exposure when considering availability of treated seed in the broader landscape. Notably, the two individuals (one tree sparrow and one yellowhammer) that had the highest CTD plasma concentrations (8,800 and 69,300 ng/mL) exhibited intoxication symptoms at time of capture, which were similar to those described in IMI-dosed eared doves [17]. These individuals also had red dye around the bill and red faeces, indicating recent ingestion of CTD-coated seeds, as has similarly been reported in NN poisoning incidents [35]. It is likely that the concentrations of CTD in the blood stream breached a toxicity threshold for these individuals, although toxicological data for CTD are not available for these particular species to confirm this.

2.4.1 Conclusion

Results here provide clear evidence that a variety of farmland birds are subject to widespread CTD exposure following normal agricultural sowing of CTD-treated cereal seed. CTD exposure was confirmed in 32% of species observed and 50% of individuals sampled in treated fields post-sowing, with levels of exposure to CTD among the highest recorded for wild birds to date. The widespread availability of seeds at the soil surface was identified as a primary source of exposure. Factors such as the variation in compound application to seed, rainfall patterns after sowing, and differences in drilling efficiency between farms are likely to have contributed to temporal and spatial variability in exposure. Overall, this information is likely to have global implications for multiple bird species where NNs are in use and may help to inform any future policy decisions related to this group of insecticides. In addition, these data are pertinent to future risk assessments through identifying consumption of treated seed as a source of exposure, and thus risk, to a wide range of species of farmland bird.

Ethics statement

All works were approved by Animal Welfare and Ethical Review Body at the University of York. The Home Office reviewed and approved the licence to take blood samples from species in the study (licence number: P3AF8F232).

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2.5 SUPPLEMENTARY MATERIAL

2.S1 Supplementary Note: LC-MS/MS protocol details

Seeds & seedling samples

In total, 111 seeds (73 pooled, 38 individual samples) and 93 seedlings (32 pooled, 61 individual samples) were tested for CTD using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample protocols were developed from the methodology used in Woodcock *et al.* (2018) [56]. Briefly, approximately 0.1 g of wet sample was weighed and spiked with labelled internal standard (Clothianidin D3; QMX, Essex. UK). Extraction was carried out in 50:50 methanol:water (v:v) containing 0.2% formic acid and vortexed briefly. Samples were centrifuged at 3000 rpm for 5 min, then 4 mL of HPLC grade water was added to 1 mL of the supernatant and the samples were vortexed for another 30 sec. 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. After passing the sample through the cartridge and drying under vacuum, the compounds were eluted with acetonitrile. The extracts were blown down using a Turbovap (Biotage, Uppsala, Sweden), re-dissolved in 1 mL mobile phase (95% phase A, 5% phase B), and analysed by LC-MS/MS.

Analysis was performed using a LC coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific; Hemel Hemsptead, UK), interfaced with ion max electrospray ionisation (ESI) and operated with Xcalibur^{TM} (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 x 2 mm ID; Phenomenex, Macclesfield, UK) using a H_2O:CH_4O mobile phase gradient. The analytes were eluted from the column using the following programme: 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 and held for 5 min, and returned to initial conditions. Mobile phase A - 0.1% acetic acid in water, and mobile phase B - 0.1% acetic acid in methanol at a rate of 0.3 mL min^{-1}. MS/MS was performed in single reaction mode using ESI in the positive mode, and two characteristic fragments (m/z 168.72 and m/z 131.56) were monitored for the compound CTD. Argon was used as collision gas.

Plasma samples

Plasma samples from 96 individual birds were tested for CTD using LC-MS/MS. Sample protocols were developed from the methodology used in Hao *et al.* (2018) [40]. Briefly, each sample (20-

50 µL) was spiked with labelled internal standard (CTD 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 again, after which the solution was evaporated using a Turbovap (Biotage, Uppsala, Sweden). The residue was reconstituted with H_2O:C_2H_3N (95% phase A, 5% phase B) mobile phase, vortexed briefly 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 a HPLC vial (Waters, Hertfordshire, UK).

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 (V4.2) software. Analyte separation was performed on a Waters Acquity BEH C18 column (1.7 µm particle size, 50 mm x 2.1 mm ID; Waters, Hertfordshire, UK) using a H_2O:acetonitrile mobile phase gradient (mobile phase A - 0.1% formic acid in water; mobile phase B - 0.2% formic acid in acetonitrile at a rate of 0.5 mL min^{-1}). The LC programme started from 95% A and 5% B, increased to 70% B in 3 min then returned to initial conditions. MS/MS was performed in multiple reaction mode, using UniSpray in positive mode. The same characteristic fragments and collision gas were used as for seed and seedling samples.

Table 2.S1. Designated species on the Home Office licence for blood-taking and details of sample numbers from the six farms in Lincolnshire.

English name	Latin	Samples obtained	Farms sampled
Dunnock	*Prunella modularis*	25	6
Chaffinch	*Fringilla coelebs*	17	6
Goldfinch	*Carduelis carduelis*	14	3
Tree sparrow	*Passer montanus*	14	3
Blackbird	*Turdus merula*	12	5
Yellowhammer	*Emberiza citrinella*	10	2
House sparrow	*Passer domesticus*	7	2
Reed bunting	*Emberiza schoeniclus*	6	3
Greenfinch	*Chloris chloris*	2	1
Robin	*Erithacus rubecula*	2	2
Starling	*Sturnus vulgaris*	1	1
Blue tit	*Cyanistes caeruleus*	*0*	*0*
Bullfinch	*Pyrrhula pyrrhula*	*0*	*0*
Collared dove	*Streptopelia decaocto*	*0*	*0*
Corn bunting	*Emberiza calandra*	*0*	*0*
Feral pigeon	*Columba livia domestica*	*0*	*0*
Great tit	*Parus major*	*0*	*0*
Long-tailed tit	*Aegithalos caudatus*	*0*	*0*
Meadow pipit	*Anthus pratensis*	*0*	*0*
Mistle thrush	*Turdus viscivorus*	*0*	*0*
Song thrush	*Turdus philomelos*	*0*	*0*
Stock dove	*Columba oenas*	*0*	*0*
Wood pigeon	*Columba palumbus*	*0*	*0*
Wren	*Troglodytes troglodytes*	*0*	*0*

Table 2.S2. Summary of data for all bird species included in the study, including: A) where each species was observed during bird abundance surveys (EA sites); **B)** whether species were observed by camera traps at or eating seeds at treated-seed piles (EA sites); and **C)** whether plasma samples tested positive for CTD residue (LN sites). Species are ordered by guild and split by diet (CTD-treated seed present or absent). Shading indicates a confirmed observation for each scenario ordered from the smallest risk of ingesting treated seed, to confirmed exposure (left to right) [49].

Species			A)			B)		C)
English name	Latin	Guild	Field boundary	Field centre	Field headland	Treated seed pile	Seed ingestion	CTD in plasma sample
Crop seed present in diet:								
Corn bunting	*Emberiza calandra*	Bunting						na
Yellowhammer	*Emberiza citrinella*	Bunting						
Collared dove	*Streptopelia decaocto*	Columbid						na
Feral pigeon	*Columba livia domestica*	Columbid						na
Stock dove	*Columba oenas*	Columbid						na
Wood pigeon*	*Columba palumbus*	Columbid						
Carrion crow	*Corvus corone*	Corvid						na
Jackdaw	*Corvus monedula*	Corvid						na
Jay	*Garrulus glandarius*	Corvid						na
Magpie	*Pica pica*	Corvid						na
Rook	*Corvus frugilegus*	Corvid						na
Chaffinch	*Fringilla coelebs*	Finch						
Greenfinch	*Chloris chloris*	Finch						
Linnet	*Linaria cannabina*	Finch						na
Grey partridge*	*Perdix perdix*	Gamebird						
Pheasant*	*Phasianus colchicus*	Gamebird						
Red-legged partridge*	*Alectoris rufa*	Gamebird						
Black-headed gull	*C. ridibundus*	Gull						na
Common gull	*Larus canus*	Gull						na
Great black backed gull	*Larus marinus*	Gull						na
Herring gull	*Larus argentatus*	Gull						na
Lesser black backed gull	*Larus fuscus*	Gull						na
Dunnock	*Prunella modularis*	Other passerine						
House sparrow	*Passer domesticus*	Other passerine						
Tree sparrow	*Passer montanus*	Other passerine	*Not observed at EA sites*					
Shorelark	*Eremophila alpestris*	Other passerine						na
Skylark	*Alauda arvensis*	Other passerine						na
Starling	*Sturnus vulgaris*	Other passerine						
Robin	*Erithacus rubecula*	Thrush						
Crane	*Grus grus*	Waterbird						na
Mallard	*Anas platyrhynchos*	Waterbird						na
Moorhen	*Gallinula chloropus*	Waterbird						na
Mute swan	*Cygnus olor*	Waterbird						na
Ruff	*Calidris pugnax*	Wader						na

*Residue found in liver, but not in plasma samples; units in ng/g. (unpublished data).
CTD: clothianidin; EA: East Anglia; LN: Lincolnshire; na: not available.

Table 2.S2 (cont). Summary of data for all bird species included in the study, including: A) where each species was observed during bird abundance surveys (EA sites); **B)** whether species were observed by camera traps at or eating seeds at treated-seed piles (EA sites); and **C)** whether plasma samples tested positive for CTD residue (LN sites). Species are ordered by guild and split by diet (CTD-treated seed present or absent). Shading indicates a confirmed observation for each scenario ordered from the smallest risk of ingesting treated seed, to confirmed exposure (left to right) [49].

Species			A)			B)		C)
English name	Latin	Guild	Field boundary	Field centre	Field headland	Treated seed pile	Seed ingestion	CTD in plasma sample
Crop seed absent in diet:								
Reed bunting	*Emberiza schoeniclus*	Bunting						
Bullfinch	*Pyrrhula pyrrhula*	Finch						na
Goldfinch	*Carduelis carduelis*	Finch						
Chiff chaff	*Phylloscopus collybita*	Other passerine						na
Goldcrest	*Regulus regulus*	Other passerine						na
Meadow pipit	*Anthus pratensis*	Other passerine						na
Pied wagtail	*Motacilla alba*	Other passerine						na
Stone-curlew	*Burhinus oedicnemus*	Other passerine						na
Treecreeper	*Certhia familiaris*	Other passerine						na
Wheater	*Oenanthe oenanthe*	Other passerine						na
Whitethroat	*Sylvia communis*	Other passerine						na
Wren	*Troglodytes troglodytes*	Other passerine						na
Barn owl	*Tyto alba*	Raptor						na
Buzzard	*Buteo buteo*	Raptor						na
Kestrel	*Falco tinnunculus*	Raptor						na
Sparrowhawk	*Accipiter nisus*	Raptor						na
Blackbird	*Turdus merula*	Thrush						
Feidlfare	*Turdus pilaris*	Thrush						na
Mistle thrush	*Turdus viscivorus*	Thrush						na
Redwing	*Turdus iliacus*	Thrush						na
Song thrush	*Turdus philomelos*	Thrush						na
Blue tit	*Cyanistes caeruleus*	Tit						na
Great tit	*Parus major*	Tit						na
Long-tailed tit	*Aegithalos caudatus*	Tit						na
Golden plover	*Pluvialis apricaria*	Wader						na
Grey heron	*Ardea cinerea*	Waterbird						na
Lapwing	*Vanellus vanellus*	Wader						na
Little ringed plover	*Charadrius dubius*	Wader						na
Little egret	*Egretta garzetta*	Wader						na
Snipe	*Gallinago gallinago*	Wader						na
Green woodpecker	*Picus viridis*	Woodpecker						na
Greater-spotted woodpecker	*Dendrocopos major*	Woodpecker						na

*Residue found in liver, but not in plasma samples; units in ng/g. (unpublished data).
CTD: clothianidin; EA: East Anglia; LN: Lincolnshire; na: sample not available.

Table 2.S3. All species observed at naturally spilt clothianidin-treated seed piles at farms in East Anglia during the autumn sowing seasons of 2015-2016. Time spent at seed pile was recorded to the nearest 0.5 min and the median was calculated from these data.

Species		Total N individuals at seed piles	Time at seed piles (mins) per visit		
English name	**Latin**		*Med*	*Min*	*Max*
Birds					
Woodpigeon*	*Columba palumbus*	**353**	0.5	0.5	11
Starling	*Sturnus vulgaris*	**256**	0.5	0.5	2
Red-legged Partridge*	*Alectoris rufa*	**167**	1	0.5	5
Carrion Crow*	*Corvus corone*	**91**	0.5	0.5	6
Pheasant*	*Phasianus colchicus*	**72**	0.5	0.5	4
Grey Partridge*	*Perdix perdix*	**52**	1	0.5	4
Magpie*	*Pica pica*	**47**	0.5	0.5	8
Rook*	*Corvus frugilegus*	**45**	0.5	0.5	11
Chaffinch*	*Fringilla coelebs*	**38**	0.5	0.5	1
House Sparrow*	*Passer domesticus*	**30**	0.5	0.5	1
Jackdaw*	*Corvus monedula*	**16**	0.5	0.5	6
Lapwing	*Vanellus vanellus*	**14**	0.75	0.5	4
Linnet	*Linaria cannabina*	**13**	0.75	0.5	1
Robin*	*Erithacus rubecula*	**11**	0.5	0.5	0.5
Feral Pigeon*	*Columba livia domestica*	**8**	3.25	0.5	9
Dunnock*	*Prunella modularis*	**5**	0.5	0.5	1
Black-headed Gull	*Chroicocephalus ridibundus*	**3**	0.75	0.5	1
Jay*	*Garrulus glandarius*	**3**	1	0.5	2
Song Thrush	*Turdus philomelos*	**3**	0.5	0.5	0.5
Blackbird	*Turdus merula*	**2**	0.5	0.5	0.5
Golden Plover	*Pluvialis apricaria*	**2**	0.5	0.5	0.5
Stock Dove*	*Columba oenas*	**2**	2.25	0.5	4
Pied Wagtail	*Motacilla alba*	**1**	0.5	0.5	0.5
Mammals					
Hare	*Lepus europaeus*	**189**	0.5	0.5	24
Mouse*	*Mus musculus*	**162**	0.5	0.5	17
Rabbit*	*Oryctolagus cuniculus*	**75**	0.5	0.5	22
Fox	*Vulpes vulpes*	**30**	0.5	0.5	3
Badger	*Meles meles*	**14**	0.5	0.5	16
Muntjack	*Muntiacus sp.*	**10**	0.5	0.5	3
Other deer	*Cervidae sp.*	**16**	0.5	0.5	3

*Species observed consuming treated seeds (see **Table 2.6**).
Max: maximum; Med: Median; Min: minimum; N: number of.

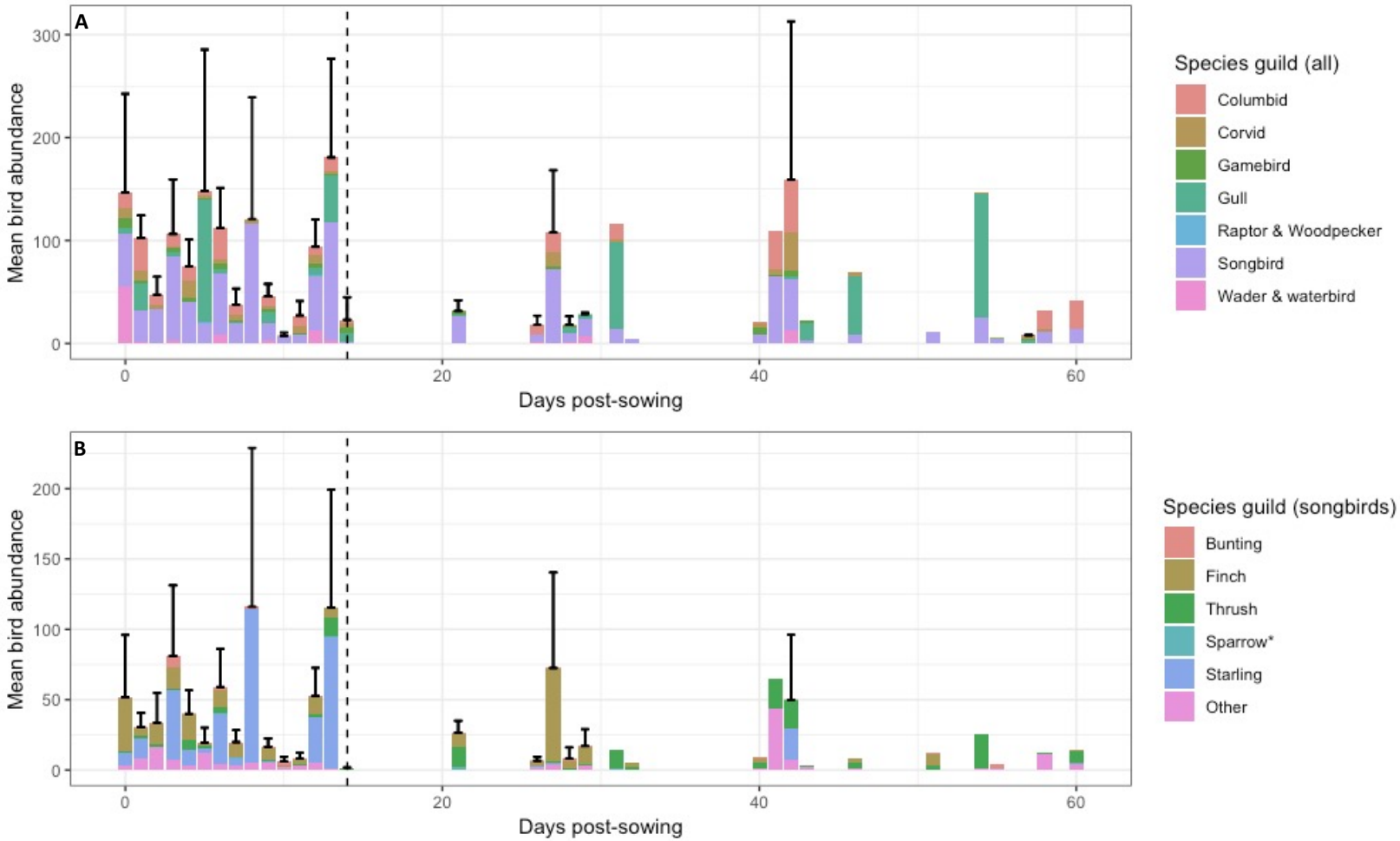

Figure 2.S1. Mean bird abundance and proportion of species guilds observed for all sites surveyed between days 0 and 60 post-sowing for (A) all species and (B) songbirds. Each bar represents the mean bird abundance (with standard error bars), calculated from all available bird abundance surveys from all sites surveyed on that day. Days with

no bars are those where no surveys took place; data points with no error bars are those where only one site was surveyed. Bars are shaded by the proportion of the total number of birds observed across all sites on each day. Dashed line: 14 days post-sowing, representing the temporal distinction between seed and seedling sample collection periods across all sites. (*) The category `Sparrow' contains the species house sparrow *Passer domesticus* and dunnock *Prunella modularis* only (**Table 2.A**). The mean number of species recorded during each bird survey was seven (range: 2 -12), whilst the mean number of birds recorded per survey was 83 (range: 5-180).

Table 2.A. Species included in each species guild in Figure S4.

Species guild	Species present in species guild
Figure S1A: All	
Columbid	Collared dove, feral pigeon, stock dove, wood pigeon
Corvid	Carrion crow, jackdaw, jay, magpie, rook
Gamebird	Grey partridge, red-legged partridge, pheasant
Gull	Black-headed gull, common gull, great black-backed gull, herring gull, lesser black-backed gull
Raptor & Woodpecker	Barn owl, buzzard, kestrel, sparrowhawk, green woodpecker, greater-spotted woodpecker
Songbird	Corn bunting, yellowhammer, reed bunting, chaffinch, greenfinch, linnet, bullfinch, goldfinch, dunnock, house sparrow, skylark, starling, chiff chaff, goldcrest, meadow pipit, pied wagtail, eurasian stone-curlew, treecreeper, wheater, whitethroat, wren, robin, blackbird, fieldfare, mistle thrush, redwing, songthrush, blue tit, great tit, long-tailed tit
Wader & Waterbird	Golden plover, grey heron, lapwing, little ringed plover, little egret, snipe, crane, mallard, moorhen, mute swan, ruff
Figure S1B: Songbirds	
Bunting	Corn bunting, yellowhammer, reed bunting
Finch	Chaffinch, greenfinch, linnet, bullfinch, goldfinch
Thrush	Blackbird, fieldfare, mistle thrush, redwing, songthrush
Sparrow*	House sparrow, dunnock
Starling	Starling
Other	Skylark, starling, chiff chaff, goldcrest, meadow pipit, pied wagtail, eurasian stone-curlew, treecreeper, wheater, whitethroat, wren, robin, blue tit, great tit, long-tailed tit

For all scientific names please refer to **Table 2.S2**.

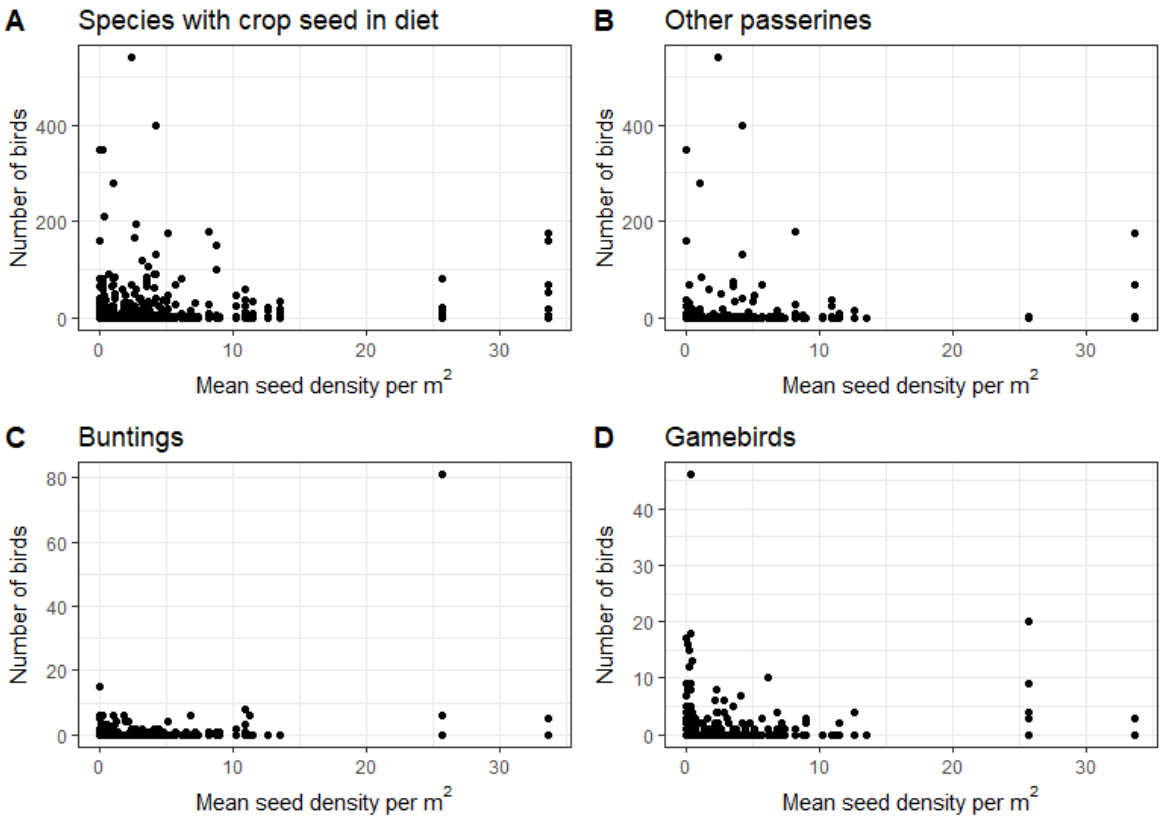

Figure 2.S2. The total number of birds and mean seed surface densities per field, per survey visit for all bird surveys in 2015 and 2016 at sites in East Anglia. Each data point represents a single species count at one field, at one survey visit and the corresponding mean seed surface density for that field at that visit. Each panel depicts a different species group where the number of birds was found to be significantly associated with seed surface densities over the course of the study period. Corresponding GLMM outputs for each of the panels can be found in **Table 2.5**.

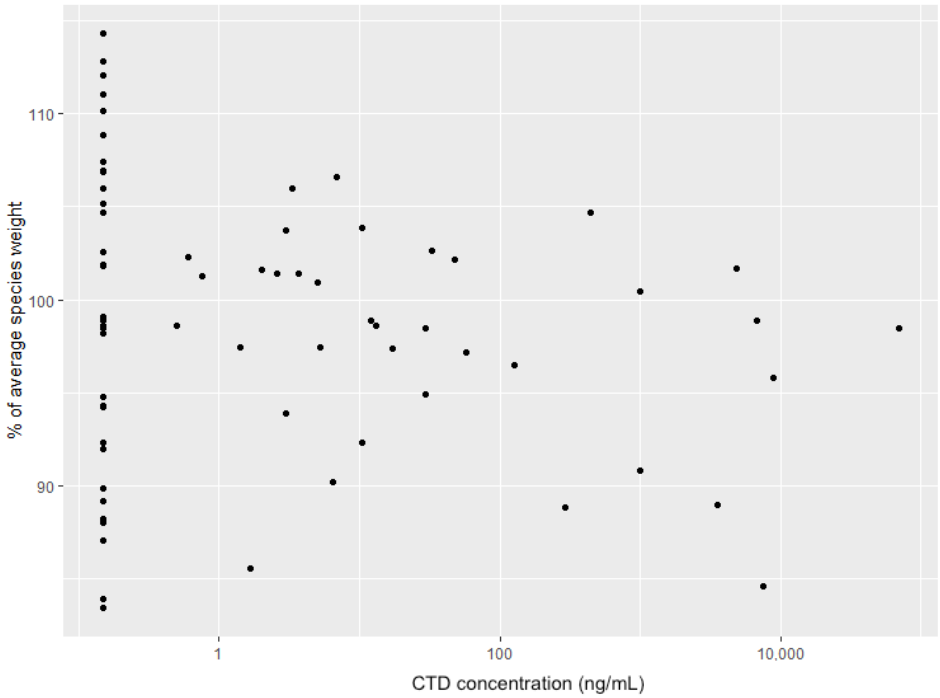

Figure 2.S3. Concentration of clothianidin (CTD) plotted against the bird weight (represented as the percentage change from average species weight) for all plasma samples collected from birds post-sowing. The LOD value (0.15 ng/mL) was entered for non-detect samples.

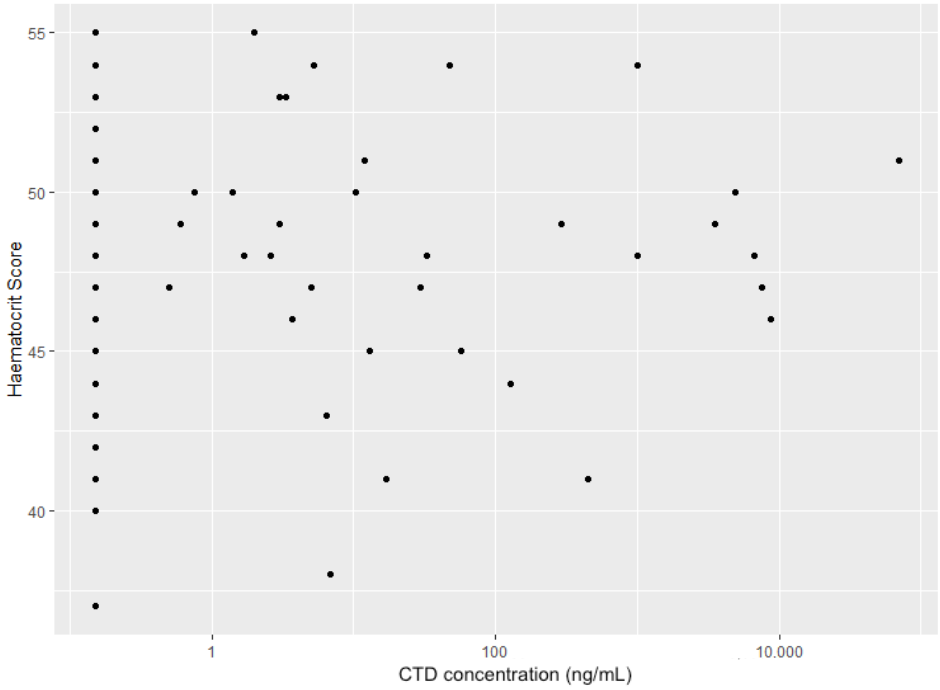

Figure 2.S4. Concentration of clothianidin (CTD) plotted against the haematocrit score for all plasma samples collected from birds post-sowing. The LOD value (0.15 ng/mL) was entered for non-detect samples.

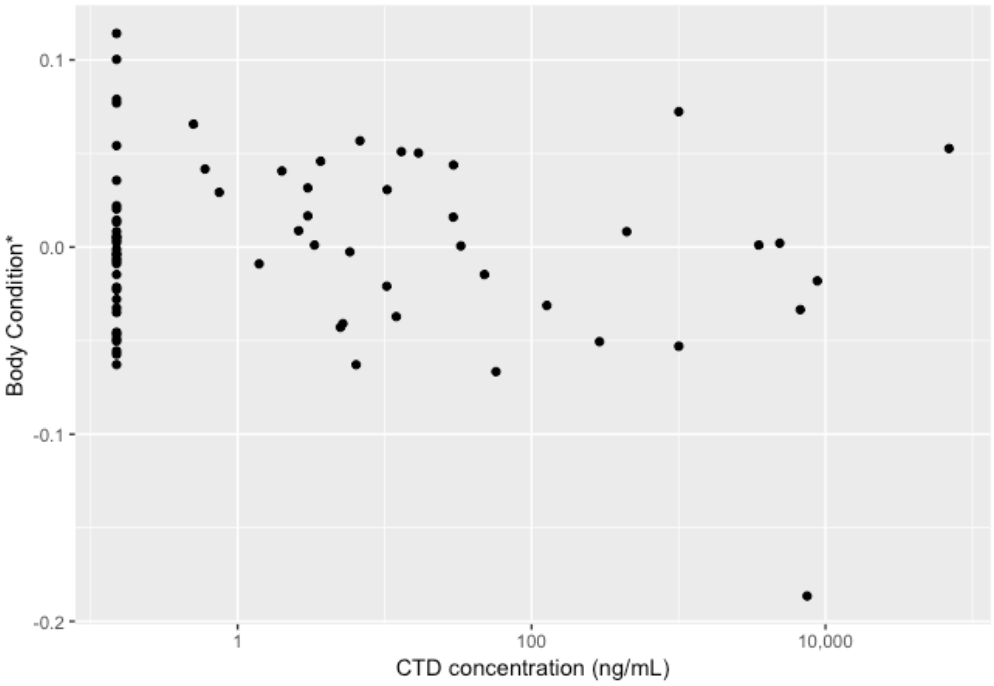

Figure 2.S5. Concentration of clothianidin (CTD) plotted against the body condition for all plasma samples collected from birds post-sowing. (*)Body condition was calculated from the residuals of log body weight modelled as a function of log wing length. Robin *Erithacus rubecula* and greenfinch *Chloris chloris* were excluded due to small species sample sizes. The LOD value (0.15 ng/mL) was entered for non-detect samples.

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

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Contribution	**Authors**						
	RJL	RFS	MGP	WJP	JCD	KEA	CDB
Conceptualization	●						
Data Curation	●						
Formal Analysis	●						
Funding Acquisition						●	●
Investigation	●						
Methodology	●	●	●	●	●		●
Resources			●		●	●	
Supervision		●	●	●	●	●	●
Visualization	●						
Original Draft Preparation	●						
Review & Editing	●	●	●	●	●		●

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, however the extent of neonicotinoid exposure in avian communities is poorly understood and very little is known about how neonicotinoid exposure may affect free-living birds.

In this study, gamebirds were used as a model species group to measure the extent of avian exposure to the neonicotinoid clothianidin via seed treatments during a typical sowing period of winter cereals. Specifically, blood and liver samples were collected simultaneously from individual birds, both pre- and post-sowing, to analyse patterns of exposure in the two sample types. Samples were analysed via LC/MS-MS and clothianidin residue data were compared to measures of bird weight, body condition, fat score and faecal parasite load to ascertain whether any of these health parameters were associated with neonicotinoid 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 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 this association was not replicated for plasma samples, nor were there any associations with fat, body condition or body weight for either sample type.

This study provides clear evidence that treated seed is a source of exposure for gamebirds following autumn sowing. Data here demonstrate that different sample types provide somewhat different measures of exposure in the field, and that a health parameter can be associated with pesticide residue in one type of sample, but not the other. These data imply that multiple species of gamebird worldwide are likely to be exposed to neonicotinoids where they are in use as seed treatments, and will aid design of any future avian biomonitoring studies for agrochemical compounds.

3.1 INTRODUCTION

Neonicotinoids (NNs) are insecticides with a specific neurotoxic mode of action via nicotinic acetylcholine receptors [1], and are the most widely used group of systemic insecticides on the global agricultural market [2]. Seed treatments are one of the most common forms of NN application [2], for which the three main compounds are: imidacloprid (IMI), clothianidin (CTD) and thiamethoxam (THX). Following the use of NNs for nearly two decades, concerns were raised regarding the safety of non-target invertebrates [3], and as a result these three compounds were banned from being applied outdoors within the European Union (EU) in 2018. Despite this, 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 [4]. 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 [3]. 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 [5], and the extent to which they frequent arable fields during the sowing season [5-8]. To date, exposure of wild galliformes to NNs has been confirmed in a handful of studies across three continents [9-12]. 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*) [9]. 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 IMI was detected in the gizzards and livers of grey partridge [9]. IMI residues have also been detected in the livers of Northern bobwhite quail *Colinus virginianus* [13] and scaled quail *Callipepla squamata* in the USA [12], wild turkeys *Meleagris gallopavo silvestris* in Canada [10], Cape spurfowl *Pternistis capensis* in South Africa [11], and in the crop and gizzard contents of red-legged partridge in Spain [8]. In addition, THX has been detected in the eggs of grey partridge in France [14]. 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 [15]. 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 [16]. 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 [17-19], whereas tissue samples are more commonly analysed for species of hunted columbid or galliforme [9-13]. Existing data suggest that the concentration of NN compounds in birds may differ depending on the type of sample that is being analysed and the time of sampling [20-22]. For example, NNs are thought to exit the blood stream 6-8 hrs post exposure [17, 20], whereas there is evidence to suggest NNs accumulate in the liver over multiple exposure events [21, 23]. 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 [24], with some NN compounds (e.g., IMI) being more toxic to birds than others [25]. For example, the no-observed-effect level in bobwhite quail is reported to be 120, 525 and 300 ppm for IMI, CTD and THX, respectively [26]. In particular, adverse changes to weight, fat stores and the immune system have been reported among species of galliforme and columbids dosed with IMI [23, 27]. With regards to the immune system, aviary studies have reported that IMI can negatively affect cell-mediated and humoral immunity [23, 28-31], both of which are important for regulating parasite burdens in birds (e.g., in the gut and blood). As yet however, the effect of NN exposure on avian parasite load has 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 IMI at a migratory stopover site [32]. Overall, there is a paucity of data to assess sub-lethal impacts of NNs 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 CTD via ingestion of treated cereal seed was measured *in situ* using both blood plasma and liver samples during the autumn sowing period.

Specifically, the objectives of the study were to: 1) assess the extent and level of exposure of gamebirds to CTD via treated cereal seed during the autumn sowing period; 2) measure the difference in the concentration of CTD recorded in liver and plasma samples collected simultaneously from individual birds to assess the sensitivity of either sample type for biomonitoring purposes; and 3) investigate whether there were any changes to physiological parameters (weight, fat or parasite load) associated with the concentration of CTD in liver and/or plasma.

3.2 METHODS

3.2.1 Study sites

Data collection took place at six farms in North Lincolnshire (UK) that were sown with Redigo-deter®-dressed wheat or barley (Bayer Crop Science Ltd., UK); these specific seed treatments only contained the compound CTD. Each site contained distinct populations of managed gamebirds and was separated from the others by an average of 16 km to ensure spatial independence.

3.2.2 Sample collection

Bird carcasses were collected between September and November in 2017. These months are within the official shooting season in the UK, which opens on 1st September and closes on the 1st February each year. Samples were collected from managed shoots (on scheduled shoot dates) once prior to the sowing of CTD-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 3.1**). Between one and eight galliforme carcasses were collected on each site visit depending on the relative success of the shoot (**Table 3.1**). 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 wood pigeon *Columba palumbus* were taken in lieu. A total of 42 bird carcasses were collected within 10 min of time of death. The remainder were collected at intervals up to a maximum of 3 hrs after time of death in order to ensure safe working within the constraints of an ongoing shoot. Carcasses were labelled, bagged and stored on ice for transportation. All carcasses were frozen at -20°C within 6 hrs of the time of death. Blood samples were obtained post-mortem via heart puncture (using an 18 G needle and 2.5 ml syringe), from individuals collected within 10 min of the time of death. 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 hrs of collection. Plasma was separated out from the sample and stored at -20°C until analysis. Whole livers were excised at necropsy and stored separately at -20°C until analysis.

Table 3.1. 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 are indicated by numbers in superscript.

Site code/ species	Number of birds collected		
	Visit 1 *Pre-sowing*	**Visit 2** *Post-sowing*	**Visit 3** *Post-sowing*
LN1*	6^{4}	0	10^{5}
LN2	6^{5}	8^{6}	6^{5}
LN3	0	4^{3}	5^{4}
LN4*	0	8^{3}	7^{3}
LN5	6	1^{1}	5^{1}
LN6	0	3^{2}	0
Red-legged partridge	17^{8}	16^{13}	27^{17}
Grey partridge	1^{1}	5^{2}	1^{1}
Pheasant	0	2	5
Woodpigeon	0	1	0
Total	18^{9}	24^{15}	33^{18}

*Sites where two shoots were attended in the visit 3 timeframe. On average, birds were collected three and 23 days post-sowing for visit 2 and visit 3, respectively.

3.2.3 Health parameter data collection

Sex, age, weight, and fat score (as per standard British Trust for Ornithology protocol [33]) 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 x 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). The total number of eggs (all species) was multiplied by the weight of the faecal sample to obtain a measure of parasite load per unit mass (g) for all individuals.

3.2.4 Residue analysis

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

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 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 mobile phase (95% phase A, 5% phase B) and transferred into LC vials.

For plasma, each sample (20-50 µL) was spiked with labelled internal standard (CTD 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 sec, after which the solution was evaporated using a Turbovap. The residue was reconstituted with 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).

Analysis

The analysis of liver samples was performed using a LC coupled to a triple quadrupole Quantum Ultra TSQ mass spectrometer (Thermo Fisher Scientific; Hemel Hemsptead, UK), interfaced with ion max electrospray ionisation (ESI) and operated with Xcalibur^{TM} (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 x 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 x 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 CTD. Argon was used as collision gas.

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 CTD and the deuterated internal standard; 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 CTD 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 CTD was calculated using the Nordtet TR537 handbook [34].

3.2.5 Statistical analysis

Concentrations of CTD in liver and plasma were not normally distributed (Shapiro-Wilk test - liver: $W = 0.36$, $p < 0.001$; plasma: $W = 0.39$, $p < 0.001$), so non-parametric analyses were used to assess general patterns of exposure, including Wilcoxon-signed rank or -rank sum test for paired and unpaired data points, Fishers exact test for count data, and Kruskal-Wallis rank sum test for grouped data. Data points included in analyses were restricted to those collected post-sowing (treatment group) unless stated otherwise. Respective LOD values (0.004 ng/g ww and 0.15 ng/mL) were entered as the concentration of CTD for liver and plasma for samples with non-detected residues. All analyses were conducted in R [35].

The residuals of from linear models, where log body weight was modelled as a function of log tarsus length, were used as a measure of body condition for each individual. Residuals were obtained for each species from separate linear models to account for significant differences between species biometrics (this was not necessary for age or sex). Wood pigeon was excluded from the analyses because only one sample was available. Generalised linear models (GLMs) were used to model fat score, body condition and body weight as a function of CTD concentration in liver and plasma samples using either a negative binomial distribution (fat score and body weight), or a Gaussian distribution (body condition). Only red-legged partridge data were included for models using bird weight because of inter-species variation for this parameter, whereas all species were included for fat score and body condition because these measures were uniform. CTD concentrations in plasma (only) were log-transformed in all GLMs to improve model fit. Model fit for all GLMs was assessed by testing for over-dispersion (using the 'overdisp' function, [36]) and by comparing modelled residuals to simulated residuals (using the 'simres' function in the 'Dharma' package [37]). Generalised linear mixed models (GLMMs) were used to assess the association between CTD 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 CTD concentration between sites. All GLMMs were assessed for model fit using the same protocol described for GLMs.

Concentrations of CTD 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). The density of blood plasma is approximately 1025 kg/m^3 [38], and so the difference in concentration values for plasma when expressed as ng/mL or ng/g is negligible.

3.3 RESULTS

3.3.1 Prevalence & levels of exposure

CTD 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 3.2**). CTD 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 CTD concentration in positive samples 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 CTD in liver and plasma were 37.0 ng/g ww and 3200.0 ng/mL, respectively. Residue concentrations in samples collected post-sowing differed significantly between sites for both liver (Kruskal-Wallis sum rank test: $\chi^2_5 = 16.2$, $p = 0.006$) and plasma samples

(Kruskal-Wallis sum rank test: χ^2_5 = 11.5, p = 0.042). The median concentration of CTD in the two sample types, collected from each site, ranged between 0.02 and 1.30 ng/g ww (liver), and 0.0 and 246.7 ng/mL (plasma), with sites LN2 and LN6 generating the largest concentrations for both sample types (**Table 3.S1**). Overall, partridges collectively had the highest frequency of detection, with 94% of individuals testing positive for CTD in plasma and/or liver; however, the concentration of CTD in liver samples was four-fold larger in pheasants compared to the other three species tested (**Table 3.2**). The concentration of CTD did not differ between male and female 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).

Table 3.2. Summary of clothianidin (CTD) detection in individuals collected pre- and post-sowing and the level of CTD recorded in liver and plasma samples obtained. The frequency of exposure is inclusive of both liver and plasma samples. The proportion of individuals that tested positive for CTD is calculated to the nearest 1%.

Species	**Latin**	**Number of individuals**		**CTD detection**	**Liver** (ng/g ww)			**Plasma** (ng/mL)		
		Sampled	*CTD 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.

3.3.2 Concentration of clothianidin in liver versus 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). CTD was not detected in either the liver or plasma samples of those birds collected pre-sowing, but 31 out of the 33 birds collected post-sowing tested positive for CTD: 13 (42%) birds tested positive for CTD 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, CTD 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 plasma (0.15 ng/mL) was applied on an equivalent basis (0.15 ng/g ww) to liver samples, CTD detection decreased from 31 to 20 birds out of the 33: two birds (10%) tested positive for CTD 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. Where CTD was detected in both the liver and the plasma, the concentration of CTD was found to be on average 98% greater in plasma samples compared to liver.

CTD was detected in significantly more plasma samples during visit 2, compared to visit 3 across all sites (Fishers exact test: OR = 0.06, p = 0.001; **Table 3.3**). Comparatively, the number of samples where CTD was detected in the liver remained similar between the two post-sowing visit groups (Fishers exact test: OR = 2.58, p = 0.261; **Table 3.3**). The concentration of CTD in samples that tested positive for the compound was significantly smaller during visit 3, compared to visit 2 for liver samples (Wilcoxon-rank sum test: W = 384.5, p = 0.042), but not plasma samples (Wilcoxon-rank sum test: W = 31, p = 0.921; **Table 3.3**; **Figure 3.S1**). These trends remained the same when only red-legged partridge data were included (**Table 3.S2**). The concentration of CTD in liver and plasma samples collected from all species (inclusive of all samples) was not found to decrease significantly with the number of days post-sowing (**Table 3.4; Figure 3.1**). This remained the same when only red-legged partridge data were used (**Table 3.S3B**).

Table 3.3. Summary of clothianidin (CTD) detection and concentrations in plasma and liver samples throughout the study for all species tested. 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 CTD 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**		**CTD detection (%)**		**CTD concentration***	
		Total	*CTD detected*	*Visit*	*Group*	*Median*	*IQR*
Plasma (ng/mL)[§] LOD: 0.15	1 (pre-sowing)	9	0	0	0	*na*	*na*
	2 (post-sowing)	15	13	87	58	56.0	357
	3 (post-sowing)	18	5	28		17.2	23.0
Liver (ng/g ww)[¶] LOD set to: 0.15	1 (pre-sowing)	18	0	0	0	*na*	*na*
	2 (post-sowing)	24	11	46	40	2.42	11.6
	3 (post-sowing)	33	11	33		0.53	0.8
Liver (ng/g ww)[¶] LOD: 0.004	1 (pre-sowing)	18	1	5	6	0.13	*na*
	2 (post-sowing)	24	19	79	85	0.51	2.7
	3 (post-sowing)	33	30	91		0.06	0.3

[§]Grey and red-legged partridge only (38 red-legged partridge, 4 grey partridge).

[¶]All species included (60 red-legged partridge, 7 grey partridge, 7 pheasant and 1 woodpigeon).

*Positive samples only.

IQR: inter-quartile range; LOD: level of detection; ww: wet weight.

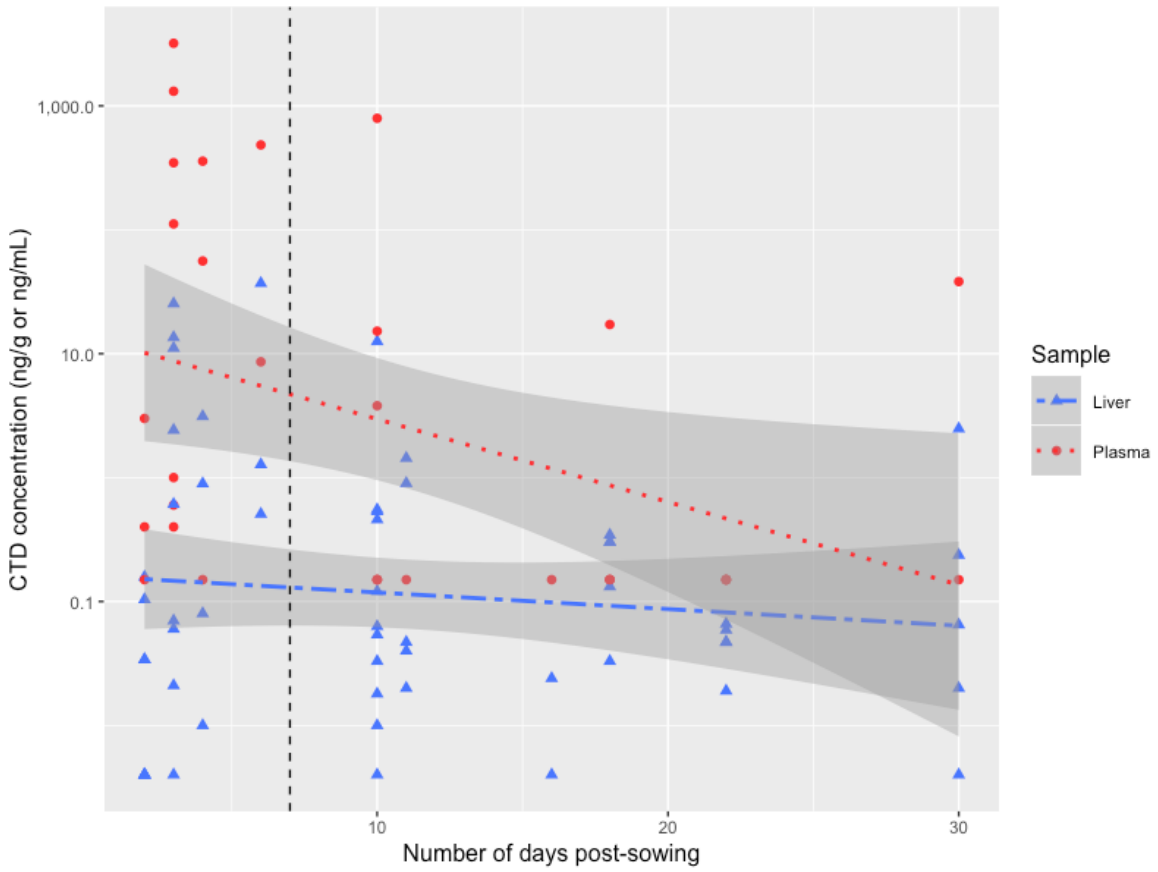

Figure 3.1. Concentration of clothianidin (CTD) in liver and plasma samples collected post-sowing. Non-detects are represented as the LOD for both sample types (liver: 0.004 ng/g wet weight; plasma: 0.15 ng/mL). Each point represents one sample type obtained from one bird per site visit, inclusive of all species. 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 either set of data points is represented by coloured lines, with 95% confidence intervals represented by the grey shading.

Table 3.4. Summary of generalised linear models and generalised linear mixed model outputs. Model outputs are inclusive of all species for which samples were available. Models were used to investigate CTD concentration in relation to the number of days post-sowing (grey shading) and health parameters (no shading), for plasma and liver samples.

Sample	Model + (random effects)	N obs	Disp	Estimate	SE	p-val
Liver	CTD conc ~ days post-sowing + (site)	75	1.86	0.016	0.021	0.439
	parasite load ~ CTD conc	42	1.63	0.037	0.019	**0.048**
	parasite load ~ CTD conc (outlier removed)	41	0.81	0.042	0.018	**0.020**
	weight ~ CTD conc[≠]	43	1.07	-0.001	0.002	0.731
	Body condition ~ CTD conc	54	0.02	-0.003	0.008	0.699
	fat ~ CTD conc	57	1.06	-0.013	0.013	0.314
Plasma	CTD conc ~ days post-sowing + (site)[¶]	42	1.57	-0.007	0.018	0.670
	parasite load ~ CTD conc[§]	26	0.80	0.090	0.069	0.192
	weight ~ CTD conc[≠]	30	1.10	0.005	0.006	0.468
	Body condition ~ CTD conc	31	0.007	0.001	0.004	0.788
	fat ~ CTD conc[§]	33	*(model did not converge)*			
All	parasite load ~ weight[≠]	43	1.25	0.003	0.002	0.270
	parasite load ~ fat	42	1.52	-0.174	0.110	0.113
	parasite load ~ fat (outlier removed)	41	0.63	-0.193	0.112	0.084

[≠]Red-legged partridges only.
[§]Grey and red-legged partridges only.
[¶]Simulated residuals were significantly different from modelled residuals.
CTD: 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.

3.3.3 Physiological parameters & exposure

There was no association between bird weight and the concentration of CTD in the liver or plasma (red-legged partridges only), nor fat or body condition and the concentration of CTD in the liver (all species; **Table 3.4; Figure 3.S2**). There was a significant positive association between faecal parasite load and the concentration of CTD in livers for all birds for which faecal samples were available (**Table 3.4**). This association remained when one outlier with very high faecal parasite load (1050 eggs/g) was removed (**Table 3.4**; **Figure 3.2**), but became weaker when the same analyses were performed using subsets of the data for partridge species, and red-legged partridge only (**Table 3.S3A & B**). There was no significant association between faecal parasite load and the concentration of CTD in plasma samples for partridge species, nor red-legged partridge alone (**Table 3.4** and **Table 3.S3B**; **Figure 3.2**). Parasite load was not significantly associated with bird weight (red-legged partridges only) or fat score (all species; **Table 3.4**). When the same outlier with high parasite load was removed, the association between faecal parasite load and weight remained unchanged, whereas the association between fat score and parasite load became slightly stronger (**Table 3.4**).

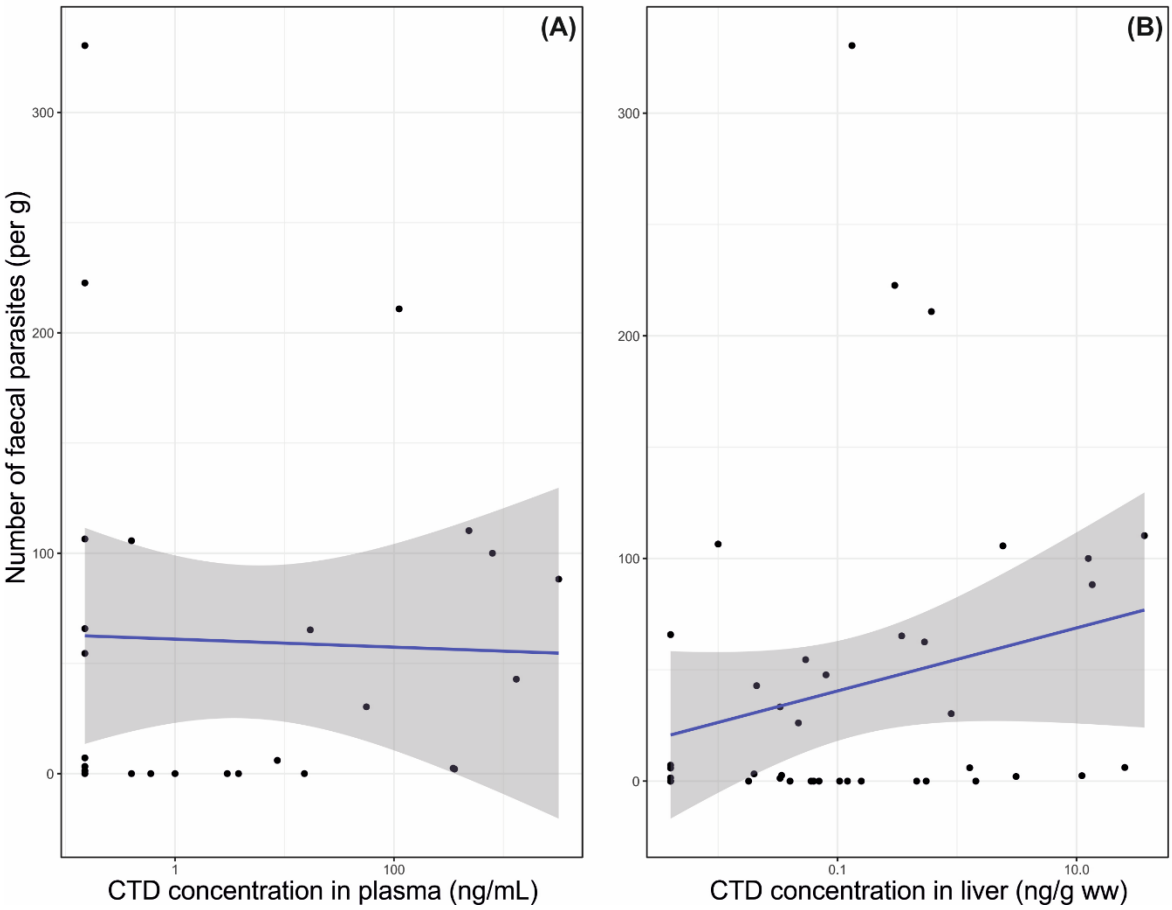

Figure 3.2. Faecal parasite load plotted against the concentration of clothianidin (CTD) in (A) plasma and (B) liver samples. One outlier was removed from panel B (1050 faecal parasites per 1 g faeces and 0.5 ng/g wet weight CTD detected in the liver). Linear best-fits for the two data sets are represented by solid lines, with 95% confidence intervals represented by the grey shading. Data for all species are presented excluding woodpigeon (no faecal samples available).

3.4 DISCUSSION

This study provides strong evidence that CTD-treated seed is a source of exposure for gamebirds following autumn sowing. The number of birds that tested positive for CTD post-sowing (89%) was high compared to the prevalence of CTD 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; IMI, CTD, THX) [13]; 17% of scaled quails (rolling plains eco-region, USA; IMI, CTD, THX) [12]; 22% of wild turkeys (Ontario, Canada; THX and CTD) [10]; and 8% of eggs collected from grey partridges (France, THX) [14]. 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 active pesticides, including NNs [7]. 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 CTD detection among individuals remains high at 77 and 70%, respectively. Notably, the prevalence of IMI 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 [9]. In the same study, IMI-related poisoning incidents were also reported more frequently during the autumn sowing season [9], which tallies with CTD prevalence observed here pre- and post-sowing.

When comparing the amount of CTD recorded in liver and plasma samples to existing data for wild birds across all NNs, 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 in liver samples collected from comparable galliforme species and excluding poisoning events ranged between 3.7 and 160 ng/g ww [10, 12]; whereas CTD concentrations recorded in this study ranged between 0.01 and 37.0 ng/g ww. Conversely, concentrations of NNs previously recorded in plasma collected from wild birds ranged between 0.0025 and 3.28 ng/mL [17, 19], which is far exceeded by the median concentration of CTD 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 [18, 19] and one migratory passerine [17]. Birds of prey are inherently more likely to experience secondary exposure (e.g., ingestion of contaminated prey items), rather than primary exposure 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, some of which inhabit non-arable habitats (e.g., those native to North America) [10, 12, 13], 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 drilled CTD-treated seed contributed to large concentrations of CTD 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 [39], which present relatively high densities of treated seed on the soil surface after drilling [8, 40], and are often adjacent to game cover strips (e.g., maize crops grown to provide shelter for released birds found at field margins). Furthermore, gamekeepers will often provision managed birds with untreated cereal seeds at the same time of year that spillages of NN-treated cereal seed occur [40].

Patterns of exposure and concentrations of CTD differed between plasma and liver samples obtained from individual birds over the course of the study. These patterns will reflect both the time course of exposure and the toxicokinetics of CTD in the birds, but no firm conclusions can be drawn because there is no available toxicokinetic information for CTD. The literature does contain information for IMI, and controlled avian experiments have shown that IMI concentrations in plasma are largest 1 hr after exposure [20], with large variation in concentrations observed up to 6 hrs after exposure [17]. Comparatively, concentrations of IMI in liver are reported to be 46-84% of that in plasma and appear to be more consistently dose-dependent across existing studies, with reported cases of bioaccumulation [20, 21]. In a field setting, where treated seeds are the only 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 CTD on remaining seeds decline. Here, the frequency of CTD detection in plasma decreased significantly between one week (visit 2) and two-to-four weeks (visit 3) post-sowing, and the concentration of CTD in liver samples significantly decreased between visit 2 and visit 3. However, the frequency of CTD detection in livers was similar between these two temporal groups and the concentration of CTD measured in plasma between these two time periods remained similar (although the range in concentrations markedly decreased among fewer individuals within the second visit; **Figure 3.S1**). It is tempting to conclude that plasma samples provide a short-term measure of exposure with more individual variation, whereas liver samples provide a more stable measure of exposure that is less dependent on the precise timing of sampling in relation to the exposure event; however, further data would be required to confirm this hypothesis in a field-based setting. Overall, data here suggest that liver samples provide a more sensitive detection method for CTD (CTD was detected in the liver of 88% of birds, compared to the plasma of 55% of birds when both samples were available), which should be taken into consideration in any future avian NN biomonitoring studies.

Faecal parasite load was the only sub-lethal parameter to be associated with CTD concentrations in samples collected, specifically the liver. 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 [41]. 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 [28, 31]. 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 3.S4**), and secretory immunoglobulin-A antibodies are thought to bind to the

coccidial surface to inhibit the protozoan parasite [42]. 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. Indeed, a weak negative association was detected here between parasite load and fat scores. Interestingly, the association between faecal parasite load and concentrations of CTD only existed for liver samples, and not for plasma samples. 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 an association is detected.

No association was detected for the remaining health parameters measured (fat, body condition or weight) in relation to CTD exposure, although fat and weight have been reported previously to be negatively affected in avian NN exposure studies. For example, a significant reduction in body weight was recorded in CTD-dosed South American eared doves *Zenaida auriculata* [27] and IMI-dosed red-legged partridges [23], whilst white-crowned sparrows dosed with IMI lost between 17 and 26% of body mass during a 3-day period, with fat scores following a similar trend [43]. It is possible that the field-based design and/or test species used here precluded any such associations. Firstly, captive birds in NN-dosing studies can exhibit reduced food consumption as a result of post-ingestion distress [44], which is something wild birds may not experience when consistent dosing is absent or natural food sources are available. Secondly, migratory passerine species (such as white-crowned sparrows) are likely to be 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.

3.4.1 Conclusion

Data presented here provide clear evidence that CTD-treated cereal seeds are a significant source of exposure for gamebirds during autumn sowing. CTD was detected in both the liver and plasma, although the difference in the amount of residue present in the two sample types suggests that they provide somewhat different measures of exposure in the field. It seems likely that, as noted for other contaminants, plasma samples provide a shorter-term labile measure of recent exposure, whereas liver samples provide a more stable measure of exposure. These data also demonstrated that a sub-lethal effect can be associated with one measure of CTD exposure, but not the other, which may be an important factor to consider in any future work relating to the sub-lethal effects of NNs on free-living birds. CTD residue in liver was found to be negatively associated with faecal parasite load, and further research is required on the interaction between NN compounds and avian

parasites and/or disease in a field-based setting. Field data collected here contribute to the growing body of evidence for NN exposure in galliformes worldwide, and are relevant to any future avian biomonitoring studies or risk assessments for insecticide seed treatments.

Ethics Statement

All works were approved by Animal Welfare Ethical Review Bodies panel at the University of York. Protocols were also approved by an independent panel at the Game and Wildlife Conservation Trust.

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3.5 SUPPLEMENTARY MATERIAL

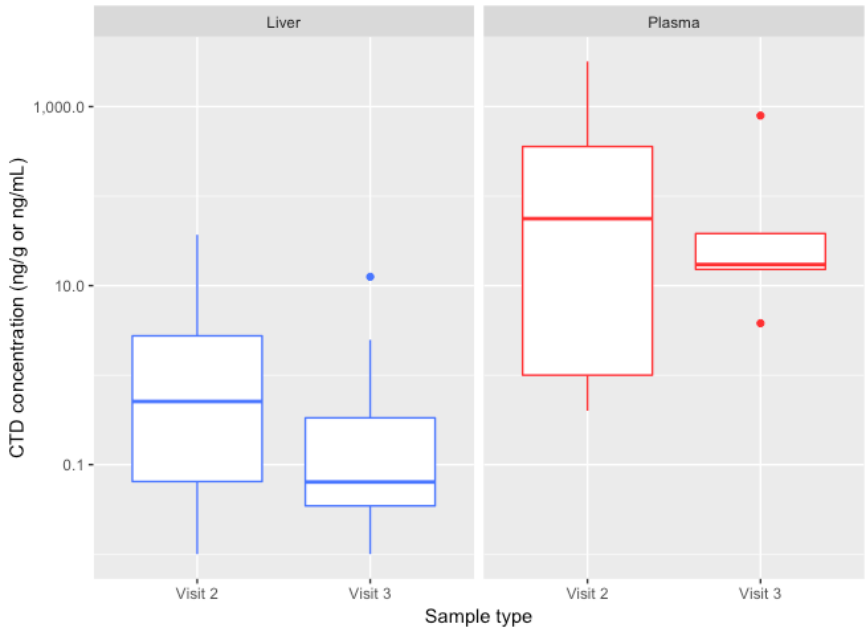

Figure 3.S1. Concentration of clothianidin (CTD) in liver and plasma samples collected from visit 2 (1-7 days post-sowing) and visit 3 (8-30 days post-sowing). Only samples that tested positive for CTD are included. Boxes represent the interquartile range for each group of samples, and the median is represented by the horizontal line within each box. Whiskers of each box denote the minimum and maximum concentrations recorded, except for outliers which are represented by individual points. Concentrations of CTD in plasma are in ng/mL, whereas the concentration of CTD in livers are in ng/g wet weight.

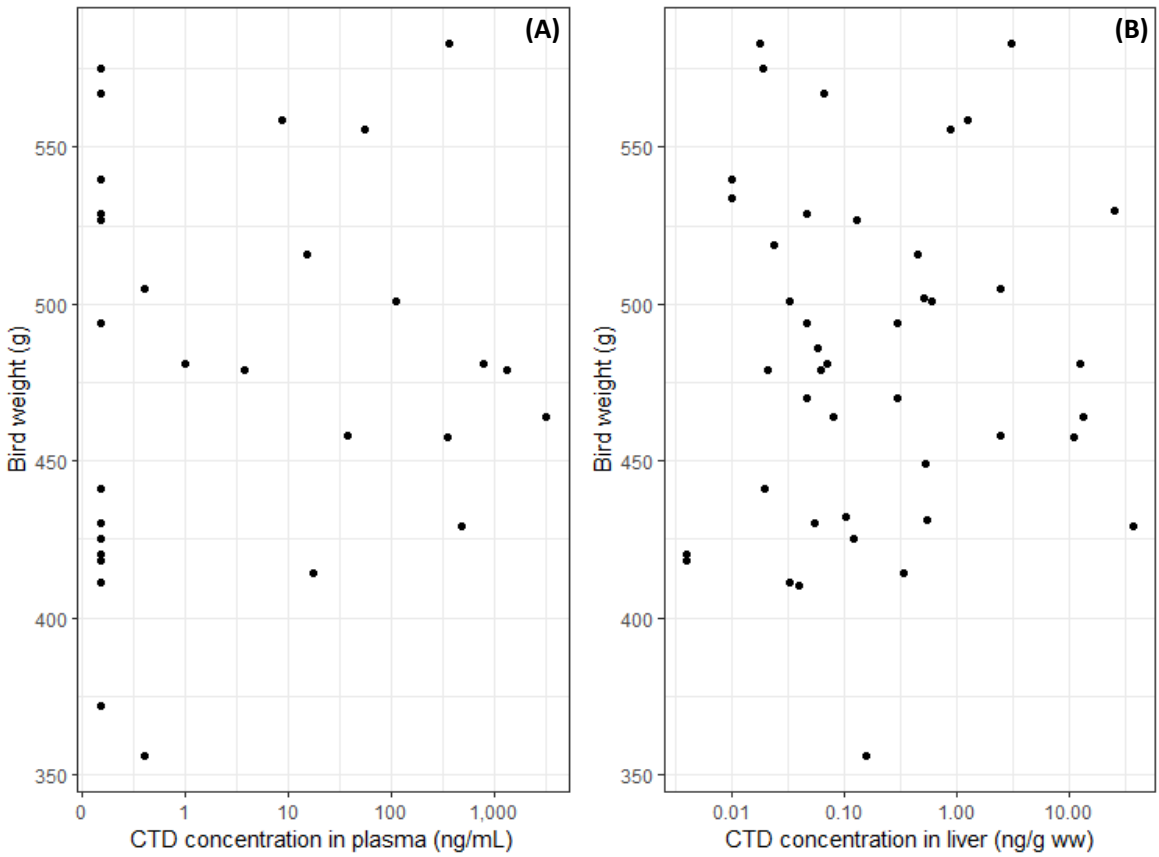

Figure 3.S2. Total bird weight plotted against the concentration of clothianidin (CTD) in (A) plasma and (B) liver samples. Data presented for red-legged partridges only.

Table 3.S1. Median concentrations of clothianidin (CTD) in liver and plasma samples collected at each site post-sowing. Inclusive of all species.

Site code	CTD in liver (median)			CTD in plasma (median)		
	N	*ng/g ww*	*IQR*	*N*	*ng/mL*	*IQR*
LN1	10	0.05	0.04	5	ND	*na*
LN2	14	0.50	0.87	11	15.2	570
LN3	9	0.30	0.26	7	0.15	36.4
LN4	15	0.02	0.08	6	0.27	2.2
LN5	6	0.04	0.66	2	0.37	0.2
LN6	3	1.30	18.3	2	247	238

N: number of samples collected at each site; IQR: inter-quartile range; ND: non detect.

Table 3.S2. Summary of clothianidin (CTD) 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 CTD 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**		**CTD detection (%)**		**CTD concentration***	
		Total	*CTD detected*	*Visit*	*Group*	*Median*	*IQR*
Plasma (ng/mL) LOD: 0.15	1 (pre-sowing)	8	0	0	0	*na*	*na*
	2 (post-sowing)	13	11	85	57	112	416
	3 (post-sowing)	17	5	29		17.2	23.0
Liver (ng/g ww) LOD set to: 0.15	1 (pre-sowing)	17	0	0	0	*na*	*na*
	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) LOD: 0.004	1 (pre-sowing)	17	1	6	6	0.13	*na*
	2 (post-sowing)	16	16	100	97	0.75	5.0
	3 (post-sowing)	27	25	93		0.06	0.3

*Positive samples only.

IQR: inter-quartile range; LOD: level of detection; ww: wet weight.

The frequency of CTD detection differed significantly between visit 2 and visit 3 for plasma samples (Fishers exact test: OR = 0.08, p = 0.004). There was no significant difference in the detection of CTD between these temporal groups for liver samples when an LOD of 0.004 ng/g ww was applied (Fishers exact test: OR = 0.00, p = 0.522), but there was a significant difference when an LOD of 0.15 ng/g ww (equivalent to plasma) was applied (Fishers exact test: OR = 0.20, p = 0.025). The concentration of CTD detected in liver samples decreased significantly between visit 2 and visit 3 when an LOD of 0.004 ng/g ww and 0.15 ng/g ww was applied (Wilcoxon-rank sum test: LOD 0.004 ng/g - W = 302.5, p = 0.006; LOD 0.15 ng/g - W = 66, p = 0.075), but this trend was not observed for plasma samples (Wilcoxon-rank sum test: W = 31, p = 0.734).

Table 3.S3A. Summary of generalised linear models and generalised linear mixed model outputs for species of partridge only. Models were used to investigate CTD concentration in relation to the number of days post-sowing (grey shading) and health parameters (no shading), for plasma and liver samples.

Sample	Model + (random effects)	N obs	Disp	Estimate	SE	p-val
Liver	CTD conc ~ days post-sowing + (site)	67	1.70	0.023	0.023	0.304
	parasite load ~ CTD conc	40	1.55	0.037	0.019	**0.054**
	parasite load ~ CTD conc (outlier removed)	39	0.77	0.042	0.018	**0.024**
	weight ~ CTD conc[≠]	43	1.07	-0.001	0.002	0.731
	body condition ~ CTD conc	*(model severely under dispersed)*				
	fat ~ CTD conc	*(model did not converge)*				
Plasma	CTD conc ~ days post-sowing + (site)	42	1.57	-0.007	0.018	0.670
	parasite load ~ CTD conc	26	0.80	0.090	0.069	0.192
	weight ~ CTD conc[≠]	30	1.10	0.005	0.006	0.468
	body condition ~ CTD conc	*(model severely under dispersed)*				
	fat ~ CTD conc	*(model did not converge)*				

[≠]Red-legged partridges only.
CTD: 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.

Table 3.S3B. Summary of generalised linear models and generalised linear mixed model outputs for red-legged partridge only. Models were used to investigate CTD concentration in relation to the number of days post-sowing (grey shading) and health parameters (no shading), for plasma and liver samples.

Sample	Model + (random effects)	N obs	Disp	Estimate	SE	p-val
Liver	CTD conc ~ days post-sowing + (site)	60	1.55	0.018	0.025	0.467
	parasite load ~ CTD conc	34	1.58	0.029	0.020	0.142
	parasite load ~ CTD conc (outlier removed)	33	0.77	0.034	0.019	**0.077**
	weight ~ CTD conc	43	1.07	-0.001	0.002	0.731
	body condition ~ CTD conc	*(model severely under dispersed)*				
	fat ~ CTD conc	*(model did not converge)*				
Plasma	CTD conc ~ days post-sowing + (site)	38	1.43	-0.007	0.021	0.725
	parasite load ~ CTD conc	24	0.79	0.068	0.066	0.301
	weight ~ CTD conc	30	1.10	0.005	0.006	0.468
	body condition ~ CTD conc	*(model severely under dispersed)*				
	fat ~ CTD conc	*(model did not converge)*				

CTD: 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.

Table 3.S4. Summary of faecal parasite data for all birds for which samples were available.

ID	Species	Sample group*	Number of faecal parasites				Faecal weight (g)	Parasite load per 1g faeces
			Coccidia	*Capillaria*	*Other*	*Total*		
S2V11	RL	Pre	2	0	0	2	0.05	40
S2V12	RL	Pre	9	0	0	9	0.08	113
S2V13	RL	Pre	0	0	0	0	0.36	0
S2V14	RL	Pre	2	0	0	2	0.21	10
S2V15	RL	Pre	76	13	3	92	0.06	1533
S2V16	RL	Pre	2	0	0	2	0.44	5
S1V12	GP	Post	0	0	0	0	1.41	0
S1V14	GP	Post	0	0	0	0	1.20	0
S1V15	GP	Post	4	0	0	4	1.55	3
S2V22	GP	Post	0	0	0	0	1.46	0
S4V28	GP	Post	0	0	0	0	0.30	0
S1V24	PH	Post	0	0	1	1	0.17	6
S1V25	PH	Post	1	0	0	1	0.68	1
S2V25	PH	Post	0	0	0	0	0.35	0
S1V11	RL	Post	0	0	0	0	1.05	0
S1V13	RL	Post	0	0	0	0	1.42	0
S1V21	RL	Post	2	0	0	2	0.28	7
S2V21	RL	Post	3	1	0	4	1.23	3
S2V23	RL	Post	0	0	0	0	1.86	0
S2V24	RL	Post	44	2	0	46	1.76	26
S3V21	RL	Post	0	0	0	0	1.25	0
S3V22	RL	Post	0	0	0	0	1.53	0
S3V23	RL	Post	0	0	0	0	0.44	0
S3V25	RL	Post	9	0	0	9	0.27	33
S4V21	RL	Post	53	1	2	56	0.53	106
S4V22	RL	Post	1	0	0	1	0.41	2
S4V23	RL	Post	0	0	0	0	0.31	0
S4V24	RL	Post	97	0	0	97	0.46	211
S4V25	RL	Post	30	0	0	30	0.34	88
S4V26	RL	Post	12	0	0	12	0.28	43
S4V27	RL	Post	3	0	0	3	0.49	6
S4V31	RL	Post	54	0	0	54	0.54	100
S4V32	RL	Post	15	0	0	15	0.24	63
S4V33	RL	Post	0	0	0	0	0.26	0
S4V34	RL	Post	24	0	0	24	0.44	55
S4V35	RL	Post	0	0	0	0	0.44	0
S4V36	RL	Post	25	0	0	25	0.38	66
S5V12	RL	Post	10	0	0	10	0.33	30
S5V13	RL	Post	33	0	0	33	0.31	106
S5V14	RL	Post	21	0	0	21	0.44	48
S5V15	RL	Post	1	0	0	1	0.48	2
S5V21	RL	Post	30	0	0	30	0.46	65
S5V22	RL	Post	1	0	0	1	0.78	1
S5V23	RL	Post	185	0	0	185	0.56	330
S5V24	RL	Post	118	0	0	118	0.53	223
S6V11	RL	Post	30	2	11	43	0.39	110
S6V12	RL	Post	3	0	0	3	0.50	6
S6V13	RL	Post	294	0	0	294	0.28	1050

*Collected 'pre'- or 'post'-sowing.

GP: grey partridge *Perdix perdix*; PH: Pheasant *Phasianus colchicus*; RL: red-legged partridge *Alectoris rufa*.

Using long-term datasets to assess the impacts of dietary exposure to neonicotinoids on farmland bird populations in England

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Short title: Impacts of neonicotinoids on farmland birds

Contribution	**Authors**								
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Conceptualization	●								
Data Curation	●							●	
Formal Analysis	●	●							●
Funding Acquisition							●		●
Investigation	●								
Methodology	●	●	●	●					●
Resources									
Supervision			●	●	●	●	●		●
Visualization	●								
Original Draft Preparation	●	●							
Review & Editing	●	●	●	●	●	●		●	●

ABSTRACT

Over the last 20 years, a new group of systemic insecticides – the neonicotinoids - has gained prominence in arable systems, and their application globally has risen year on year. Previous modelling studies using long-term data have suggested that neonicotinoid application has had a detrimental impact on bird populations, but these studies were either limited to a single species or neglected to analyse specific exposure pathways in conjunction with observed population trends.

Using bird abundance data, neonicotinoid usage records and cropping data for England at a 5x5 km resolution, generalised linear mixed models were used to test for spatio-temporal associations between neonicotinoid use and changes in the populations of 22 farmland bird species between 1994 and 2014, and to determine whether any associations were explained by dietary preferences. We assigned farmland bird species to three categories of dietary exposure to neonicotinoids based on literature data for species diets and neonicotinoid residues present in dietary items.

Significant estimates of neonicotinoid-related population change were obtained for 13 of the 22 species (9 positive effects, 4 negative effects). Model estimates for individual species were not collectively explained by dietary risk categories, so dietary exposure to neonicotinoids via ingestion of treated seeds and seedlings could not be confirmed as a causal factor in farmland bird declines. Although it is not possible to infer any generic effect of dietary exposure to neonicotinoids on farmland bird populations, our analysis identifies three species with significant negative estimates that may warrant further research (house sparrow *Passer domesticus*, skylark *Alauda arvensis* and red-legged partridge *Alectoris rufa*).

We conclude that there was either no consistent effect of dietary exposure to neonicotinoids on farmland bird populations in England, or that any over-arching effect was not detectable using our study design. The potential for indirect effects of insecticide use on bird populations via reduced food availability was not considered here and should be a focus for future research.

4.1 INTRODUCTION

Agricultural intensification is thought to be the largest threat to global avifauna [1]. Significant declines in farmland birds have been well documented over the past 30 years and have been attributed to many aspects of agricultural intensification, including habitat loss, seasonal shifts in cultivation practices and the increased use of agro-chemicals [2, 3]. A recent review of farmland bird declines in North America found that pesticide use was the most commonly reported driver of population declines in farmland birds (42% of all studies, 93% of which reported negative impacts), followed by habitat loss and alterations [2]. Similarly, insecticide application was found to be one of the higher ranking variables to explain farmland bird declines during agricultural intensification in the UK between 1962 and 1995 [3] and has been cited in multiple reports as one of the key agricultural practices that has contributed to avian population change [4-6].

Over the last 20 years, the neonicotinoid (NN) group of systemic insecticides has gained prominence in arable systems, and their application globally has risen year on year [7]. Over 90% of NN applications in the UK (based on area treated) have been in the form of coated seed [8] with imidacloprid (IMI), clothianidin (CTD) and thiamethoxam (THX) the three most commonly used compounds [9]. In the UK there has been a significant shift in the main compound of use during the period of NN application. Prior to 2008, IMI was the main compound applied as seed treatment, but from 2008 onwards CTD took precedence. NN compounds also differ in their toxicity to birds [10]; in bobwhite quail *Colinus virginianus* IMI is over 13-times more toxic than CTD [11]. As a result, the hazard posed by both acute and chronic toxicity to birds in the UK (theoretically) peaked in the mid-2000s (**Figure 4.1A** and **Figure 4.1B**, respectively), rather than mirroring the net weight of NN applied (**4.S1 Figure**). Patterns of NN usage corrected for either acute or chronic toxicity are identical through to mid-2000s, but there is a slower decline from that peak when correcting for chronic toxicity (**Figure 4.1B**) because the difference in toxicity between IMI and the other NNs is smaller for chronic exposure than for acute exposure.

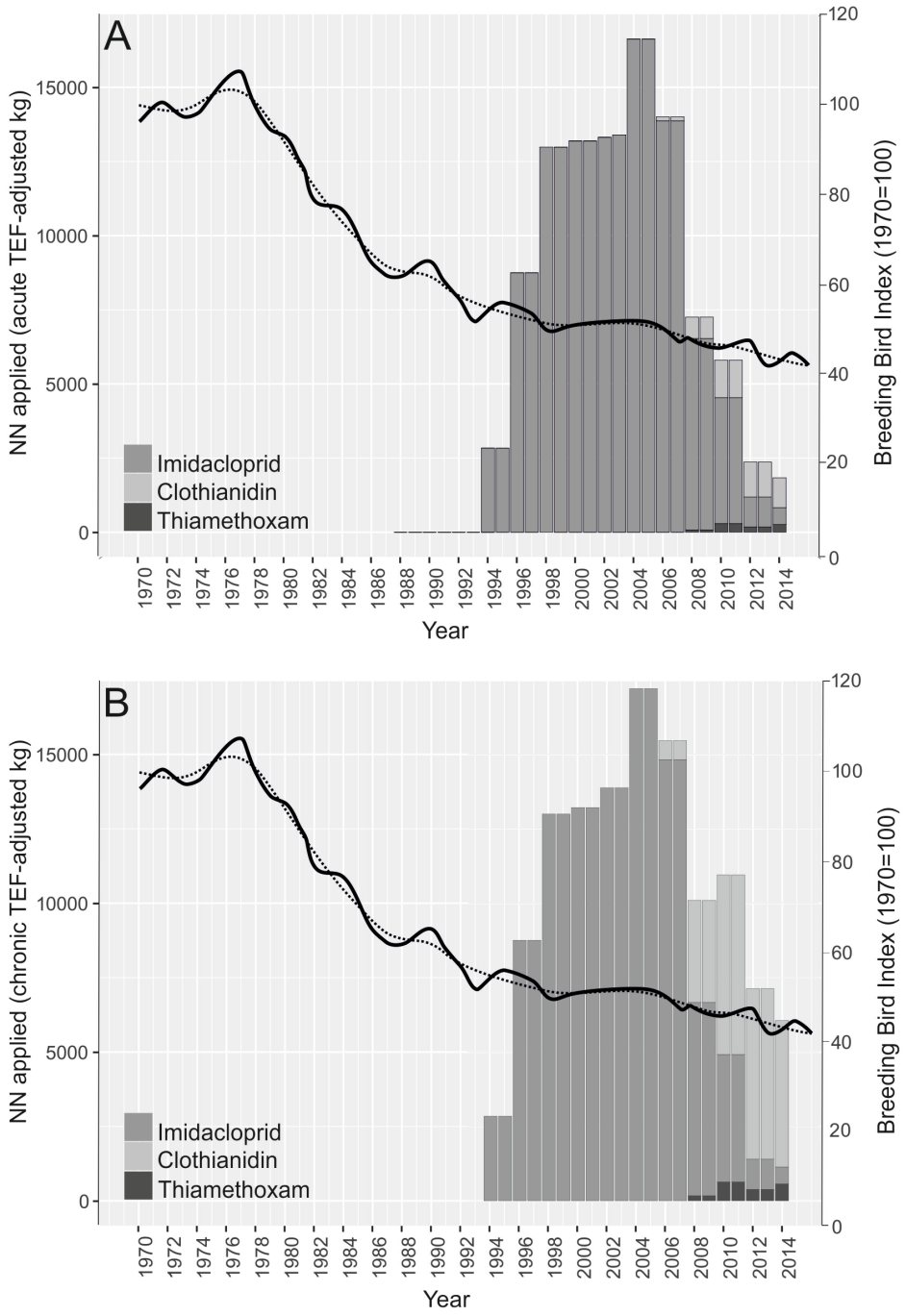

Figure 4.1. **Change in NN application and change in farmland bird abundance for the UK between 1970 and 2014.** Bars: Pesticide Usage Survey data for annual weight (kg) of NN applied, moderated by a toxicity equivalency factor (TEF) to account for differences in the acute (**A**) or chronic (**B**) toxicity of each NN compound to birds (see Methods for details) [9]. Lines: breeding bird index for farmland birds based on 19 farmland indicator species (solid: unsmoothed trend; dotted: smoothed trend), reproduced from the Defra report 'Wild bird populations in the UK, 1970 to 2014: Annual statistical release' (Figure 2) [12]. NN: neonicotinoid.

UK farmland bird populations declined substantially between 1970 and 2013. Of the 19 farmland indicator species (those deemed dependent on farmland habitat), 12 experienced population declines of between 23 and 97% [13]. The steepest declines took place between the mid-1970s and the early-1990s (**Figure 4.1**) when the amount of farmland hedgerow had decreased significantly, a widespread switch to autumn sowing occurred, and the number of commercial pesticides in use (including DDT up until it was banned in 1986) rose from 137 to 344 as a result of agricultural intensification [14]. NNs were first used as agricultural plant protection products in Britain in 1994 [15] at a time when farmland bird declines appeared to slow. Nevertheless, there are growing concerns within the scientific community regarding the availability of NNs to birds and the potential for effects of NNs on avian physiology and behaviour [11, 16-21].

According to manufacturers' instructions, NN-treated seeds should be efficiently incorporated at drilling to minimise exposure to non-target species [22]. However, recent research in Spain found a mean (± SE) of 43.4 ± 5.5 seeds per m^2 on field headlands within the first two weeks following NN applications [16]; this suggests that the risk posed from availability and subsequent ingestion of seeds by birds may have been underestimated. Furthermore, NN residue has also been detected in crop seedlings, which are thought to take up approximately 1-15% of compound applied to seed coatings [23, 24], and wild plants at field boundaries [25]. Crop seedlings and vegetation at agricultural margins provide food for a number of farmland bird species, suggesting another potential pathway of exposure to NNs.

Thus far, only a handful of studies have investigated pathways of exposure to NNs for farmland birds, and the primary focus for granivorous birds has been on ingestion of NN-treated seeds. Prosser (2001) recorded a total of 18 species foraging on seed types that are regularly treated with NNs as part of agricultural practice [26] and Lopez-Antia *et al.* (2016) observed 30 species consuming NN-treated seeds in recently drilled fields [16]. Furthermore, NN residues have been detected in two wild passerine species [20, 27], and in the eggs, crops and livers of wild partridges [28, 29]. A detailed review conducted by the American Bird Conservancy calculated that as few as 3.9 and 1.3 imidacloprid-coated wheat seeds could produce lethal and sub-lethal (reproductive) effects, respectively, if ingested by a 15-g bird [11]. There is also potential for direct ingestion of NN-contaminated insects as many granivorous bird species will switch to an insectivorous diet during the breeding season; however, the relatively small concentrations of NNs on insects [30] means that ingestion of NN-treated seeds and seedlings is likely to be a much more significant source of exposure. Various aviary experiments have found that birds dosed with environmentally-

relevant concentrations of NNs can suffer changes to the immune system, oxidative stress, impaired navigational ability and the accumulation of NN residues in the liver [18, 21, 31]. Thus not only is it possible for birds to be exposed to NNs, but the likely levels of exposure may be sufficient to produce sub-lethal effects and these may in turn affect survivorship, reproduction and consequently, populations.

Even though the literature identifies the potential for effects of NNs on farmland birds, there is a sparsity of evidence on whether bird populations have actually been impacted. In 2014, a Dutch study investigated the spatial correlation between surface water concentrations of NNs and insectivorous bird population trends, and reported that in areas where IMI concentrations in water were >20 ng/L, bird populations experienced average annual declines of 3.5% across 15 insectivorous species [32]. The study postulated that the observed trends were a result of depleted insect food resources, occurring as a result of NN-usage. However, despite the thorough statistical approach used for these analyses, the causative link between surface water concentrations and population level impacts remained hypothetical. A separate study evaluated effects of historic NN use on abundance of bobwhite quail in Texas by developing models structured by time period (pre- or post-NN use) and eco-region, including potential confounding variables such as temperature, land use and precipitation (32). NN use was found to be the variable that most commonly exhibited a negative association with quail abundance (62% of all post-NN use models), although a causative pathway by which NN use may have impacted quail populations was not defined. As yet, there are no long-term studies that investigate explicitly whether dietary exposure to NNs has been associated with population-scale effects on birds.

In the present study, we hypothesise that dietary exposure to NNs via ingestion of treated seed and/or crop material is associated with population declines of granivorous farmland birds. To gain adequate power to test this hypothesis, we construct a model with 21 years of pesticide usage and bird abundance data for England expressed at a 5x5 km resolution. This model is used to test: 1) whether spatio-temporal variation in NN use over a 21-year period is correlated with changes in the abundance of 22 individual farmland bird species; and 2) whether any correlations that exist are associated with potential dietary exposure to NNs based on known dietary preferences of the individual bird species. This is the first analysis of its kind to focus on farmland bird populations with regards to the long-term application of a specific pesticide group and a specific dietary route of exposure.

4.2 METHODS

Three datasets comprising bird abundance, NN usage and cropping data (each resolved to a 5x5km resolution) were used to build the model to test our hypotheses. These data were obtained from the British Trust for Ornithology (BTO) Breeding Bird Survey (BBS) [33], the pesticide usage surveys (PUS) [9] and the EDiNA agcensus (AgC) dataset [34], respectively. An overview of the data manipulation process used in producing the data frame for analysis is given in **Figure 4.2**.

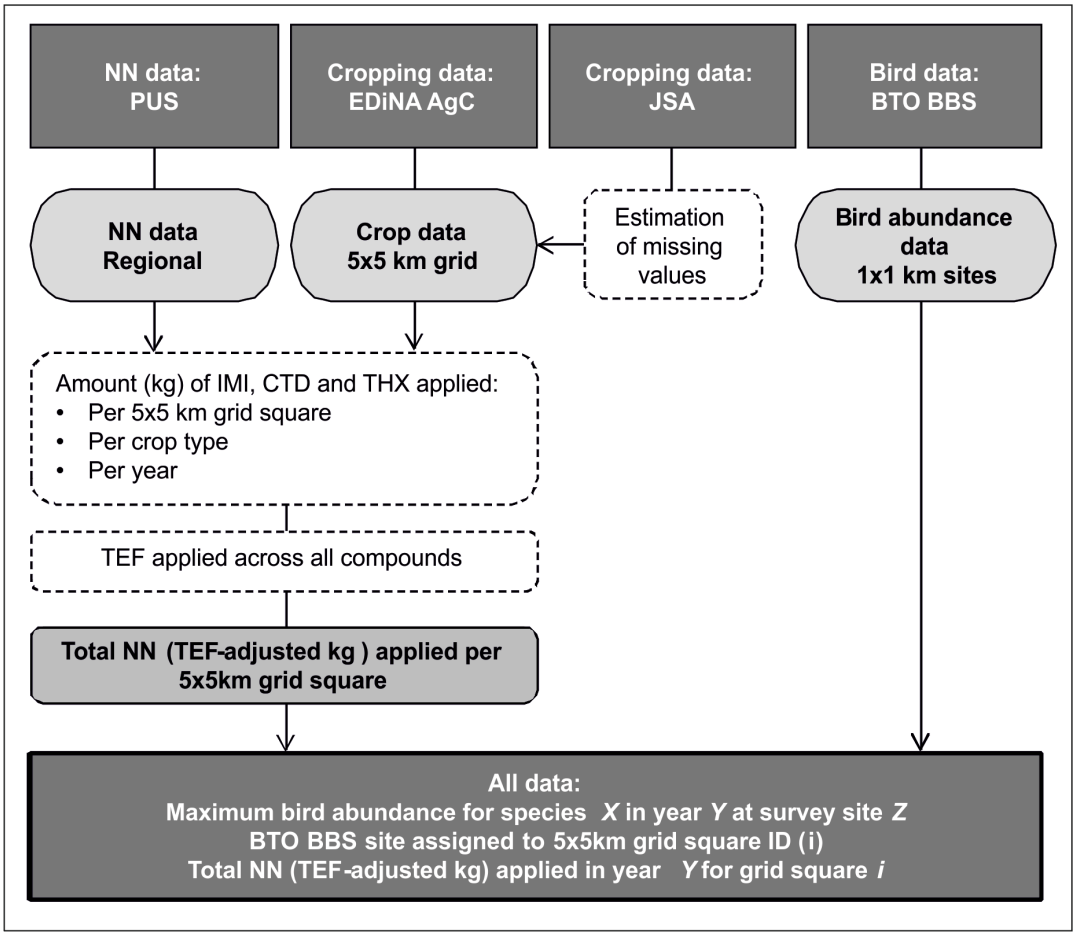

Figure 4.2. **Overview of the manipulation process used to combine independent data sources to build the final model data frame.** AgC: EDiNA agcensus; BBS: breeding bird survey; BTO: British Trust for Ornithology; CTD: clothianidin; IMI: imidacloprid; JSA: June Survey of Agriculture; NN: neonicotinoid; PUS: Pesticide Usage Survey; TEF: toxicity equivalency factor (used to adjust for the differences in toxicity of each compound to birds); THX: thiamethoxam.

4.2.1 Calculating spatial NN application rates for England: 1994-2014

Pesticide usage data were only available at a regional level (approximately 20,000 km^2). Here annual NN application at a 5x5 km scale was interpolated using spatial cropping data [35].

Cropping data

Cropping data were obtained for England from the EDiNA AgC resource at a 5x5 km scale. Data were obtained for all available years from 1994 to 2014, and for all crops identified by the PUS as receiving NN applications as a seed coating. Sufficient data were available for all major arable crop types except rye (*Secale sp.*; **Table 4.1**). A total of 9221 AgC 5x5km grid squares were available for England. Each grid square was assigned a 'NUTS' region based on level 1 of the Nomenclature of Territorial Units for Statistics (NUTS; 9 regions for England), and a 'Defra' region (5 regions for England) to match with the two types of region categories used in the PUS dataset (1994-2002: Defra regions; 2004-2014: NUTS regions; **4.S2 Figure**).

Table 4.1. Availability of EDINA agcensus data for each crop type in England.

Crop	Genus	Missing Years	Interpolation method for missing years*
Sugar beet	*Beta*	1998;1999;2001;2002;2006-2009; 2011-2014	Linear
Oilseed rape	*Brassica*	1998;1999;2001;2002;2006-2009; 2011-2014	Linear, Regional JSA
Wheat	*Triticum*	1998;1999;2001;2002;2006-2009; 2011-2014	Regional JSA
Winter Barley	*Hordeum*	1998;1999;2001;2002;2006-2009; 2011-2014	Regional JSA
Linseed	*Linum*	1998;1999;2001;2002;2006-2009;2011-2014	Regional JSA, National JSA
Oats	*Avena*	1998;1999;2001;2002;2006-2009; 2011-2014	Regional JSA
Rye	*Secale*	1998-2014	None: excluded from analysis

*No interpolation for 1998 due to non-availability of JSA and agcensus data across all crop types.
JSA: June Survey of Agriculture (Defra).

As there was a significant number of consecutive missing years for cropping data, regional data obtained from the June Survey of Agriculture (JSA) were used to estimate the areas of individual crops within each grid square for all missing years (national JSA data were also used for linseed [*Linum sp*.] where regional data were not available). Where JSA data were not available for a missing year, linear interpolations were used to estimate cropping areas per grid square (**Table 4.1**). Details of interpolation methods can be found in **4.S1 Supplementary Note**. Cropping data were not available from either AgC (at a 5 x 5 km resolution) or JSA (at a regional resolution) for any crop type in 1998; this year was therefore excluded from the analysis.

NN data

Regional NN usage data were obtained from the PUS provided by FERA Science Ltd [9]. These data provided the weight (kg) of NN applied as seed treatments by crop type, year, and region, with the

survey year denoting the year of harvest (i.e., autumn sowings of winter crops in year *n*-1 and spring sowings of spring crops in year *n* would both be counted in the survey for year *n*). Data were available for all arable crops in England at a two-year resolution from 1994 to 2014. For odd years (those with no data) pesticide usage values for each region and each crop type were calculated by taking the mean of values for the preceding and following years. The sensitivity of the model to this approach was tested using an alternative assumption that NN use in a year without data was the same as in the preceding year when data were collected.

NN application rate per grid square

Total compound application per 5x5 km grid square was calculated using **Equations 1-3**:

$$\left(\frac{x}{y}\right) \times 100 = Z \qquad \textbf{(Eqn. 1)}$$

$$\left(\frac{A}{100}\right) \times Z = \mathrm{B} \qquad \textbf{(Eqn. 2)}$$

$$\sum \mathrm{B}\ (\text{all crop types}) = \mathrm{C} \qquad \textbf{(Eqn. 3)}$$

where *x* = total crop area in grid square (ha), *y* = total crop area in region (ha), *Z* = percentage of total crop in region that the grid square contains, *A* = total amount of compound applied in region per crop (kg), *B* = total compound application per crop type (kg per grid square), and *C* = total NN application per grid square (kg).

A toxicity equivalency factor (TEF) was applied to account for differences between compounds in either their acute or chronic toxicity to birds. The acute TEF was based on the oral acute toxicity (LD_{50}) for bobwhite quail for each compound (152, 2000 and 2716 ng/kg body weight for IMI, CTD, and THX, respectively [11]). The TEF for IMI was set at 1, and the TEFs for CTD and THX were calculated as 152/2000 (0.08) and 152/2716 (0.06), respectively. The chronic TEF used critical intake values for a sensitive bird at the 5% tail of the acute sensitivity distribution published by Mineau and Palmer [11] based on lowest observed adverse effect levels (2820, 7380 and 12660 ng/kg body weight/day for IMI, CTD and THX respectively, giving TEF values of 1, 0.38 and 0.22, respectively. Acute TEFs were multiplied by the application rates for each compound per grid square per year, and the values for each compound were summed to give the total TEF-adjusted NN (kg) applied per grid square for use in the primary analysis. A repeat analysis was undertaken using the chronic TEF values to investigate the impact that this had on model results.

4.2.2 Bird data

BTO BBS data were obtained for 22 farmland species for the period 1994 to 2014 (**4.S1 Table**). The BBS consists of two visits per year (April/May and May/June) to a series of 1x1 km^2 survey sites where all species seen and heard are recorded across 10 transects within the survey square. Here, the maximum species count from either visit was extracted per site and per year as the measure of bird abundance. Both audible and visual records were included across all BBS distance categories, including fly overs. All birds on the farmland bird indicator list (19 species native to the UK [36]) were included, as well as red-legged partridge (*Alectoris rufa*), which is a non-native farmland specialist. Data for house sparrow (*Passer domesticus*) and chaffinch (*Fringilla coelebs*) were also included due to the availability of appropriate dietary data for these granivorous species.

Only BBS sites for which the level 1 habitat type was specified as farmland (code: 'E'), for the grid reference location of the BBS site, in one or more surveys during the time series were included in the analyses. A block of 343 sites in the North-West of England for which level 1 habitat type was not recorded were also included. Each BBS survey location (the central point of the 1 km square in which the BBS was undertaken) was assigned to the 5x5km grid square in which it fell. The analysis was restricted to BBS squares within mainland England to match the available pesticide and cropping data. All BBS data for 2001 were excluded from the analysis due to anomalies caused by site access restrictions during an outbreak of foot-and-mouth disease. Total change in each species population growth for England between 1995 and 2016 (referred to as 'BBS trends') was also independently obtained for each species from existing BTO BBS data sources [37] (**4.S1 Table**).

Defining NN exposure category for each species

The majority of bird species have heterogeneous diets [38-41], so data on dietary preferences were used to generate an index of likelihood of exposure. **Table 4.2** presents data for NN residue in potential food items, and a resulting categorisation of food items into low-level and high-level residue categories. Treated seed and crop seedlings represent food items with 'high' NN residue, while exposed birds (as prey items), eggs laid by exposed birds and exposed wild plant species were categorised as food items with 'low' NN residue (<0.01% of highest concentration). Invertebrates were found to have negligible NN residue (see **4.S2 Supplementary Note** for details) and were added to the 'low'-level residue category.

Table 4.2. Reported concentrations of NN residues in avian dietary components.

Dietary component	Data source	Residue of NN (ng/g)	Compound	Residue level
Crop seed	RSPB (*pers. com*)	555,600	CTD	High
Crop seedlings	RSPB (*pers. com*)	3,425	CTD	High
Exposed birds (<50g)	Lopez-Antia *et al.* (2015)	56	IMI	Low
Eggs (exposed bird)	Bro *et al.* (2016)	28	IMI	Low
Wild plants (at field margins)	Biotas *et al.*(2016)	0.51	CTD	Low
Invertebrates*	Chauzat *et al.* (2011)	0.3-11.1	IMI	Low

*Concentrations recorded in field-sampled honeybees (Apis mellifera) [30]; see **4.S2 Supplementary Note**). CTD: clothianidin; IMI: imidacloprid; NN: neonicotinoids.

Mean proportions of plant families in species diets were extracted from a quantitative literature review of European farmland bird diets reported by Holland *et al.* for 16 species [38] (**Table B** in **4.S3 Supplementary Note**). Where available, data were extracted for plant families *Cruciferae* (crops only), *Poaceae* (cereals only) and *Amaranthaceae* (all), which cover the main crop types associated with NN application (wheat, barley, sugarbeet, oilseed rape, rye and oats). Data were extracted separately for breeding and non-breeding adult birds and for chicks. Where specific plant family data were not available, values were estimated from data for the total percentage of plant material in species diets at each life stage (**4.S3 Supplementary Note**). Due to the variety of dietary assessment methods used in the studies reviewed in Holland *et al.* [38], extracted proportion values across multiple plant species were summed for each bird species to provide a measure of high-level residue food items in the diet (i.e., NN-treatable crop seed and seedling) and to capture the potential exposure from multiple crop types (**Table 4.3**).

Table 4.3. Relative quantity of high-level residue food items in species diet and dietary exposure groups assigned to each species.

Bird species	Latin name	Plant families treated with NN that are present in species diet	Relative value* (based on summed proportions) of plant families in diet at each life stage			Exposure group
			Adult BR	*Adult NB*	*Chicks*	
Chaffinch	*Fringilla coelebs*	*Poaceae (O)*	44	25	n/a	Medium
Corn Bunting	*Miliaria calandra*	*Poaceae*	44	75	16	High
Goldfinch	*Carduelis carduelis*	None	0	0	n/a	Low
Greenfinch	*Carduelis chloris*	*Poaceae*	16	11	21	Medium
Grey Partridge	*Perdix perdix*	*Poaceae*	12	28	21	Medium
House Sparrow	*Passer domesticus*	*Poaceae (O)*	37	23	^24	Medium
Jackdaw[+]	*Corvus monedula*	(Cereal grain)	n/a	n/a	(11)	Medium
Kestrel[+]	*Falco tinnunculus*	None	(0)	(0)	(0)	Low
Lapwing[+]	*Vanellus vanellus*	None	(0)	(0)	(0)	Low
Linnet	*Carduelis cannabina*	*Cruciferae; Poaceae (O)*	0	0	71	High
Red-legged Partridge	*Alectoris rufa*	*Amaranthaceae; Poaceae (O)*	n/a	44	^29	Medium
Reed Bunting	*Emberiza schoeniclus*	*Amaranthaceae (O); Poaceae*	0	69	^0	High
Rook	*Corvus frugilegus*	*Poaceae*	38	58	34	High
Skylark	*Alauda arvensis*	*Amaranthaceae; (Poaceae[+])*	[#]22	36	^2	Medium
Starling~	*Sturnus vulgaris*	(Grain)	(0)	(51)	(0)	Medium
Stock Dove	*Columbus oenas*	*Cruciferae; Poaceae*	61	22	5	High
Tree Sparrow	*Passer montanus*	*Amaranthaceae; Poaceae (O)*	22	36	^15	Medium
Turtle Dove	*Streptopelia turtur*	*Amaranthaceae (O); Cruciferae; Poaceae*	99	n/a	70	High
Whitethroat[+]	*Sylvia communis*	None	(0)	(0)	(0)	Low
Woodpigeon	*Columbus palumbus*	*Cruciferae; Poaceae (O)*	50	45	^47	High
Yellow Wagtail[+]	*Motacilla flava*	None	(0)	(0)	(0)	Low
Yellowhammer	*Emberiza citrinella*	*Poaceae*	92	32	4	High

*Extracted from Holland *et al.,* 2006 [38], with the exception of;

Values in brackets extracted from: ([+]) Birds of the Western Palearctic [39] and (~) Tait *et al.,* 1973 [40].

Values estimated from Holland *et al*., 2006 [38] are indicated as follows: ([#])Breeding value extrapolated from non-breeding value based on percentage of plant material in breeding vs. non-breeding season; (^) chick value extrapolated from available adult diet data based on percentage of plant material in breeding vs. non-breeding season (**4.S3 Supplementary Note**).

[+]Adult skylark are also known to feed on leaves of cereal plants (*Poaceae*) [41], but representative mean proportions are not shown here.

(O): Data includes percentage occurrence, as well as percentage items and percentage biomass.

AV: average; BR: breeding; NB: non-breeding.

Holland *et al*. [38] did not provide diet composition data for jackdaw (*Corvus monedula*), kestrel (*Falco tinnunculus*), starling (*Sturnus vulgaris*), lapwing (*Vanellus vanellus*), yellow wagtail (*Motacilla flava*) or whitethroat (*Sylvia communis*). For these species, dietary data were extracted from relevant volumes of Birds of the Western Palearctic [39]. Lapwing, yellow wagtail and whitethroat are insectivorous species, and kestrel a predatory species, so do not consume either crop seed or seedlings and were therefore assigned values of zero for these food items. Data extracted for adult jackdaw, nestling jackdaw and nestling starling were preferentially taken from studies with the largest available sample size, comparable sample type, sampling location within the UK and annual (rather than seasonal) data [39] (**4.S3 Supplementary Note**). Data for adult starling were extracted from Tait *et al.,* 1973 [40] (**4.S3 Supplementary Note**).

Species were broadly assigned to one of three dietary exposure categories (high, medium and low) based on the relative proportions of high-level residue food items in the diet. 'High' potential for exposure was assigned where high-level residue food items comprised >50% of the diet at any life stage (i.e., chick, breeding adult, non-breeding adult), 'medium' if diet comprised between 1 and 49% high-residue food items, and 'low' if those items were not present in the diet across any life stage. Comparable dietary data (e.g., summed proportion values of individual plant families in the diet) were not available for jackdaw and starling; however data obtained from sources outside of Holland *et al.* confirmed that crop seed is present in the diets of both species [39, 40] and therefore both were conservatively assigned to the medium exposure group.

4.2.3 Statistical modelling

A total of 3774 grid squares were used in the analysis, containing 5729 BBS sites (413 BBS sites were excluded from the analysis due to lack of cropping data and 6377 grid squares were excluded due to lack of BBS data). All models were run in R using the '`glmmTMB`' function in the 'glmmTMB' package [42]. A separate model was fitted for each species, then the parameter estimates from each species model were compared to test our hypotheses.

Species specific model: NN application & species population growth

Individual generalised log-linear mixed models (adapted from Freeman and Newson 2008) were used to estimate the effect of NN application on population growth for each of the 22 species (**Equation 4**):

$$\ln(\mu_{g,t,s,r}) = \beta_0 + \beta_1 \sum_{j=1}^{t-1} P_{g,r} + \beta_2 \sum_{j=1}^{t-1} R_j + x_g + y_s + z_r \qquad \textbf{(Eqn. 4)}$$

where the response variable μ is the count of birds in a given grid square *g* (at 5x5 km resolution), in year *t*, at BBS site *s* and within region *r*. The expected value of $\mu_{g,t,s,r}$ was modelled as a function of NN application (*P*; TEF-adjusted kg) and the 'background' species population growth (β_2) in the absence of NNs as fixed effects. Grid square number (*x*), BBS site (*y*) and region (*z*) were modelled as normally-distributed random effects with zero mean. Issues related to density dependence were circumvented by using raw abundance data as the response variable to calculate population growth [43].

In detail, β_0 represents the estimate of the log abundance for the relevant bird species in 1994 (the baseline year: *P* = 0), for the average grid cell, region and survey site (with distribution errors and log link). *R* was entered as a binary matrix, the columns of which indicate the time period across which species population growth is calculated, where *j* is an index of year. β_2 therefore represents a vector of parameters, one for each year from 1995 to 2014, each of which is an estimate of the population growth rate for that year (i.e., the 'background' population growth rate); for example, the estimated log abundance for μ for 1996, at an 'average' site is given by $\beta_0 + \beta_{2(1995)} + \beta_{2(1996)}$. The variable *P* denotes the pesticide, measured as 'cumulative' NN (TEF-adjusted kg) from the baseline year (1994) up to and including the year of observation, indexed by *j*; note that 'cumulative' in this instance refers to the pesticide term within the model that is used to track year-on-year change in NN use, and does not imply multi-year accumulation of pesticide in the environment. Parameter β_1 introduces the effect of NN application on the population growth (β_2), in a similar way to the model used in Baker *et al.* [44]. Entering NN application (*P*) as a cumulative value allows β_1 to be interpreted as the change in population growth rate per unit application of NN (adjusted for toxicity of each NN compound to birds). Simply put, the model tests the relationship between the change in bird abundance between years *t-1* and *t* (β_2) and the NN application in to crops harvested in year *t-1* (β_1), with the estimate represented as a decimal fraction. Therefore under the study hypothesis a negative impact of NN application on species of farmland birds would be indicated by negative estimates for NN-related population growth (β_1) for species in the high exposure category.

NN applications to spring crops (particularly sugar beet) predominated in terms of total mass applied during the first half of the study period (1994-2004), whereas NN applications in the second half of the study period (2005-2014) were greatest for winter oilseed rape and winter cereals. As such, the possible demographic mechanisms through which NN exposure would affect our

modelling of BBS counts include both reduced productivity, and overwinter survival or subsequent recruitment into breeding populations.

Model fit

All species models were initially run using a Poisson distribution and tested for over-dispersion (ratio of sum of squares residuals: residual degrees of freedom > 1.5; '`overdisp`' function [45]) and zero-inflation (root mean squared error comparison, log-likelihood tests and the '`testzeroinflation`' function in DHARMAa [46]). Residual QQ-plots were visually inspected for each species model to check uniformity, and simulated residuals were plotted ('`simulateResiduals`' function in DHARMAa) to check model fit.

All species except kestrel and woodpigeon were modelled using a quasi-Poisson distribution to account for over-dispersion in the count data, although data for lapwing and starling remained over-dispersed despite this adjustment (over-dispersion ratio = 1.68 and 1.90, respectively). Kestrel was modelled using a Poisson distribution and woodpigeon a negative binomial distribution. The fitted residuals were sigmoidal for all species models with non-uniform residual tails. The residuals for the grey partridge model were the only exception in that the residuals significantly deviated from the fitted trend for over 60% of the predicted values. It was not possible to use scaling to address these issues for this species.

Multispecies models: dietary exposure

β_1 estimates and their standard errors were extracted from each species-specific model. The difference in β_1 estimates between dietary exposure groups (high, medium, low) were analysed using Kruskal–Wallis one-way analysis of variance ('`kruskall.test`', [47]). In order to account for differences in dietary preferences at each individual life stage, weighted linear regressions were used to model β_1 as a function of the proportion of high-level residue food items for adult diet during the breeding season, adult diet outside of the breeding season, and chick diet for each species. A weighted linear regression was also used to assess whether there was any association between NN-related population change and overall population trends in England (BBS 1995-2016) across all species. Estimate values for β_1 were weighted by their corresponding standard errors. Linear regressions were run in R using the '`lm`' function [47].

4.3 RESULTS

Individual model estimates for the change in species population growth per unit (TEF-adjusted kg) of NN applied (β_1 - represented as a decimal fraction and referred to hereafter as 'NN-related population change') were obtained for all 22 study species (**4.S1 Table;** refer here for all Latin names hereafter), calculated across all years and all available grid squares. Estimates of NN-related population change (β_1) ranged between -0.2 and +0.2%, and were significant for 13 out of the 22 species ($p < 0.05$) (**Figure 4.3** and **4.S1 Table**). There were significant positive estimates for nine species (chaffinch, greenfinch, grey partridge, linnet, rook, starling, tree sparrow, woodpigeon, yellowhammer), and significant negative estimates for four species (house sparrow, red-legged partridge, skylark, turtle dove). Standard errors in the estimate of β_1 were largest for those species with fewest observations per survey event, in particular corn bunting, turtle dove and tree sparrow. BBS population trends for England (1995-2016) and NN-related population change were directionally matched for only seven of the 22 species (three species with negative BBS trends and β_1 estimates, and four species with positive BBS trends and β_1 estimates) (**Figure 4.3**). The root mean squared error was >10 for the majority of flocking species (jackdaw, rook, starling, woodpigeon) and <10 for those that are usually recorded in small numbers during the summer months (**4.S1 Table**). Overall, BBS site was the largest source of variance in the model for 18 of the 22 species, followed by grid square and region. For grey partridge, red-legged partridge, wood pigeon and yellow wagtail, grid square ID was the largest source of variance. Model outputs were almost identical when an alternative approach was used to estimate NN use in years without data (i.e., when data were repeated from the preceding year, rather than calculating the mean of the preceding and following years; **4.S2 Table**). Similarly, model outputs were almost identical when chronic TEFs were used to account for differences in toxicity between compounds rather than acute TEFs; there was a roughly equal split between species where the results shift towards a slightly more positive model estimate for NN effects on population size and those where the reverse was true (**4.S2 Table**); the estimate of negative impacts for the skylark changed to being non-significant in the analysis based on chronic TEFs, and the positive estimate for the reed bunting became significant.

Where NNs were applied, the median estimated value of application per grid square was 0.28 kg, with a maximum application of 69.98 kg (with TEF applied). The East region had the largest mean and total NN application over the entire study period, whilst the North West had the smallest (**4.S3 Table**).

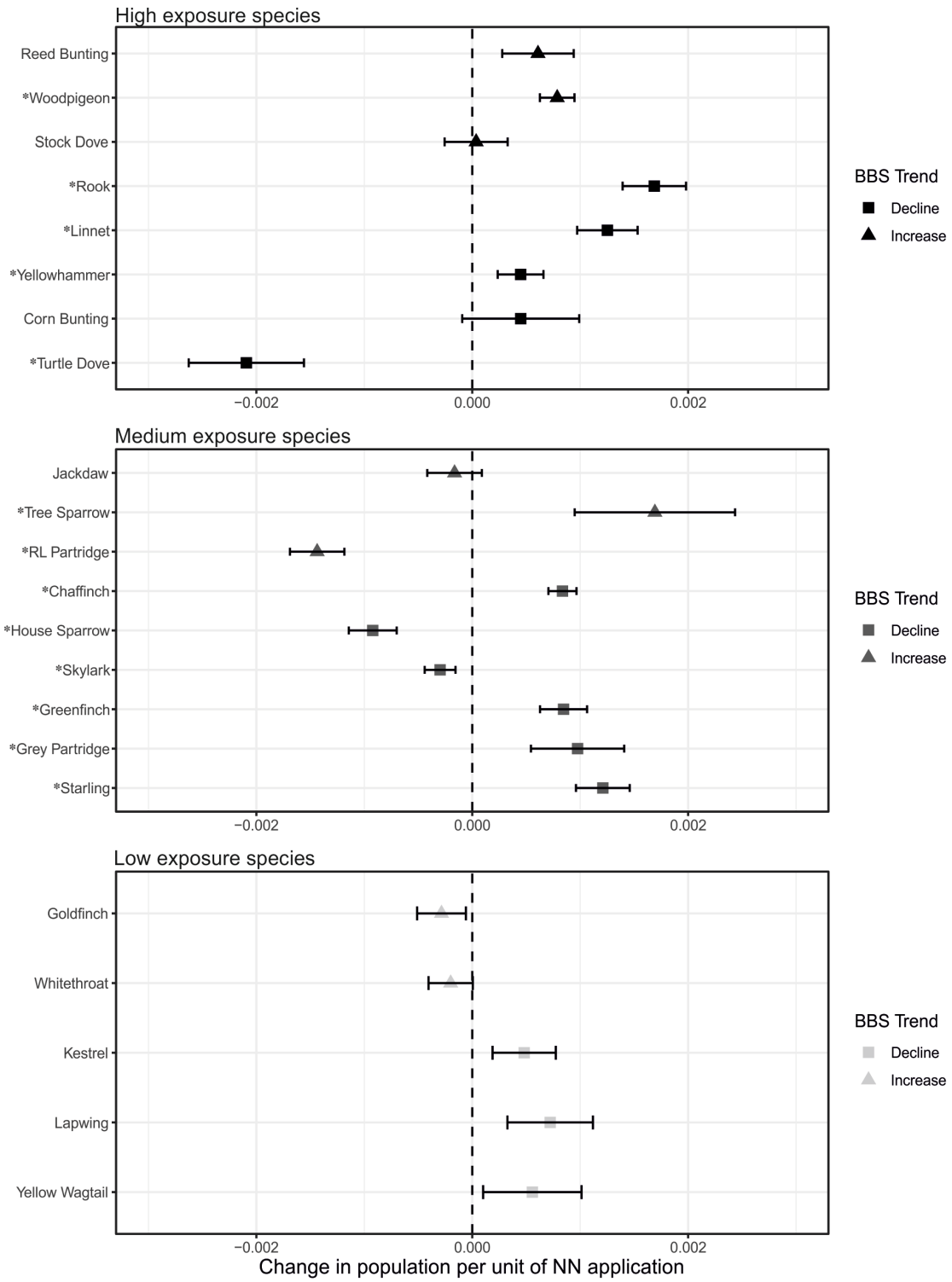

Figure 4.3. Model estimates plus standard error bars for change in species population per unit (TEF-adjusted kg) of NN applied for each species included in the analysis. Species are split by dietary exposure group and are ordered in each plot by rate of overall population change ('BBS Trend') according to BTO BBS data for England between 1995 and 2016 [37] with the largest population increase at the top and the largest decline at the bottom of each plot (see **4.S1 Table** for values). Species marked with (*) indicate significant ($p<0.05$) estimates of change in population per unit NN applied. BBS: breeding bird survey; BTO: British Trust for Ornithology; NN: neonicotinoid; TEF: toxicity equivalency factor.

4.3.1 Dietary exposure & population change as a result of NN application

NN-related population change did not differ significantly between dietary exposure groups (Kruskall-Wallis chi-squared = 0.55, 2 d.f., p = 0.75; **Figure 4.4**). Furthermore, estimates of NN-related population change were not correlated with the relative values of high-residue food items in the diet of breeding adults, non-breeding adults or chicks extracted from Holland *et al.* (breeding adults: adjusted R^2 = -0.029, $F_{1,17}$ = 0.47, p = 0.49; non-breeding adults: adjusted R^2 = -0.053, $F_{1,17}$ = 0.08, p = 0.77; chicks: adjusted R^2 = -0.021, $F_{1,17}$ = 1.38, p = 0.25). There was also no correlation between NN-related population change and BBS trends (overall change in species population in England between 1995 and 2016) across all species in the study (adjusted R^2 = -0.03, $F_{1,20}$ = 0.27, p = 0.60).

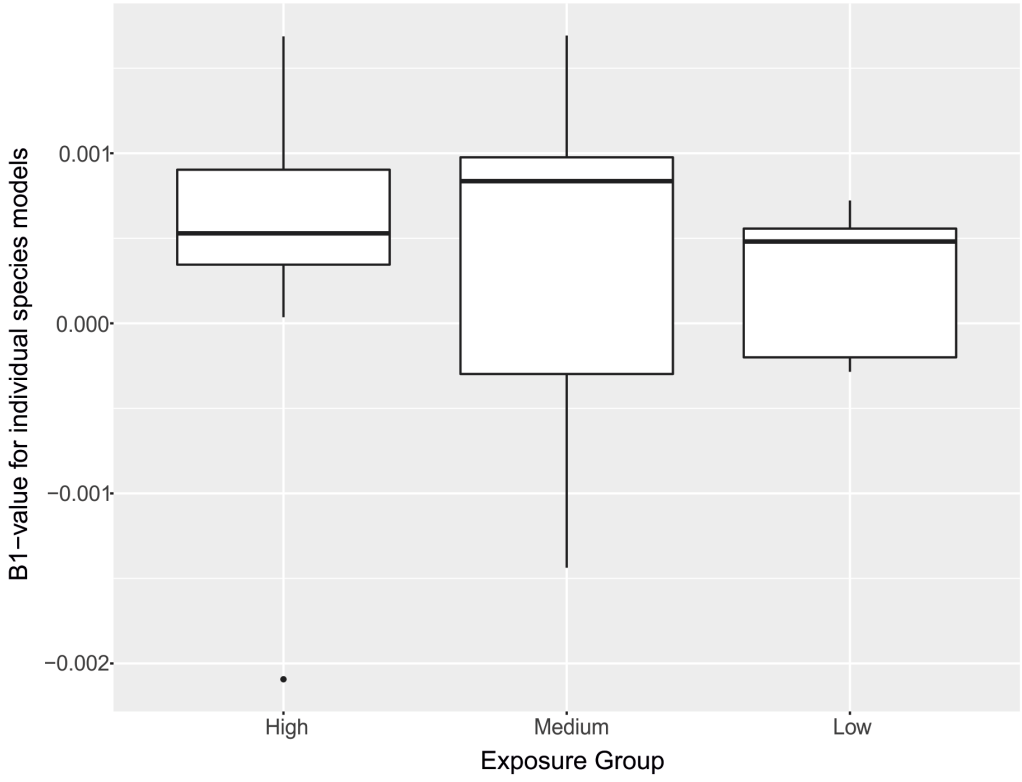

Figure 4.4. Distribution of β_1 values (change in species population growth per unit [TEF-adjusted kg] of NN applied) obtained for each species across dietary exposure groups. The mean is represented by the black lines through the centre of each bar, the upper and lower quartiles are contained within the box and the range is represented by the whiskers. The estimate for turtle dove (*Streptopelia turtur*) is displayed as an outlier (represented by the single point) for the high exposure group. TEF: toxicity equivalence factor.

4.4 DISCUSSION

Overall, our findings provide no consistent evidence for impacts of dietary exposure to NN insecticides on the abundance of farmland birds in England. Individual estimates of NN-related

population change for each species varied considerably within the range of model outputs, but were noticeably smaller than annual 'background' changes in population for each species. Across all species, significant population change associated with spatial and temporal variation in NN application were mostly positive (9 out of 22), with a smaller number of negative relationships (4 out of 22). Under the study hypothesis, species in the high and the medium exposure groups were expected to have a higher proportion of significant negative estimates for NN-related population change compared to species in the low exposure group. Species in the low exposure group did not have any significant estimates of NN-related population change, which lends some support to the hypothesis. However, only one species in the high exposure group and three in the medium exposure group exhibited significant negative estimates. Moreover, nine species from these groups had significant positive estimates.

4.4.1 Individual species

Of the nine species that had significant positive estimates for NN-related population change, four were in the high exposure category (linnet, rook, wood pigeon, yellowhammer), whilst the remaining five belonged to the medium exposure group (chaffinch, greenfinch, grey partridge, starling, tree sparrow). Seven of these nine species experienced population declines in England between 1995 and 2016. The most notable of these were grey partridge, linnet, and rook, (estimated declines of -58, -19, and -13%, respectively [37]). The remaining two species experienced population increases (tree sparrow: +64% and woodpigeon: +36%). However, estimates for rook, starling and woodpigeon had associated root mean squared error values (the number of birds per grid square by which the model estimate could vary) between 21 and 28, compared to <10 for the majority of other species. Rook, starling and woodpigeon in particular tend to form flocks, which may have added to the noise associated with the data for these species, especially with regard to 'fly over' records that may have recorded long-distance traveling flocks rather than local populations in each grid square. Furthermore, the model for starling was over- dispersed and the model fit for grey partridge was poor compared to all other species models. Thus, only five of the nine models reporting positive estimates for β_1 were without confounding issues.

Positive estimates of NN-related population change for these nine species do not support the study hypothesis of adverse population change in response to dietary exposure to NNs. Currently, there is little evidence of a positive effect of NNs on birds in existing literature, and there is no known mechanism by which this could occur. One plausible explanation for these observed trends is that the overall availability of seeds/grain as a food resource within arable landscapes may have been strongly correlated with NN application, particularly at the height of NN use when a large

proportion of crop types and large cropping areas were treated with NNs [9], resulting in greater granivorous species abundance at these sites. This theory is one that the present study cannot substantiate, but may be important to note as a potential paradox in NN exposure-population modelling of this type.

The four species that had significant negative estimates for NN-related population change were house sparrow, skylark, red-legged partridge and turtle dove. Of these, one was placed in the high exposure group (turtle dove), three belonged to the medium-exposure group (house sparrow, red-legged partridge, skylark), and all except red-legged partridge experienced overall population declines in England between 1995 and 2016. It is possible that the negative estimates for these species may be indicative of a true negative relationship between NN application and population change; indeed, a recent study reported widespread exposure of house sparrow to NNs in the field [27], but the implications of this exposure for fitness and/or survival were not assessed. However, other ecological factors may have also been important drivers. For instance, turtle dove populations are estimated to have undergone the greatest population decline of any species included in the study (-94%); however, turtle doves are migratory and unlikely to be exposed to NNs during the autumn sowing period as most individuals depart the UK in September at latest [48], and peak NN application occurs during late September and October [9]). Thus far, turtle dove population declines in the UK have primarily been attributed to the loss of weed seeds due to herbicide usage, resulting in an increased reliance on cultivated species such as cereals [49, 50].

The model output for red-legged partridge is also of note. Partridges (as well as other game birds) are one of the most commonly studied species in relation to NNs and exposure of various partridge species to NN-dressed seeds has been recorded [28, 29, 51, 52]. Sub-lethal impacts on red-legged partridge have been found when individuals have been given environmentally-relevant doses of IMI [53] while a long-term study found a significant negative impact of NNs on the population of the Northern bobwhite quail - another ground-dwelling galliform [54]. Our finding of a negative impact on red-legged partridge populations arising from NN use is therefore plausible when considered alongside previous research. However, there was a small population increase over the study period (+3% between 1995 and 2016 [37]) that indicates that other factors were likely to have been more important in determining population dynamics. Furthermore, this species is highly managed as part of the shooting industry, which may obscure natural changes in population numbers.

Collectively, our model outputs did not provide any consistent evidence that dietary exposure to NNs has had a negative impact on farmland bird populations in England at a 5x5 km spatial scale. We found that there were both significant positive and negative changes to individual species population growth where NNs were applied. It is unlikely that positive NN-related changes were directly related to NN use as there is no apparent mechanism by which NN ingestion is likely to be beneficial to birds (either individually or at a population scale). However, there is a substantial body of literature that provides evidence of NN-exposure to wild birds, and that NN ingestion results in adverse effects on avian physiology and behaviour [55]. We therefore cannot rule out the possibility that NN use had a negative effect on some species populations (particularly house sparrow, red-legged partridge and skylark) where negative changes were observed in areas where NNs were applied.

4.4.2 Direct ingestion of NNs as an exposure pathway

Our exposure categories did not predict the magnitude of estimates for NN-related population change across the set of species included in the study; results here suggest that dietary exposure to NNs via treated seed and seedlings is unlikely to be associated with changes to farmland bird populations across England. Estimates of NN-related population change were both positive and negative within high and medium dietary risk groups and relative values of high-residue food items in the diet of adults and chicks did not explain population changes in the context of NN application. In addition, model estimates for four species in the high and medium risk groups were not significant (high risk group: corn bunting, stock dove and reed bunting; medium risk group: jackdaw), despite a large proportion of their diets consisting of high-residue food items. Corn bunting in particular has been cited in the literature as being a candidate species for studying the effect of NNs on small song birds due to the frequency with which it has been observed foraging in fields of treated seed [16], but this does not tally with our findings. The distribution of significant estimates between high and medium exposure categories suggests that NN-treated seed and seedling ingestion is not a strong driver of population change at this spatial scale (e.g., effects of NNs may be highly localised), and that NNs are uninfluential compared to other population drivers for the species included, such as food availably and habitat provision.

4.4.3 Modelling approach

This analysis was undertaken with 19 years of pesticide usage and bird abundance data across 94,350 km^2 (72%) of England. A key advantage in using these data is that the spatial and temporal variation in NN usage during the study period maximised the statistical power needed to test our hypotheses. Furthermore, our model verification process followed ‘best practice’ guidelines for

fitting generalised linear mixed models [56]. Well-fitted models were difficult to achieve as is typical for many ecological studies using 'real-world' data collected from complex ecosystems. Nevertheless, the approach used is arguably one of the most powerful available to test our hypotheses; it is of note that the use of smaller datasets (e.g., the analysis of data using individual compounds or splitting the time series at the time point where the primary compound applied in England switched from IMI to CTD) was not effective due to the reduction in statistical power, attributable to the loss in variation of NN application.

In common with previous studies [32, 35], the spatial matching of NN usage data to records of non-target species required some interpolation of usage data. The model was shown not to be sensitive to the approach used to estimate NN usage in alternate years when pesticide usage data were not collected (**4.S2 Table**), but the interpolation step still introduces uncertainty into the analysis. The model structure also assumes that bird populations at each BBS site will only be affected by NN applications within the encompassing 5 x 5 km^2 grid square. The hypotheses tested in this study related specifically to the ingestion of treated-crop material, whereas there are multiple exposure pathways that wild birds may be subject to. The decision to quantify NN in our model using weight of seed treatment applied means that exposure pathways associated with the much smaller usage of NNs as spray applications (~11% of applications in the UK during the study period [9]), such as direct overspray of birds or insects, were excluded from this study. However, these alternative pathways are expected to result in comparatively lower exposure than direct ingestion of treated seed or seedlings (**Table 4.2** and **4.S2 Supplementary Note**). Many granivorous birds switch to and/or feed their young an insectivorous diet during the breeding season [38] meaning there is also a potential impact on breeding success from reduced food availability [32]. This potential indirect impact from insecticide use was explicitly not considered within the current study and results should be interpreted in this context. The potential for indirect effects via reduced food availability would be a priority for future investigation and would require different measurements of NNs in the environment (e.g., residue in non-crop material or the impact of NNs on non-target invertebrate species). Finally, the analysis did not consider any particularly sensitive timings for NN application. As such, sub-lethal effects during the reproductive period were not specifically targeted, but were rather considered alongside the multiple sub-lethal endpoints proposed to result from neonicotinoid exposure in wild birds [19, 21, 31, 53] and which may affect both survival and productivity.

The overall number of species used in this study is both an advantage and a disadvantage. Modelling multiple species within one system allows for dietary exposure routes to be assessed through cross-

species comparisons and is useful for pinpointing specific species from a large number of those potentially affected, which warrant further research attention. It also gives a full picture across a range of species with different physiologies, and different patterns of habitat use. The risk associated with modelling just one species is that, if a significant effect is found, it cannot be placed into context with either similar or dissimilar species, and that a finding for one species may be extrapolated to all species within that taxa. Conversely, the disadvantage of modelling multiple species is that the 'one size fits all' approach to the model structure may not be suitable across the board and may therefore contribute to poor model fit. Specifically tailored variables for each species may produce higher quality outputs (such as the approach used in Ertl *et al.*, 2018), but at the cost of considerably narrowing the study spectrum.

4.4.4 Conclusion

Here we found no evidence to suggest that dietary exposure to NNs via ingestion of treated seed and/or crop material has been associated with population declines of farmland birds in England over the period 1994 to 2014. We conclude that overall, there has either been no consistent effect of NN application on farmland bird populations, or any over-arching effect has been so small that it was not detectable by this modelling approach, which was limited by the spatial availability of pesticide usage data. The potential for indirect effects of insecticide use on bird populations via reduced food availability was not considered within our study design and should be a focus for future research. This study highlights some of the issues in isolating specific causal factors for population dynamics from the 'noise' of other agricultural processes and underlying species population trends; this is particularly challenging when attempting to analyse a specific toxicant exposure route with regards to population-scale outcomes. Although it is not possible to infer any direct role of NNs on farmland birds collectively from these analyses, our results identify house sparrow, red-legged partridge and skylark as species that may warrant further research attention.

Data sources

Agcensus Cropping data

The grid square agricultural census data, as converted by EDiNA at the University of Edinburgh and available through their AgCensus service (http://agcensus.edina.ac.uk), are derived from data obtained for recognised geographies from the Department of Environment, Food and Rural Affairs (DEFRA), the Welsh Assembly Government, and the Scottish Government (formerly SEERAD), and are covered by Crown Copyright.

British Trust for Ornithology Breeding Bird Survey data

The Breeding Bird Survey (https://www.bto.org/volunteer-surveys/bbs/bbs-publications/bbs-reports) is run by the British Trust for Ornithology (BTO) and is jointly funded by the BTO, the Joint Nature Conservation Committee (JNCC) (on behalf of the statutory nature conservation bodies: Department of Agriculture, Environment and Rural Affairs - Northern Ireland, Natural England, Natural Resources Wales and Scottish Natural Heritage), and the Royal Society for the Protection of Birds (RSPB).

Pesticide Usage Survey data

Fera Science Ltd is commissioned to conduct agricultural, horticultural and amenity pesticide usage surveys by the Chemicals Regulation Division (CRD) of the Health and Safety Executive. The surveys are funded from the pesticides charge on turnover, and the costs are paid to Fera Science Ltd by CRD. The Pesticide Usage Survey Teams of Fera Science Ltd, a joint venture between Capita PLC and the Department for Environment, Food & Rural Affairs (Defra), Science & Advice for Scottish Agriculture (SASA), a division of the Scottish Government's Agriculture, Food and Rural Communities Directorate and the Agri-Food & Biosciences Institute (AFBI), a Non-Departmental Public Body of the Department of Agriculture and Rural Development, Northern Ireland (DARD) conduct a series of UK surveys of pesticide usage in the major sectors of agriculture and horticulture. Reports from these surveys are published on Fera's website (https://secure.fera.defra.gov.uk/pusstats/surveys/index.cfm).

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4.5 SUPPLEMENTATRY MATERIAL

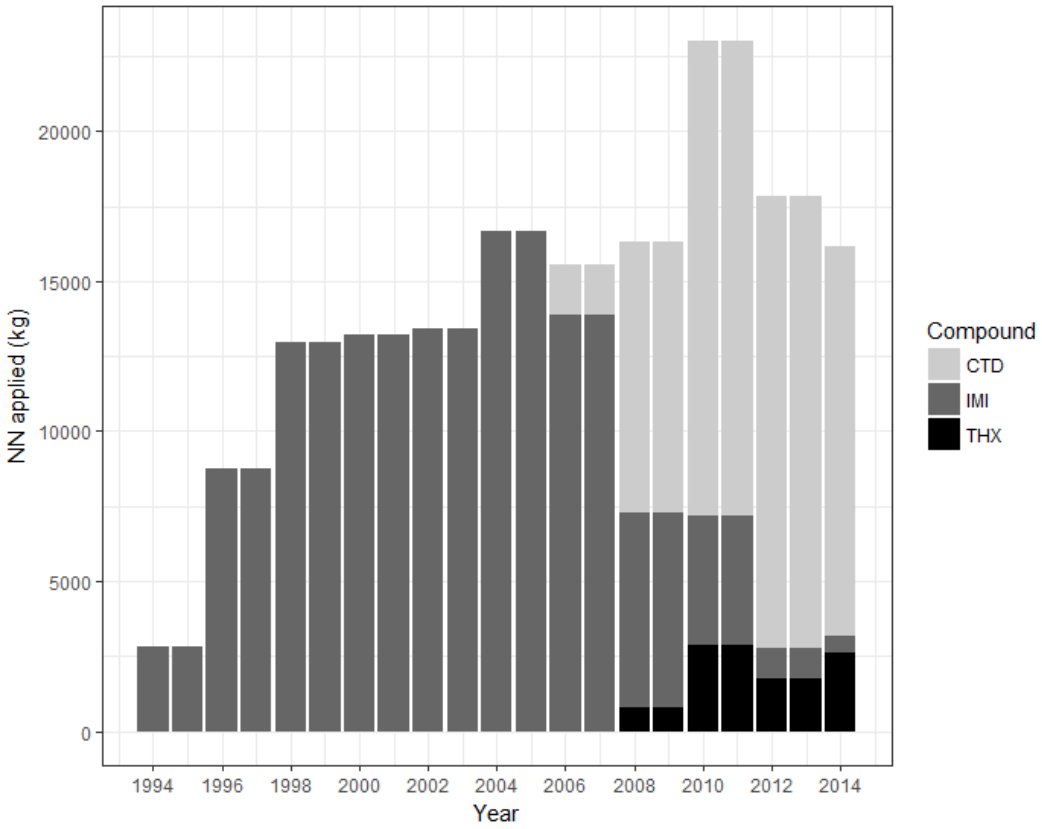

Figure 4.S1. **Pesticide Usage Survey data for annual weight (kg) of NN applied in the UK between 1994 and 2014 (without toxicity equivalency factor applied) [9].** Bars are shaded according to amount of each NN compound annually applied.CTD: clothianidin; IMI: imidacloprid; THX: thiamethoxam; NN: neonicotinoid.

Figure 4.S2. Pesticide usage survey regions. A) Eight 'NUTS regions' (NUTS level 1) used in the pesticide usage survey from 2004 to 2014 (C: North East; D: North West; E: Yorkshire & Humber; F: East Midlands; G: West Midlands; H: Eastern; I&J: London & South East; K: South West). **B)** Five 'Defra regions' (originally MAFF [Ministry of Agriculture, Fisheries & Food] regions) used in the pesticide usage survey from 1994 to 2002 (1: Northern; 2: Midlands & Western; 3: Eastern; 4: South East; 5: South West).

4.S1 Supplementary Note. Interpolation method and validation

To create a dataset to input into the model, cropping data for each of the five main crop types (wheat *Triticum sp.*, winter barley *Hordeum sp*., sugar beet *Beta sp.*, linseed *Linum sp.*, oilseed rape *Brassica sp.*) were required on an annual basis at a 5x5 km resolution for the whole of England. The AgCensus (AgC) data (provided by EDiNA) has these data at a 5x5 km resolution for England, however, it does not provide data for all years between 1994 and 2014 (**Table A**). For years where these data were missing, two different interpolation approaches were used to estimate cropping areas within each 5x5 km grid. PUS data assigned the cropping category 'set aside' were excluded from the analysis due to the ambiguity of the crop type they were applied to.

Approach 1: June Survey of Agriculture

Regional data from the June Survey of Agriculture (JSA) were used to estimate yearly increases and decreases per crop type from the last available annual AgC data set. Missing years were then interpolated by multiplying the last available AgC annual data ('the baseline') by the increase or decrease in cropping area from the baseline to the JSA data for that missing year. Only the years that had available JSA data and no AgC data were interpolated using this method. Due to an anomaly in the algorithm applied by EDiNA, the regional 2010 AgC cropping data values were consistently below the regional JSA figures (EDiNA, *pers. comm.*). Therefore all AgC data for 2010 were also adjusted (using the same interpolation method), so that it was in accordance with JSA regional cropping data.

Oilseed rape was the only exception to the interpolation protocol in that all AgC years from 2000 (inclusive) onwards were adjusted so that regional AgC data for those years matched JSA regional data. This was due to mis-matches between AgC data and JSA data, which was most likely caused by changeable groupings of oilseed rape categories (e.g., some years all oilseed rape was summed, whereas other years it was divided into two categories based on whether it was winter or spring sown).

Approach 2: linear interpolation

Cropping areas for individual grid squares for any remaining years that had neither AgC nor JSA data were estimated using a linear interpolation using the '`na.approx`' function in the 'zoo' package.

Limitations

For each crop type, two 'dummy' years (1995 from 1994 baseline, 2010 from 2004 baseline) that had available AgC data were interpolated using JSA data ('Approach 1'). Interpolated cropping areas

were compared to actual AgC cropping areas to assess the effectiveness and accuracy of interpolation methods. We found that grid squares with higher cropping areas produced poorer interpolations, as did missing years that were further away from the last available baseline year. For example, r^2 values for wheat and oilseed rape when comparing actual AgC data to interpolated AgC data for 1995 (created from a 1994 baseline) were 0.93 and 0.85 respectively, whereas the r^2 values for wheat and oilseed rape when comparing actual AgC data to interpolated AgC data for 2010 (created from a 2004 baseline) were 0.77 and 0.64. To account for this, the most recent baseline available was always used and the regional totals for interpolated data for missing years for all crop types were subsequently checked to ensure that they tallied with regional JSA totals for that year. If any grid squares exceeded a total cropping area of 25 km^2, all crops were evenly scaled down so that the total cropping area was capped at 25 km^2. This only occurred for 42 grid squares (range: 2502 – 3453 ha) in any one year across the whole interpolated dataset.

Table A. Data source and interpolation method used for each year of cropping data included in the study (excluding 1998 and 2001).

Crop	Year	AgC	JSA	Baseline	Linear
Wheat & Winter Barley	1994	X			
Wheat & Winter Barley	1995	X			
Wheat & Winter Barley	1996	X			
Wheat & Winter Barley	1997	X			
Wheat & Winter Barley	1998				X
Wheat & Winter Barley	1999		X	1997	
Wheat & Winter Barley	2000	X			
Wheat & Winter Barley	2001		X	2000	
Wheat & Winter Barley	2002		X	2000	
Wheat & Winter Barley	2003	X			
Wheat & Winter Barley	2004	X			
Wheat & Winter Barley	2005		X	2004	
Wheat & Winter Barley	2006		X	2004	
Wheat & Winter Barley	2007		X	2004	
Wheat & Winter Barley	2008		X	2004	
Wheat & Winter Barley	2009		X	2004	
Wheat & Winter Barley	2010	X	X*	2010	
Wheat & Winter Barley	2011		X	2010	
Wheat & Winter Barley	2012		X	2010	
Wheat & Winter Barley	2013		X	2010	
Wheat & Winter Barley	2014		X	2010	
Sugarbeet	1994	X			
Sugarbeet	1995	X			
Sugarbeet	1996	X			
Sugarbeet	1997	X			
Sugarbeet	1998				X
Sugarbeet	1999				X
Sugarbeet	2000	X			
Sugarbeet	2001				X
Sugarbeet	2002				X
Sugarbeet	2003	X			
Sugarbeet	2004	X			
Sugarbeet	2005		X	2004	
Sugarbeet	2006				X
Sugarbeet	2007				X
Sugarbeet	2008				X
Sugarbeet	2009				X
Sugarbeet	2010	X	X*	2010	
Sugarbeet	2011		X	2010	
Sugarbeet	2012		X	2010	
Sugarbeet	2013		X	2010	
Sugarbeet	2014		X	2010	

(*) Indicates which dataset was used where both JSA and AgC data were available.

AgC: agcensus data (5x5 km grid square resolution); JSA: June Survey of Agriculture data (regional resolution); Baseline: AgC data used for JSA interpolation.

Table A (cont.). Data source and interpolation method used for each year of cropping data included in the study (excluding 1998 and 2001).

Crop	Year	AgC	JSA	Baseline	Linear
Oilseed rape	1994	X			
Oilseed rape	1995	X			
Oilseed rape	1996	X			
Oilseed rape	1997	X			
Oilseed rape	1998				X
Oilseed rape	1999		X	1997	
Oilseed rape	2000	X			
Oilseed rape	2001		X	2000	
Oilseed rape	2002		X	2000	
Oilseed rape	2003	X	X*	2003	
Oilseed rape	2004	X	X*	2004	
Oilseed rape	2005		X	2004	
Oilseed rape	2006		X	2004	
Oilseed rape	2007		X	2004	
Oilseed rape	2008		X	2004	
Oilseed rape	2009		X	2004	
Oilseed rape	2010	X	X*	2010	
Oilseed rape	2011		X	2010	
Oilseed rape	2012		X	2010	
Oilseed rape	2013		X	2010	
Oilseed rape	2014		X	2010	
Linseed	1994	X			
Linseed	1995	X			
Linseed	1996	X			
Linseed	1997	X			
Linseed	1998				X
Linseed	1999				X
Linseed	2000	X			
Linseed	2001				X
Linseed	2002				X
Linseed	2003	X			
Linseed	2004	X			
Linseed	2005		X	2004	
Linseed	2006				X
Linseed	2007				X
Linseed	2008				X
Linseed	2009				X
Linseed	2010	X	X*	2010	
Linseed	2011				X
Linseed	2012				X
Linseed	2013				X
Linseed	2014				X

(*) Indicates which dataset was used where both JSA and AgC data were available.

AgC: agcensus data (grid square resolution); JSA: June Survey of Agriculture data (regional resolution); Baseline: AgC data used for JSA interpolation.

4.S2 Supplementary Note. Concentrations of neonicotinoid in invertebrates

Although a significant amount of neonicotinoid (NN) is taken up into plants from treated seed, the majority of applied compound is available for transfer out of the seed coating into the surrounding soil. Therefore there is potential for NN to leach into the surrounding substrate and water table when using seed coating as an application method. Using standardised CTD application rates for wheat, calculations were made as to the amount of uptake in soil-dwelling invertebrates, based on earthworms as a model species. The number of seeds sown per unit area (m^2), the ingestion rate of soil for earthworms and type of soil were taken into account. Results from these calculations estimated that the concentration of CTD in earthworms would be <0.0001 ng/g.

Currently, field data for concentrations of NNs measured in 'above-ground' invertebrate samples are largely limited to honeybees (genus: *Apis*), which do not predominate the majority of farmland bird diets [1]. One such study reported imidacloprid concentrations in honeybees to be between 0.3 and 11.1 ng/g [2]. Data are also available for the concentration of imidacloprid found on multiple species of insect (ground- and canopy-dwelling) as part of the European Food Safety Authority bird and mammal risk assessment for NNs; however these data refer to concentrations of NN measured in insects after imidacloprid was applied via spray treatment, rather than as a seed treatment [3]. As over 90% of NN applications in the UK are in the form of seed treatments [4], these data did not inform our study.

Routes of exposure of NNs to birds via invertebrates would be confined to those insects that feed on treated and/or contaminated plants (whether these be wild or crop species), and restricted by the level of residue within each individual plant and the ecology of the insect species [5], which would mediate the level of NN taken up within the invertebrate (e.g., ingesting plant material vs. use as a habitat only). Furthermore, the concentration of NN the bird is subject to would also be dependent on the ratio of exposed:non-exposed invertebrates consumed, the proportion of the diet that consists of invertebrate species and seasonal/daily changes in foraging habits. Based on the information available with regards to seed treatments and NN concentrations in insect prey items for birds, the ingestion of either above-ground or soil-dwelling invertebrates was considered to be negligible in terms of NN exposure and therefore categorised as a low-residue food item.

References

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4.S3 Supplementary Note. Data extraction protocol to inform dietary risk categories

All values in **Table B** were extracted from Tables 1-4 of Holland *et al*., 2006 (*A review of invertebrates and seed-bearing plants as food for farmland birds in Europe*) [1]. Interpolated values for chick diet were calculated by finding the change in total plant material (%) between chicks and adults (breeding or non-breeding, depending on availability), and then estimating the percentage of NN plant material in chick diet based on the change in total plant material from adult to chick. The same approach was used to calculate breeding adult values for skylark.

Where data were unavailable in Holland *et al.,* data were extracted from the relevant volumes of Birds of the Western Palearctic [2]. For jackdaw (*Corvus monedula*) and starling (*Sturnus vulgaris*) data were extracted as follows - jackdaw (adult): percentage wet weight of cereal grain in 439 stomachs collected in Spain (all year round) [vol. 8, pg. 126, Table A, Soler *et al.,* 1990] [2]; jackdaw (chick): percentage volume of cereals in 357 collar samples collected in Wales [vol. 8, pg. 126, Table B, Richford 1978] [2]; starling (chick): absence of crop material in chick diet across multiple studies [vol. 8, pg. 244, Table B] [2]). For kestrel (*Falco tinnunculus*), lapwing (*Vanellus vanellus*), yellow wagtail (*Motacilla flava*) and whitethroat (*Sylvia communis*) the full list of items listed in the 'Food' section for each species were examined. If crop plant material (seed and seedling) did not appear in this list, then a value of zero was given to that species for each life stage.

Data for adult starling were not available from the Birds of the Western Palearctic, so were extracted from Tait *et al.,* 1973 [3] (Appendix II). Percentage values for non-breeding adults were averaged for months outside of April-July, and averaged for months April-July for breeding adults to provide the final values presented in **Table 3** of the main text.

References

1. Holland JM, Hutchison MAS, Smith B, Aebischer NJ. A review of invertebrates and seed-bearing plants as food for farmland birds in Europe. Ann Appl Biol. 2006;148(1):49-71. doi: 10.1111/j.1744-7348.2006.00039.x.
2. Cramp S. Handbook of the Birds of Europe the Middle East and North Africa. The Birds of the Western Palearctic (Vol. I-IX). Oxford University Press, UK; 1985.
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Table B. Extracted and interpolated dietary values from Holland *et al.,* 2006 (extracted from tables 1-4).

Species included in study	Proportion values extracted for each plant family for each life stage											T1: Total plant material in diet (%)		
	T2: BR Adults				T3: NB Adults				T4: Chicks (%)					
	AM	*CR*	*PO*	*Total*	*AM*	*CR*	*PO*	*Total*	*CR*	*PO*	*Total*	*Br*	*NB*	*N/C*
Chaffinch	0	0	44	**44**	0	0	25	**25**			**n/a**	85	95	
Corn Bunting	0	0	44	**44**	0	0	75	**75**		16	**16**	85		13
Goldfinch	0	0	0	**0**	0	0	0	**0**			**n/a**	95	99	
Greenfinch	0	0	16	**16**	0	0	11	**11**	0	21	**21**	95	99	95
Grey Partridge	0	0	12	**12**	0	0	28	**28**	0	21	**21**	88	100	30
House Sparrow	0	0	37	**37**	0	0	23	**23**			***24**	6		4
Linnet	0	0	0	**0**	0	0	0	**0**	51	20	**71**	99	99	99
RL Partridge				**n/a**	11	0	33	**44**			***29**		100	65
Reed Bunting	0	0	0	**0**	17	0	52	**69**			***0**	39	100	0
Rook			38	**38**			58	**58**		34	**34**	42	78	18
Skylark				***22**	36	0	0	**36**			****2**	60	100	^6
Stock Dove	0	29	32	**61**	0	0	22	**22**	0	5	**5**			100
Tree Sparrow	0	0	22	**22**	14	0	22	**36**			***15**	4	60	5
Turtle Dove	27	41	31	**99**				**n/a**	32	38	**70**	100		100
Woodpigeon	0	18	32	**50**	0	7	38	**45**			***47**	98	95	97
Yellowhammer			92	**92**			32	**32**		4	**4**	23		35

Grey shading indicates where values are not available.

*Interpolated values calculated from the difference between total plant material (%) in chick and NB *or* Br diet for each species using extracted values for plant families.

**Interpolated using NB value.

^Range given in literature as 0-6.

Plant families data were extracted for: AM: *Amaranthacae* - includes sugarbeet; CR: *Cruciferae* (crops) - includes oilseed rape; PO: *Poaceae* (crops) - includes wheat, barley, oats, rye.

BR: breeding; NB: non-breeding; N/C: nestlings/chicks; T: table; RL: red-legged.

4.S1 Table. Summary of species diet (related to high-residue food items), species traits, model input and model output for each of the 22 species included in the analysis.

Species	Latin	Dietary data related to high-residue food items				Species traits			Model input		Model output					
		Adult BR (%)	Adult NB (%)	Chick (%)	Exp. group	Weight (g)	Status^	BBS trend* (%)	BBS sites (N)	Grid square (N)	Model	Estimate	SE	p-value	ODR	RMSE
Chaffinch	*Fringilla coelebs*	44	25	n/a	Med	21	Green	-11	3716	2478	QP	0.000836	0.000130	**<0.001**	0.93	4.58
Corn Bunting	*Miliaria calandra*	44	75	16	High	46.5	Red	-33	**635**	**533**	QP	0.000449	0.000542	0.407	1.25	2.25
Goldfinch	*Carduelis carduelis*	0	0	n/a	Low	15.5	Green	132	3476	2386	QP	**-0.000285**	0.000226	0.207	0.98	3.30
Greenfinch	*Carduelis chloris*	16	11	21	Med	28.5	Green	-51	3355	2327	QP	0.000846	0.000218	**<0.001**	1.04	3.59
Grey Partridge	*Perdix perdix*	12	28	21	Med	400	Red	-58	1387	1130	QP	0.000976	0.000432	**0.024**	0.67	1.11
House Sparrow	*Passer domesticus*	37	23	24	Med	22	Red	-17	2967	2140	QP	**-0.000922**	0.000222	**<0.001**	0.93	7.98
Jackdaw	*Corvus monedula*	n/a	n/a	11	Med	245	Green	68	3408	2333	QP	**-0.000164**	0.000253	0.517	1.24	**10.24**
Kestrel	*Falco tinnunculus*	0	0	0	Low	245	Amber	-20	2952	2095	P	0.000481	0.000293	0.100	0.81	0.60
Lapwing	*Vanellus vanellus*	0	0	0	Low	225	Red	-26	2343	1715	QP	0.000722	0.000396	0.069	**1.68**	6.44
Linnet	*Carduelis cannabina*	0	0	71	High	17.5	Red	-19	2997	2145	QP	0.001252	0.000280	**<0.001**	1.15	4.84
Red-legged Partridge	*Alectoris rufa*	n/a	44	29	Med	475	Green	3	2122	1593	QP	**-0.001437**	0.000252	**<0.001**	0.74	1.75
Reed Bunting	*Emberiza schoeniclus*	0	69	0	High	18.5	Green	44	1641	1287	QP	0.000609	0.000331	0.066	0.84	1.25
Rook	*Corvus frugilegus*	38	58	34	High	490	Green	-13	3209	2242	QP	0.001687	0.000294	**<0.001**	1.14	**27.25**
Skylark	*Alauda arvensis*	22	36	2	Med	39	Red	-23	3347	2293	QP	**-0.000298**	0.000143	**0.038**	0.97	3.46
Starling	*Sturnus vulgaris*	n/a	n/a	0	Med	82.5	Red	-61	3271	2288	QP	0.001210	0.000249	**<0.001**	**1.92**	**20.71**
Stock Dove	*Columbus oenas*	61	22	5	High	310	Amber	22	2654	1969	QP	0.000036	0.000292	0.903	1.47	3.01
Tree Sparrow	*Passer montanus*	22	36	15	Med	22	Red	64	**772**	**666**	QP	0.001692	0.000743	**0.023**	0.83	2.56
Turtle Dove	*Streptopelia turtur*	99	n/a	70	High	155	Red	-94	**775**	**635**	QP	**-0.002093**	0.000534	**<0.001**	0.74	0.95
Whitethroat	*Sylvia communis*	0	0	0	Low	15	Green	25	3035	2157	QP	**-0.000200**	0.000205	0.328	0.85	1.94
Woodpigeon	*Columbus palumbus*	50	45	47	High	515	Green	36	3698	2482	NB	0.000787	0.000160	**<0.001**	1.11	**21.06**
Yellow Wagtail	*Motacilla flava*	0	0	0	Low	20	Red	-42	**851**	**723**	QP	0.000557	0.000456	0.221	0.75	1.42
Yellowhammer	*Emberiza citrinella*	92	32	4	High	27	Red	-28	2676	1918	QP	0.000448	0.000212	**0.035**	0.85	2.34

Numbers in bold indicate those species with significantly fewer data points (BBS sites, Grid Square), species with negative estimates (Estimate), models that are over dispersed (ODR) and species with estimates that have a p-value of < 0.05 (p-value), and species with RMSE >10 (RMSE).

^Status of UK birds as defined by the RSPB according to Birds of Conservation Concern.

*BBS Trend: change in species populations in England between 1995 and 2016 obtained from 'BTO / JNCC / RSPB Breeding Bird Survey Trends 2017 – England'.

BR: breeding; Exp.: exposure; NB: non-breeding; BBS: Breeding bird survey; SE: Standard error; ODR: Over dispersion ratio; RMSE: root mean squared error; QP: quasi-Poisson; P: Poisson; n/a: not available.

4.S2 Table. **Alternative model outputs for each of the 22 species included in the study. A) 'Stepped' interpolation for those years where pesticide surveys did not take place (all odd years).** Neonicotinoid usage was assumed to be the same in years where no data were available, as the preceding year (rather than being estimated by a linear interpolation). These data are presented as a means to test the sensitivity of the model to interpolation approaches used. **B) Chronic toxicity values used to calculate the toxicity equivalency factor (TEF).** Chronic LOAEL values (at the 5% tail of acute sensitivity distribution for avian species) were used (rather than acute LD50 values for bobwhite quail *Colinus virginianus*) to calculate the TEF for the three compounds included in the study. Calculations were based on information provided in Table 3.2 of Mineau & Palmer (2013). These data are presented as a means to test the sensitivity of the model to differences between acute and chronic TEFs.

Species	**Latin**	**Model**	**Model output (A): stepped interpolation**					**Model output (B): chronic TEF**				
			Estimate	*SE*	*p-value*	*ODR*	*RMSE*	*Estimate*	*SE*	*p-value*	*ODR*	*RMSE*
Chaffinch	*Fringilla coelebs*	QP	0.000802	0.000126	**<0.001**	0.94	4.58	0.000841	0.000124	**<0.001**	0.93	4.58
Corn Bunting	*Miliaria calandra*	QP	0.000397	0.000529	0.453	1.25	2.25	0.000483	0.000530	0.362	1.25	2.25
Goldfinch	*Carduelis carduelis*	QP	**-0.000307**	0.00022	0.163	0.98	3.3	**-0.000283**	0.000215	0.189	0.98	3.30
Greenfinch	*Carduelis chloris*	QP	0.000840	0.000212	**<0.001**	1.04	3.59	0.000795	0.000209	**<0.001**	1.04	3.59
Grey Partridge	*Perdix perdix*	QP	0.000907	0.000425	**0.033**	0.67	1.11	0.001015	0.000420	**0.016**	0.67	1.11
House Sparrow	*Passer domesticus*	QP	**-0.000920**	0.000214	**<0.001**	0.93	7.98	**-0.000923**	0.000214	**<0.001**	0.93	7.98
Jackdaw	*Corvus monedula*	QP	**-0.000172**	0.000245	0.481	1.24	10.24	**-0.000188**	0.000240	0.433	1.24	**10.24**
Kestrel	*Falco tinnunculus*	P	0.000470	0.000289	0.104	0.81	0.6	0.000546	0.000282	0.053	0.81	0.60
Lapwing	*Vanellus vanellus*	QP	0.000663	0.000388	0.088	1.68	6.44	0.000605	0.000380	0.111	**1.68**	6.44
Linnet	*Carduelis cannabina*	QP	0.001217	0.000273	**<0.001**	1.15	4.84	0.001409	0.000268	**<0.001**	1.15	4.84
Red-legged Partridge	*Alectoris rufa*	QP	**-0.001422**	0.000243	**<0.001**	0.74	1.75	**-0.001407**	0.000244	**<0.001**	0.74	1.75
Reed Bunting	*Emberiza schoeniclus*	QP	0.001488	0.000335	**<0.001**	0.84	1.25	0.000661	0.000320	**0.039**	0.84	1.25
Rook	*Corvus frugilegus*	QP	0.001615	0.000285	**<0.001**	1.14	27.25	0.001617	0.000280	**<0.001**	1.14	**27.25**
Skylark	*Alauda arvensis*	QP	**-0.000308**	0.000139	**0.027**	0.97	3.46	**-0.000245**	0.000138	0.076	0.97	3.46
Starling	*Sturnus vulgaris*	QP	0.001207	0.000242	**<0.001**	1.9	20.71	0.001177	0.000239	**<0.001**	**1.92**	**20.71**
Stock Dove	*Columbus oenas*	QP	**-0.000045**	0.000283	0.874	1.4	3.01	**-0.000010**	0.000280	0.973	1.47	3.01
Tree Sparrow	*Passer montanus*	QP	0.001687	0.000728	**0.021**	0.83	2.56	0.001649	0.000713	**0.021**	0.83	2.56
Turtle Dove	*Streptopelia turtur*	QP	**-0.002318**	0.000526	**<0.001**	0.74	0.95	**-0.002071**	0.000524	**<0.001**	0.74	0.95
Whitethroat	*Sylvia communis*	QP	**-0.000215**	0.000198	0.276	0.85	1.94	**-0.000152**	0.000195	0.437	0.85	1.94
Woodpigeon	*Columbus palumbus*	NB	0.000769	0.000155	**<0.001**	1.11	21.06	0.000735	0.000153	**<0.001**	1.11	**21.06**
Yellow Wagtail	*Motacilla flava*	QP	0.000507	0.000444	0.254	0.75	1.42	0.000623	0.000445	0.162	0.75	1.42
Yellowhammer	*Emberiza citrinella*	QP	0.000390	0.000206	0.058	0.85	2.34	0.000511	0.000204	**0.012**	0.85	2.34

Numbers in bold indicate those species with negative estimates (Estimate), models that are over dispersed (ODR) and species with estimates that have a p-value of < 0.05 (p-value), and species with RMSE >10 (RMSE). LOAEL: lowest-observed-adverse-effect level; SE: Standard error; ODR: Over dispersion ratio; RMSE: root mean squared error; QP: quasi-Poisson; P: Poisson; n/a: not available.

4.S3 Table. Estimated total application of NN (weight and TEF-adjusted weight) in each region for the entire study period (1994-2014).

Region	NN compound applied (kg)			NN compound applied (TEF-adjusted kg)			Total NN applied		Mean NN applied per grid square
	IMI	*CTD*	*THX*	*IMI*	*CTD*	*THX*	*(kg)*	*(TEF-kg)*	*(TEF-kg)*
East	995972	544676	171654	995972	43574	17165	1712302	1056712	4.40
East Midlands	426371	234438	26709	426371	18755	2671	687518	447797	2.87
London & South East	279468	259986	0	279468	20799	0	539454	300267	0.85
North East	25567	76540	0	25567	6123	0	102107	31690	0.54
North West	11149	51217	0	11149	4097	0	62366	15246	0.09
South West	105335	221653	0	105335	17732	0	326987	123067	0.48
West Midlands	209659	210885	0	209659	16871	0	420544	226530	1.75
Yorkshire & Humber	125050	142407	3288	125050	11393	329	270745	136772	1.14

CTD: clothianidin; IMI: imidacloprid; NN: neonicotinoid; THX: thiamethoxam; TEF: toxicity equivalency factor.

Discussion

Overview & assimilation of data

Data obtained as part of this thesis have identified that insecticide seed treatments are a significant source of neonicotinoid (NN) exposure to farmland birds in the UK. The study design implemented in chapters two and three allowed for measurements of exposure pre- and post-sowing, which revealed that the incidence of exposure among individuals rose from 9% (5/54) to 68% (87/128) after NN-treated seeds were sown. Moreover, it is likely that those 5 birds in the pre-sowing group where CTD was detected (3 blackbirds *Turdus merula*, 1 starling *Sturnus vulgaris*, 1 red-legged partridge *Alectoris rufa*), were exposed at sites outside of those sampled, particularly as two out the three species can be migratory. Comparatively, the prevalence of exposure in other studies that have measured NN residue in individual free-living birds is on average 32% [1-7]. The concentrations of the NN compound clothianidin (CTD), detected in blood plasma were also found to be among the highest of any NN recorded in avian samples to date. Overall, 72% of positive plasma samples were above the highest known concentration of NN recorded in avian plasma (3.28 ng/mL [3]), and 43% of plasma samples contained CTD at concentrations above those reported for imidacloprid in bobwhite quail *Colinus virginianus* (26.2 ng/mL), when birds were dosed with 30 imidacloprid-treated wheat seeds as part of a toxicokinetic study [8]. It is likely that exposure recorded here is significantly higher, both in terms of frequency and magnitude, than that reported in previous studies because existing data were collected at indiscriminate times of year with regards to the sowing season and therefore would not have captured this type of exposure specific to seed treatment applications. Collectively, these findings suggest that there is a period of peak exposure immediately post-sowing, with incidences here being detected up to 30 days after treated seeds were sown. As the study here was restricted to this timeframe, it is possible that this period of peak exposure could extend beyond this time point. Indeed a study by Roy *et al.* found small concentrations of imidacloprid on treated soybean seed left on the soil surface for more than 30 days [9].

Previous studies of avian NN exposure have always focused on one species when measuring NN compounds in avian biological samples collected from the field. Prior to data presented here, exposure was confirmed in 12 bird species across multiple countries using various biological sample types, such as feather, blood and liver [1-7, 10-13]. In addition, exposure was confirmed in a further 38 species by observations of treated seed ingestion (as well as two species for which biological samples are also available) [9, 14]. One of the main aims of this thesis was to gain an understanding of the frequency of NN exposure within a typical agricultural avian community, and the level of

exposure within individuals across multiple species. In chapters two and three, exposure was confirmed in a total of 21 species of bird (7 via observational data, 14 via biological samples) and 68% of individuals (via biological samples only) post-sowing. It is pertinent to compare data collected here with the largest avian single-species NN exposure study to date, undertaken by Humann-Guilleminot *et al.* (2018). In that study, NN exposure was detected in 100% of pooled feather samples collected from 617 individual house sparrows *Passer domesticus* across an area of ~15,000 km^2 in Switzerland [11]. Although the pooling of feather samples may have inflated the frequency at which NNs were detected, the study provided evidence that a large proportion of individuals belonging to one species can be subject to exposure. Comparatively, data presented here confirms exposure in a significant portion (30%) of farmland bird species sampled from Eastern UK. NN application methods in these two different countries are very similar, as are the species that reside in farmland habitat - including house sparrow, which tested positive for CTD in both sets of data. Ultimately, if one species out of the 21 that tested positive here for NNs can be subject to exposure on such a large scale elsewhere, it is also possible that this is occurring for other species within farmland bird communities for which large datasets have not yet been collected. When these data are examined collectively, there is evidence that NN exposure is widespread among multiple species where NN seed treatments are in use, and that this has the potential to affect a large proportion of individuals belonging to those species affected.

Overall, the study design used here to examine environmental NN exposure to birds via treated seed was effective and provided a clear distinction in patterns of exposure pre- and post-sowing. However, for practical reasons much of the data for elements of the exposure pathway (e.g., seed density, seed and seedling residue data) were collected from different sites to those where avian samples (biological) were obtained. A basic measure of surface seed densities was recorded at sites used for avian sampling (see **Appendix**), but these could not be incorporated effectively into the analyses presented in chapters two and three due to the variability and resolution of the data. In any future studies using a similar design it would be useful to collect a more detailed seed density dataset that is directly comparable to avian exposure data to better understand inter-site variation in patterns of exposure among individual birds, particularly where large NN concentrations in plasma are observed. Likewise it would also be pertinent to collect data from a smaller sub-set of sites for a longer period of time to establish the temporal extent of avian exposure to NNs from treated seed.

The rate of avian exposure recorded as part of this thesis, subsequently gives rise to the possibility that many wild birds are vulnerable to the potential sub-lethal effects of NNs on avian physiology and behaviour, which have previously been reported in aviary studies [15]. Here, an attempt was made to investigate whether sub-lethal effects were measurable under field conditions, where NNs applied as part of standard agricultural practices were the only source of exposure. Of the five physiological parameters investigated, only one had a significant association with NN exposure (faecal parasite load), providing some evidence for sub-lethal effects of NN in the field. This area of research is one that requires further attention, particularly as the size of the data sets for each species obtained in chapter two, and the nature of managed galliforme populations as examined in chapter three, may have precluded any further associations between NN exposure and bird health from being detected. Comparatively, the only other study to investigate sub-lethal effects of NNs in the field did so by dosing a migrant bird species at a stopover site before release [16]. By using this hybrid dosing-field experimental design, Eng *et al.* were able to confirm that migratory behaviour can be altered with exposure to NNs; however, that study did not confirm that this occurs in the wild as a result of exposures experienced by free-living birds via agricultural NN applications. Measuring sub-lethal effects of toxicants in the field when the rate and time of exposure is unknown, remains a major challenge within ecotoxicological studies. Some headway has been gained here with NNs by using seed treatment sowing dates as a means of providing a temporal control group to measure the effects of exposure, and there is potential for this type of study design to be refined and expanded in the future to further investigate sub-lethal effects under field conditions.

Data collection for avian health parameters in relation to NN exposure was limited by the NN ban that came into force in 2018, which resulted in all biological avian samples (passerines and galliformes) being collected during one autumn field season. As such, it was not possible to refine protocols or gather more data during a second field season and therefore several suggestions can be made here for further research. Firstly, a more in-depth single- or dual-species study that uses a similar pre/post-sowing design would allow for inter-species variation to be controlled for and avian biometric data to be more efficiently analysed in conjunction with exposure data. Data gathered as part of chapter two suggest that good candidate species would be house sparrow *Passer domesticus* and dunnock *Prunella modularis*, due to the rate at which they were exposed and the numbers of individuals caught at NN-treated sites. Comparatively, data presented in chapter three suggest that managed gamebirds would not be suitable for further analyses of this kind due to confounding factors associated with their hybridised ecology between captive and free-

living conditions (e.g., supplementary feed). Secondly, any future study would need to better account for diurnal variability in exposure and biometric data (e.g., weight) by collecting data consistently at the same time of day. This was not possible here due to a lack of resources and could not be statistically controlled for within the data obtained due to sample size, but patterns within the data obtained did suggest that both exposure and health parameters differed within a 6-hr period (e.g., the rate of exposure is likely to increase with feeding time, in addition to bird weight). Thirdly, the range of biometric data and related sub-lethal effects could be expanded beyond those used here. In aviary studies many different biometric data have been gathered from birds in relation to NN exposure; however here, we only collected data for haematocrit, body condition, weight, fat score and parasite load. Blood slides were also made from all available passerine and galliforme blood samples, with the aim to perform white-blood-cell counts, but unfortunately the quality of the slides was not sufficient. These health parameters were selected for several reasons, including the practical limitations of field-based data collection, associated costs and obtainable sample volumes. Of these, the main limiting factor was the amount of blood that could be obtained. Passerine samples were limited to the equivalent of 1% of the total weight of the bird, whereas clotting prevented large volumes of blood being collected from galliformes post-mortem. As a result, each blood sample only provided enough plasma for one round of chemical analysis, which was essential for confirmation and quantification of exposure for each individual bird. Any future studies would need to either consider using a different method to measure exposure, so that a wider variety of tests could be performed with blood samples (e.g., hormone assays or mechanised white-blood-cell counts) or sampling from live species of a larger body weight; however, the latter would exclude many vulnerable passerine species from this type of analysis. Overall, there is a lot of work still to be done to fully understand the impact of environmental NN exposure on individual free-living birds, but these data will be important if we are to understand the biological pathways by which exposure may translate to population-scale effects for wild birds.

Although NN exposure via seed treatments was confirmed in a large proportion of bird species sampled, this did not translate to population-scale effects when long-term data were modelled for key indicator species over the last 21 years of NN use in England. Overall, there was no evidence to suggest that dietary exposure to NNs via seed treatments has had any consistent impact on farmland bird populations. However, the populations of four species – house sparrow, skylark *Alauda* arvensis, turtle dove *Streptopelia turtur* and red-legged partridge *Alectoris rufa* – were found to be negatively associated with the mass of NN applied as seed treatments. When each species is considered separately, all but turtle dove warrant further investigation, as this species

has experienced dramatic population declines over the last three decades [17] that are likely to have contributed significantly to the extreme model estimate obtained. Of the remaining three species, biological samples were available as part of chapters two and three for house sparrow and red-legged partridge. Both of these species were observed consuming treated seeds at seed piles, had a high frequency of CTD detection across all biological samples available, and large concentrations of CTD in plasma relative to other species sampled. Indeed, house sparrow had the largest median CTD plasma concentration out of all species for which samples were available. It would therefore appear that these data tally between individual and population scales. It is also of note that house sparrow is the species described previously in Humann-Guilleminot *et al.* with high detection rate of NNs in feather samples [11], and that red-legged partridge have been cited in other reports as being susceptible to the ingestion of NN-treated seed in the field [14, 18, 19]. As yet, no exposure data have been obtained for skylark, but these would be important to collect to confirm whether exposure occurs in this species. It is unclear why negative impacts were not detected in more species, given the number of individuals for which exposure was confirmed in chapter two. These results suggest that the level, timing or perhaps period of exposure individuals are subject to does not significantly influence survival or reproduction for the majority of species and therefore does not affect population trends. It may also be that differences in species sensitivity to NN exposure [20] may produce varying population-scale effects so that no consistent effect of exposure via treated seed among multiple species is observed. Alternatively, it could be argued that the modelling approach used here could benefit from further methodological development and/or expansion to cover a larger number of potential exposure pathways.

One important aspect to consider in relation to the population modelling undertaken in this thesis is that the approach focused only on population changes in relation to dietary exposure to NNs via treated seed. This chapter did not assess the possibility that bird populations could be affected via loss of insectivorous prey items, as has been hypothesised in other population-scale studies [21]. The approach used in chapter four could be adapted in the future for this purpose (e.g., proportion of insect food items in diets assessed instead of crop material), which may provide important and comparable data for other potential NN exposure pathways for species of farmland bird. Equally, it may be useful to single out two species for which opposing model estimates were obtained, and to create a more detailed model that includes a wider variety of environmental factors that may affect population trends such as climate, land use [22] and species mobility (e.g., territorial vs resident vs migratory species). This approach may be useful to better understand the model estimates obtained from the ‘one size fits all’ approach presented for multiple species in chapter four, and

allow for a more in-depth investigation into those species for which a negative association between NN use and population change was observed. Even if these methodological alterations were implemented, one of the key issues that remains with this modelling approach is the spatial resolution at which pesticide usage data is available. If pesticide data could be supplied at a field-level resolution that did not necessitate complex extrapolation from a regional scale, then this would provide better spatial matching of avian abundance data to localised pesticide use. Furthermore, the UK is one of the few countries that has collected long-term pesticide usage data, therefore the areas of NN use where this technique can be applied are limited at present.

Data presented as part of this thesis has in many ways provided a crucial initial quantification of the exposure pathway and potential effects of NN seed treatments and farmland birds *in situ*, using many types of data collected from the field. These data have given scope for future work outside of aviary systems that can focus on environmental NN exposures at both an individual- and population-scale level. Although further work is required to fully understand how NN exposure affects individual farmland birds, data presented here confirms that seed treatments are a significant source of exposure for avian multiple species during periods of sowing. These data are relevant to any future risk assessments for seed treatments and current agricultural policies in countries where NN seed treatments are in use.

Implications for risk assessment & policy

Data presented here confirm that regulatory risk assessments and NN product safety labels have not been effective in safeguarding wild birds from significant NN exposure via seed treatments. One of the main reasons for the high exposure rates observed is the availability of NN-treated seed on the soil surface after sowing, which was commonplace among all farms sampled in chapter two. High densities of treated seed were also found on the soil surface at Lincolnshire sites that were sampled for passerines and galliformes (see **Appendix**); an average of 3.9 ± 0.6 (SE) treated seeds per m^2 were recorded on the soil surface, with seeds present at 23 out of 24 fields sampled. Seed treatment product labels specify that individual seeds and spillages should be incorporated back into the soil or cleared up so that wildlife cannot access treated seed [23]; however, in practice this often does not occur [9]. Here data were not gathered to assess why this might be, but time pressures associated with the sowing season, inefficiency of farm machinery, or a lack of understanding of key product safety issues are potential causes [9]. Aside from the risk posed by the ingestion of available treated seed, data collected here and elsewhere also suggest that the solubility of NN compounds and the systemic application of NN seed treatments may also create

additional exposure pathways for farmland birds [9, 24, 25]. For example, the presence of NNs in non-agricultural plant material, surface water bodies and/or vertebrate prey items (for predatory bird species) have been evidenced in the literature [4, 6, 25-28], all of which could cause secondary exposure if ingested. This is something that needs to be better addressed in risk assessments, as changes to agricultural practices are unlikely to prevent the hazards associated with these pathways when seed treatments are applied *in situ*.

Finding effective methods to improve current risk assessment protocols is difficult given the complexities of risk associated with agrochemical use. It has recently been suggested that long-term data could be used as a method of biomonitoring for agrochemicals in wildlife, which could complement existing regulatory risk assessments and/or act as an early warning system for adverse effects in non-target organism populations [29]. In chapter four, this approach was implemented to assess the impact of NN usage on bird species over the last 21 years, and the advantages and disadvantages were discussed. One of the key issues identified was the availability and spatial resolution of pesticide usage data, which is perpetuated by the need for anonymity that may restrict the accuracy of model outputs. However, there are many sources of population data for non-target organisms that have not been utilised in this manner, and with improved availability of pesticide usage data, this approach has the potential to be extremely useful for assessing the impact of agrochemicals over long periods of time. Comparatively, biomonitoring could also be used over shorter periods to ground-truth the estimated frequency and rate of exposure to agrochemicals in non-target organisms. This is currently something that is only undertaken by research groups within the scientific community rather than regulatory risk assessment bodies, but can provide valuable real-world data for patterns of exposure in the field. In chapters two and three of this thesis, the NN compound CTD was measured in multiple sample types from the exposure pathway from NN seed treatments to farmland birds (crop seed, crop seedlings, bird plasma, bird liver), and the density of treated seed on the soil surface was recorded. Collectively these data validated the hypothesis that seed treatments were a source of exposure for farmland birds and provided a measure of exposure in this group of non-target organisms. In the last few years refinement steps for the European Food Safety Authority risk assessment for birds and mammals have moved towards a field-based setting (e.g., the use of radio-tracking data to estimate exposure) [30], and there have been calls for real-world data to be used so that better quality risk assessment decisions can be made [31]. As such, collecting similar field data as those presented in this thesis from test sites could conceivably be used to assess risk, or the significance of a particular exposure pathway.

This may be effective on a case-by-case basis in the final stages of risk assessment to address issues such as those observed with NNs.

With an increasing list of risks associated with NN use and the environment, it is worth considering the efficacy and necessity for this group of insecticidal compounds. Over the last two decades the reliance on prophylactic seed treatments has increased significantly [32]; however, it is debated whether this is beneficial to overall crop yields [33-35]. The literature regarding this topic was renewed for NNs and fipronil in the latest Worldwide Integrated Assessment (WIA) for systemic insecticides, and several key findings were put forward [33]. Firstly, there is evidence from multiple studies that cropping yields are not positively associated with NN applications, and that actually, the adverse effects of NNs on invertebrate fauna may limit yields of pollinated crops [33]. Comparatively, additional evidence from studies that have investigated the distribution of NNs in agricultural plants after seed applications suggests that the protection afforded to crops against target pests may be minimal [36]. These data are consistent with a recent study by Humann-Guilleminot *et al*., which reported that up to 12.5% of beneficial invertebrate species may be exposed to sub-lethal concentrations of NNs under a field-realistic, worst-case scenario (based on hazard quotients), whereas no pest species would be subject to lethal exposures [26]. Secondly, the WIA advised that the use of prophylactic seed treatments should be stopped as a priority because the majority of preventative applications make very little difference to overall cropping yields and integrated pest management strategies are likely to be much more effective [33]. Indeed, a detailed study by Lechenet *et al.* estimated that pesticide application could be reduced by 42% in 59% (of 946) of farms sampled, without productivity or profitability being negatively affected [34]. Furthermore, one large case study in Italy found that the economic cost of applying insecticides was greater than the cost of providing an insurance-based pay-out for areas of land that are vulnerable to pest damage, which can be effectively predicted on a year-by-year basis using environmental data [33]. Thirdly, the WIA reported that the dramatic increase in NN applications since their introduction to the agricultural market has led to resistance being developed by many pest species at a faster rate than predicted [33]. This resistance is specific to the neural mode of action via nicotinic acetylcholine receptors, which means that any future NN compounds (or similar) to be developed will inherit this issue caused by the apparent overuse of NNs [33].

Agricultural policies for NN usage worldwide are inconsistent and are reflective of the ongoing debate surrounding NN efficacy and ecotoxicological safety. Thus far, the only data to have impacted agricultural policies are those collected for the adverse effects of NN use on pollinator

species, a group crucial to ecosystem functioning. In the EU this led to a ban on all NN seed treatment compounds in 2018, and Fiji have followed suit with a ban on IMI as of January 2020. Canada is the only other country to consider similar action, whereas policies in the USA have been changeable with the political climate. Elsewhere, the use of NNs continues with no signs of change, and one of the more toxic compounds to birds (imidacloprid) is still commonly applied [32]. As avian research within this area continues, it is becoming apparent that bird species may be facing a similar threat to that experienced by pollinators, with evidence of exposure and adverse effects accumulating along a similar trajectory. Indeed, data gathered as part of this thesis have provided evidence of widespread NN exposure in free-living farmland bird communities, and some evidence of the impact of seed treatments on avian populations and individual health. It is of note that product safety labels used on seed treatments in the UK are similar to those employed elsewhere, and in particular the USA and Australia rely on these labels to prevent the availability of treated seed to wildlife and subsequent NN exposure [37, 38]. Here, we provide evidence that the drilling process does not comply with these label stipulations that are therefore ineffective, and as such, it could be argued that policies relating to NN use should be changed to reflect this. Ultimately, data here pinpoint seed treatments as a significant source of exposure for farmland bird communities, the full impact of which remains to be understood. Changes to NN usage policies that prohibit the use of seed treatments will therefore not only benefit pollinator species, but also prevent seed-related exposure and its potential impacts on farmland birds.

In the UK the use of NNs has been banned since 2018 in accordance with EU regulations; however, data presented in this thesis are relevant to aspects of current agricultural policy in relation to general seed treatment use. In 2018, fungicides (such as fludioxonil, prothioconazole and tebuconazole) were the most common seed treatments in the UK after CTD, and these applications are set to continue [39]. Similarly to NNs, fungicides have been found to adversely impact avian biology [40] and therefore seeds treated with these compounds could also theoretically a pose a risk to farmland birds. At present the UK cross-compliance document for farmers does not contain any specific guidance on the use of pesticide-treated seed [41], but data presented here suggests that it is advisable to include guidance on the proper use of seed treatment applications. Arguably, it may be that an effective method of seed treatment usage is beyond the scope of current farming practices because of the specific time constraints during the sowing season and variability in machinery and environmental factors. If it is not possible to ensure that all treated seeds are buried after drilling, it may not be tenable to retain seed treatments as a safe way to apply pesticides. Indeed, the cycle of market approval followed by withdrawal for many seed treatment compounds

appears to support this theory. The Pesticide Action Network has lobbied the government to reduce and sustainably use the amount of pesticide applied as part of the new Agricultural Bill (2019-2021), and has suggested that a pesticide tax be implemented [42]. If a pesticide reduction and/or tax were to be actioned in the UK, other options for pesticide management or compensation would be needed to sustain either cropping yields or income. At present the Department of Agriculture and Rural Affairs is developing a new agri-environmental scheme that will be rolled out by 2024 [43]. The Environmental Land Management Scheme plans to include some financial compensation for integrated pesticide management, biological control and precision pesticide application [43]. Although this scheme is voluntary, it would appear that UK agricultural policy is set to encourage the overall reduction in pesticide usage. However, in the advent of Brexit and with increasing pressure to improve cropping yields there is the potential for compounds such as NNs to be reintroduced, the use of seed treatments to continue and for pesticide applications to move away from an EU approach. In addition, the UK will also need to perform its own regulatory processes for agrochemical registration, rather than the continued use of the European Food Safety Authority protocols. Therefore, it is important that field data relating to seed treatments specifically (such as those collected here) is disseminated accordingly to inform any future changes to UK policy, in the hope that adequate protection is afforded to farmland birds and other wildlife taxa.

Conclusions

There is a delicate balance to be struck between efficacy and safety of agrochemicals, particularly when large swathes of the wider landscape are used for agricultural purposes. These landscapes are central to the ecologies of large numbers of wild species, some of which provide crucial services within the ecosystem, and others that rely solely on agricultural habitats. Farmland birds are a species group that have undergone significant losses worldwide over the past few decades, with common and rare species alike experiencing population declines [44-47]. Data presented here provide evidence at an individual and community scale of widespread NN exposure to wild birds via seed treatments, and some evidence at an individual and population scale of the impacts of this on bird species. The future of NN use outside of the EU remains unclear; however, in light of the increasing weight of evidence for NN exposure in free-living birds, adverse effects on avian physiology and behaviours and the increasing availability of NNs in the wider environment, it is perhaps time for non-chemical alternatives to seed treatments to be implemented, before the tipping point for avian ecological safety is discovered.

Summary of key findings

Data collected as part of this thesis provide evidence that:

- There is a viable exposure pathway for NNs to farmland birds, via seed treatments
- This pathway causes widespread exposure in UK farmland bird communities, among species and individuals
- The application of seed treatments is likely to cause a period of peak exposure immediately following sowing, but the temporal extent of this has not yet been established
- Exposure experienced after the sowing of NN treated seed can result in a increased faecal parasite load at an individual level
- Data and subsequent modelling approaches currently available provide no consistent evidence that dietary exposure to NN-treated seed has impacted farmland bird populations in England, but some species populations were found to be negatively associated with the application of NNs
- The availability of pesticide usage data at a finer spatial scale would improve our understanding of the long-term impacts of NN use on non-target species populations
- Risk assessments may be improved and/or supplemented by the use of field data for the purposes of long- or short-term biomonitoring using methods similar to those presented here

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Appendix

Surface seed densities at Lincolnshire sites

As part of data collection for chapters two and three, the density of neonicotinoid (NN)-treated seed was recorded at sites in Lincolnshire, from which biological passerine and galliforme samples were obtained. Due to time and manpower constraints, these surveys were basic; however, they give some indication of the presence of treated seed in fields from which avian samples were collected.

For each sampling event post-sowing when either passerine blood samples or galliforme carcasses were collected, the densities of NN-treated seed on the soil surface were recorded. Where possible, these surveys took place within 48 hrs of each post-sowing avian sampling event. Between one and four fields in the surrounding area to the ringing site or shoot location were sampled (depending on the availability of NN-treated cereal fields in the area immediately adjacent to the sampling location), and between 10-20 quadrats readings taken per field, per sampling event (depending on time constraints). Within each of the 1x1 m^2 quadrats, the number of visible NN-coated seeds was recorded. Quadrats were randomly placed (e.g., thrown so that no placement bias occurred) and evenly spaced along field headlands and a diagonal transect of the field centre. Data were collected evenly from the field headland and field centre.

During passerine sampling (avian blood plasma data presented in chapter two), 11 fields were sampled (400 quadrats). The mean density (±SE) of treated seed on the soil surface among these fields was 2.5 ±0.5 seeds/m^2 (headland: 4.2 ±0.9/m^2; centre: 0.9 ±0.1/m^2). Seeds were present at the soil surface at all 11 fields sampled, but the density differed between sites (**Table A1**). Inter-site difference in seed surface density somewhat mirrored the median concentration of clothianidin (CTD) detected in avian samples, with the site presenting the highest mean seed surface density, also presenting the largest CTD concentration (**Table A1**).

Table A1. The number of clothianidin (CTD)-treated seeds on the soil surface and median concentrations of CTD recorded in avian plasma recorded at Lincolnshire sites sampled in chapter two.

Site	Surface seed density (per m^2)			CTD plasma concentration (ng/mL)		
	N quadrats	*Mean*	*SE*	*N birds*	*Median CTD*	*IQR*
1	80	1.9	1.2	18	5.80	12.6
2	120	1.9	0.3	9	0.15	2.45
3	80	0.5	0.2	19	0.15	37.0
4	20	0.4	0.2	13	0.15	0.35
5	80	6.4	1.9	10	647	3881
6	20	3.9	1.6	2	0.15	0.00

N: number of; SE: standard error; IQR: inter-quartile range.

During galliforme sampling (data presented in chapter three), 13 fields were sampled (280 quadrats). The mean density (±SE) of treated seed on the soil surface among these fields was 5.7 ±1.3 seeds/m^2 (headland: 10.2 ±2.7/m^2; centre: 1.3 ±0.3/m^2). Seeds were present at the soil surface at 12 out of the 13 fields sampled, but the density differed between sites (**Table A2**). Inter-site difference in seed surface density did not reflect the median concentration of CTD detected in avian samples (**Table A2**). This may have been due to the fact that seed densities were recorded for fields where carcasses were collected, but birds may have been driven from areas outside of the carcass collection point.

Table A2. The number of clothianidin (CTD)-treated seeds on the soil surface and median concentrations of CTD recorded in avian liver and plasma recorded at Lincolnshire sites sampled in chapter three.

Site	Surface seed density (per m^2)			CTD liver concentration (ng/g)			CTD plasma concentration (ng/mL)		
	N quadrats	*Mean*	*SE*	*N birds*	*Med*	*IQR*	*N birds*	*Med*	*IQR*
1	20	0.1	0.1	10	0.05	0.04	5	0.15	0.00
2	80	5.4	3.0	14	0.50	8.87	11	15.2	570
3	80	9.2	3.2	9	0.30	0.26	7	0.15	36.5
4	41	3.5	2.3	15	0.02	0.08	6	0.28	2.20
5	20	15.6	5.4	6	0.04	0.66	2	0.38	0.23
6	40	0.1	0.1	3	1.28	18.26	2	246	238

N: number of; SE: standard error; Med: median; IQR: inter-quartile range.