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Risk assessment of lead poisoning and pesticide exposure in the declining population of red-breasted goose (*Branta ruficollis*) wintering in Eastern Europe

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ABSTRACT

The red-breasted goose *Branta ruficollis* is a globally threatened species (IUCN Vulnerable) and the only European goose species currently in decline. Working on the wintering grounds on the Black Sea Coast, we address two potential causes of decline of this species for the first time: lead poisoning, and contamination from pesticides. We quantified the densities of spent Pb shot in three wetlands used by the geese in north-east Bulgaria, and analysed the Pb concentration in the faeces of red-breasted geese and the more abundant greater white-fronted geese *Anser albifrons*, using Al concentration as an indicator of soil ingestion. Pb shot densities in sediments were low, and we found no evidence for Pb shot ingestion in red-breasted geese. On the other hand, we found that the geese were feeding on wheat whose seeds were treated with four fungicides: thiram, tebuconazole, difenoconazole and fludioxonil, and the two first were even detected in geese faecal samples. Using data on the daily food intake, we estimated the exposure levels of the geese to these fungicides, both by measuring the concentrations remaining on seeds and by estimating the amount used to coat the seeds at the time of sowing. We found that the exposure rates estimated during the sowing period for both geese species can exceed the recognized hazardous doses for thiram, and to a lesser extent for tebuconazole, which indicates that some pesticides may be playing a previously overlooked role in the decline of red-breasted geese.

1. Introduction

The red-breasted goose *Branta ruficollis* is a globally threatened species (IUCN category: Vulnerable) and is one of the most threatened goose species in Eurasia (Birdlife International, 2015). Red-breasted geese breed on the Taimyr, Gydan and Yamal peninsulas in Russia. In recent decades, the entire world population of this Arctic breeder has wintered on the north-western Black Sea coast in Ukraine, Romania and Bulgaria (Dereliev, 2006). The population declined dramatically in the early 2000s, dropping to fewer than 40,000 birds (Dereliev, 2006; Cranswick et al., 2012). A recent assessment concluded that this is currently the only goose species in decline in the Western Palearctic (Fox et al., 2010), although there are some indications of recovery in recent years (Aarvak et al., 2012; Petkov, 2013). The reasons for recent declines are not very clear, although likely to include the effects of hunting, changes in land-use and climate change (see Section 4). In this paper, we study the possible impact of pollutants on this species in wintering grounds in Bulgaria. We have focused our investigation on those chemicals that may represent a risk for red-breasted goose because of their feeding habits and habitat use, namely lead poisoning and pesticide exposure.

Lead poisoning is a major conservation problem for many European Anatidae (Mateo, 2009). Lead contamination of wetlands with spent shot pellets was shown to be a serious problem in Greece (Pain and Handrinos, 1990), a neighbouring country for Bulgaria. Ingestion of spent lead shot and of lead from mining waste have been shown to affect an important proportion of the population of greylag geese *Anser anser* wintering in southern Spain (Mateo et al., 2007). However, no previous information is available about the prevalence of lead contamination in Anatidae wintering along the Black Sea Coast. Hunting in the wetlands of this area can be intensive, as in other European countries (Thomas

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and Guitart, 2010), and to date Bulgaria has only banned the use of Pb shot 200 m around wetlands (MoEW, 2007). The level of compliance of this ban is unknown and may be limited if not accompanied by an effective law enforcement (Cromie et al., 2010; Mateo et al., 2014). The AEWA action plan for the red-breasted goose highlighted the need to assess whether or not lead poisoning is a problem (Cranswick et al., 2012).

The second group of chemicals that can affect the red-breasted goose population are the pesticides currently used in agriculture and known to affect farmland birds (Mineau et al., 2001). Goose species could be especially at risk of being exposed to pesticides used for seed treatment, for several reasons (EFSA, 2009; Goulson, 2013; Lopez-Antia et al., 2016). First, geese could ingest pesticide-treated seed in recently sown fields. Second, geese feeding on early grown shoots of wheat and barley could also ingest the germinated seed remaining with the plant. Third, geese commonly grazing on cereal shoots could be exposed to systemic pesticides transferred from seeds to leaves throughout the wintering season. Because of this, pesticide exposure in geese species has frequently been reported in farmland areas (Hamilton et al., 1976; Stanley and Bunyan, 1979; Blus et al., 1984; Madsen, 1996). Seed coating, especially with insecticides, was estimated to be responsible for up to 50% of cases of lethal poisoning of wildlife caused by approved pesticides in European countries (De Snoo et al., 1999). Moreover, rodenticides can also be a threat when the treated bait (i.e. cereal seed) is spread on the soil surface for the control of vole plagues (Olea et al., 2009).

In this paper we study the Pb shot densities in the main Bulgarian wetlands used by red-breasted geese and greater white-fronted geese *Anser albifrons* for roosting. Although the geese spend most of the daytime grazing on cereals in the surrounding fields, they visit the wetlands regularly during the day for drinking, for roosting or as a refuge from disturbance. In order to study the ingestion of Pb shot in both geese species, and because of the difficulties of capturing them in large open fields, we used a non-invasive method based on the analysis of geese excreta as used in previous studies (Mateo et al., 2006; Martínez-Haro et al., 2013; Aloupi et al., 2015). This method involves measuring Pb concentration in faeces and relating it to aluminium content (Al), which is a good marker of soil ingestion (Martínez-Haro et al., 2010). The relationships between Pb and Al levels in excreta and in soil samples from the study area are used to identify faecal samples in which other sources of Pb (i.e. Pb shot) are likely to explain observed Pb levels. At the same time, samples of the winter wheat grazed by geese were taken for pesticide analysis, in particular the root containing the remains of the seed (often with an evident red staining indicative of pesticide coating). Moreover, the non-invasive sampling of faeces was used to monitor pesticide exposure in geese. The potential effect on geese of the detected pesticides was evaluated according to the expected concentration in seeds at sowing time and to the concentrations measured in plants in winter.

2. Material and methods

2.1. Study area

Bulgaria is situated at the southern end of the red-breasted goose flyway, forming a major part of its wintering range. The core wintering range is located around the N and NW Black Sea Coast, covering several large lake complexes in Ukraine (Sivash lake complex, Danube Delta and Azov sea area), Romania (the Danube Delta and some polder areas around the Danube) and Bulgaria (the so called Coastal Dobrudzha). Dobrudzha in NE Bulgaria comprises a major winter site for this species, with roosts at the coastal lagoons of Shabla and Durankulak and on the adjacent Black Sea (Dereliev et al., 2000). In recent decades, the highest winter concentrations of the species have been recorded in this area. Total goose numbers at these roosts peak at up to 300,000 individuals in some years (mainly greater white-fronted geese, Kostadinova and Dereliev, 2001; BSPB, unpublished data). Both Shabla and Durankulak lakes are Special Protection Areas (SPAs) and part of the Natura 2000 network in Bulgaria. When geese numbers are particularly high they also roost in the sea bays along the Black Sea coast in the Kaliakra SPA. Our study area included arable fields used for grazing by the geese around Durankulak Lake SPA, Shabla Lake Complex SPA and Kaliakra SPA (Fig. 1). Larger numbers of greater white-fronted geese were also grazing in these fields. At the time of our sampling (17–20 January 2012), 1000 red-breasted geese and 7000 greater white-fronted geese were present in the study area.

2.2. Sampling

Lead shot densities were studied in shallow water areas (where geese could reach the sediments and be at risk of Pb shot ingestion) near the shorelines of Durankulak Lake, the adjoining Eagle Marsh and Shabla-Tuzla Lake (Fig. 1). For this purpose, sediments at 5–15 points separated at least 10 m apart in each site were sampled along transects with a metallic corer of 6 cm diameter. At each point, 5 samples were collected from the upper 15 cm of sediment, washed and sieved in situ with a 1 mm mesh, and the retained material was pooled and stored in a Ziploc plastic bag until further laboratory examination and metal analysis.

In order to interpret the results of Pb and Al analysis in geese faces we collected sediment and soil samples of the sites used by geese. Sediment samples of Durankulak Lake (n=3) and Eagle Marsh (n=3) were collected with the corer (upper 5 cm) to determine Pb and Al levels. Sediment samples from Shabla-Tuzla Lake were not taken for Pb and Al analysis because geese were not using this wetland during our sampling period. Soil samples (n=16) were collected with a small shovel at 5 winter cereal fields where the red-breasted geese were grazing (Fig. 1). Samples were taken at 3 points along a transect in each field with a separation of 20 m between points. At each point, 5 soil samples were taken and pooled in a plastic bag to reach an overall mass of approximately 100 g. One additional soil sample was collected in a cereal field around Durankulak Lake.

Faecal samples of greater white-fronted and red-breasted goose were collected for metal analysis (Pb and Al) at fields where monospecific flocks could be detected. Fifty samples from each species were individually taken in plastic bags and stored frozen at −20 °C until metal analysis. In order to avoid repeated sampling of faeces from the same bird, samples were taken at a minimum distance of 5 m from each other.

During the collection of faecal samples we could observe that cereal shoots (mostly wheat) being eaten by geese still showed the seed attached to the root with the red-staining characteristic of pesticide treated seeds. Therefore, in order to evaluate the pesticide exposure in geese we plucked out cereal shoots from different fields. As the amount of seeds remaining in the shoots was very limited we had to make a pooled sample from different fields composed of 102 germinated seed remains with a total mass of 1 g. Another ten shoot samples from individual fields, including leaves, roots and a few seeds were analysed individually (mass from 0.5 to 1 g). Moreover, 66 additional geese faecal samples were collected in fields where both species where feeding in order to determine the presence of cereal seed remains with a binocular microscope (×45) that could confirm seed ingestion in winter; and these samples were also used to determine the presence of pesticide residues. Plants and faeces were stored frozen at −20 °C

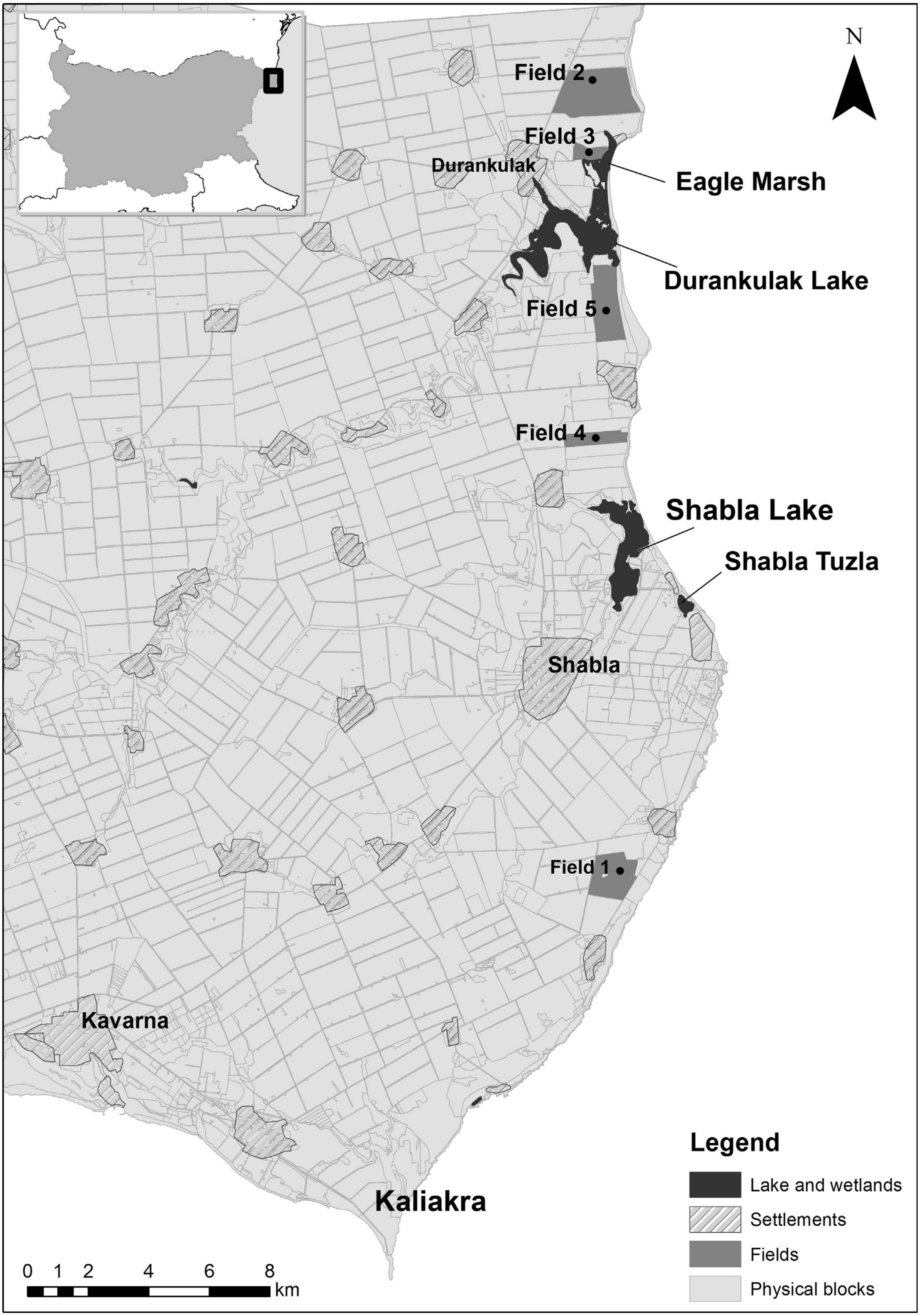

Fig. 1. Study area in the Bulgarian Dobrudzha on the Black Sea Coast. The map shows the five winter cereal fields (dark grey) where faecal samples were collected and the three wetlands where lead shot density was studied (Eagle Marsh, Durankulak Lake and Shabla-Tuzla). Physical blocks (light grey) show parcel boundaries of arable fields in the study area.

until pesticide residue analysis.

2.3. Lead shot density and grit availability

The presence of lead shot was visually searched for in the material retained during field sieving of sediment samples. The number of Pb shot was used to calculate the density in shot pellets/m^2 according to the surface area sampled. Grit availability was measured as the mass of available particles (> 1 mm) per m^2, and the type of material (mollusc shells, rock or pebbles) was recorded.

2.4. Analysis of Pb and Al in sediment, soil and faeces

Faeces (n = 100), sediment (n = 6) and soil samples (n = 16) (~0.25 g dry weight; d.w.) were analysed for Pb and Al concentrations after being freeze-dried, following methods described previously (Martinez-Haro et al., 2010). Briefly, samples were digested with HNO_3 in Pyrex tubes in a heating block (Selecta) and finally diluted to 50 mL with deionized H_2O. Then, Pb was determined by graphite furnace atomic absorption spectroscopy and Al was analysed by flame atomic absorption spectroscopy (AAnalyst800; Perkin Elmer). The limit of detection (LOD) was 0.062 μg/g for Pb and 47.8 μg/g for Al, dry weight (d.w). Samples with values below the LOD were assigned values of half the LOD for statistical purposes. Blanks and a reference material (bush, branches and leaves, NCS DC 73,349) with a certified level of Pb were processed in each batch of digestions to provide quality control data. Mean percentage Pb recovery (± RSE) of the reference material was 108.6 (± 1.5%; n = 6).

2.5. Pesticide analysis in cereal seeds

The determination of pesticides in germinated seeds, shoot plants and geese faecal samples (prior to examination for the detection of cereal seed remains) was performed by LC-MS following the method described by Lopez-Antia et al. (2016). This method has been optimized for the analysis of nine pesticides (imidacloprid, fipronil, flutriafol, metalaxyl, fludioxonil, thiram, triticonazole, tebuconazole, difenoconazole) used for seed treatment plus piperonyl butoxide, a synergist added to some formulations. About 0.5–1 g of sample was extracted with 5 mL of acetonitrile during 1 min of vortex, 5 min of sonication and 1 min again of vortex. The extract was filtered through a nylon syringe filter of 0.2 μm and was analysed by LC-MS. Pesticides were detected using positive and negative ions monitored with the following MM-ESI source settings. Nebulizer pressure was set at 35 psi, drying gas flow was 8 L/min, drying gas temperature was 250 °C, vaporizer temperature was 200 °C, capillary voltage was 3500 V in positive and 3000 V in negative, and charging voltage was 1000 V for both. The monitored ions for each pesticide along with the retention time and the fragmentation voltage for each ion are given in Lopez-Antia et al. (2016). Recoveries obtained varied between 90.9% and 108.7%. Stock solutions of pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Calibration curves were performed with concentrations of the four detected pesticides ranging from 0.25 to 2 μg/mL in acetonitrile.

2.6. Pesticide risk assessment

Geese species can feed on different aquatic and terrestrial plants, including their leaves, roots and seeds (Amat et al., 1991; Ely and Raveling, 2011). Geese wintering in farmland areas feed on seeds and shoots of growing plants, so they could be at risk of exposure to pesticides used to treat seeds during the sowing period (Hamilton et al., 1976; Madsen, 1996) and later on to those pesticides with systemic distribution in the plants (Goulson, 2013; Gibbons et al., 2015). In order to assess the risk of pesticide exposure in geese from our study area, we calculated the estimated daily intake (EDI) of pesticides due to ingestion of treated seeds by red-breasted geese and greater white-fronted geese. According to data on food intake obtained for pink-footed goose *Anser brachyrhynchus* (229 g of seeds /day and a body mass of 3.2 kg), daily food intake in this goose species is around 71.6 g of seeds/kg body weight (Madsen, 1996). The body masses of red-breasted goose (1150–1625 g) and greater white-fronted goose (1700 – 3000) g are lower than that of pink-footed goose (2170–3500 g) (Cramp and Simmons, 1977). Thus, based on the allometric relationship of energy expenditure in birds (Nagy et al., 1999), we estimated that the daily food intake was 95 g/kg b.w. for red-breasted goose and 82 g/kg b.w. for greater white-fronted goose (of body mass). These values were calculated according to the mean body mass values of 1304 g for red-breasted goose (Mitchell et al., 2015) and 2049 g for greater white-fronted goose trapped in our study area (BSPB, unpublished data). The theoretical pesticide concentrations in treated seeds were obtained from recommended application rates and the obtained EDI values were compared with the threshold values of acute hazardous dose 5% (HD_5) and chronic NOEL as described by Lopez-Antia et al. (2016). The HD_5 value corresponds to the dose of pesticide (mg/kg_{bw}) estimated to cause 50% mortality in a species in the top 5% of a species sensitivity distribution model (Mineau et al., 2001). Chronic NOEL is the highest dose level (mg /Kg_{bw}/day) at which no effects were seen after a long-term exposure (see Lopez-Antia et al., 2016). Risk assessment was also performed with the concentrations of pesticides measured in the germinated seeds.

2.7. Statistical analysis

Lead shot densities and grit availability were not normally distributed and were compared among wetlands using Kruskal-Wallis tests. Al and Pb levels and the ratio Pb: Al in sediment/soil and faecal samples were logarithmically transformed to fit a normal distribution. The log-transformed levels of Al and Pb in sediment/soil and faeces were compared among areas using one-way ANOVAs with post-hoc Tukey tests. These levels were also compared between faeces of red-breasted goose and white-fronted goose using Student *t* tests. Outliers from a linear regression between Al and Pb concentrations in sediment/soil and goose faeces were identified and considered to be those samples exposed to Pb shot, hence explaining their abnormally high Pb: Al ratio. Significance was set at $p \leq 0.05$. Statistical analyses were performed with SPSS version 22.

3. Results

3.1. Lead poisoning

The maximum density of Pb shot was found at Durankulak with 23.6 shot/m^2 and the site with lowest grit availability was Shabla-Tuzla Lake with 0.9 kg/m^2 (Table 1). No significant differences were detected among sites for Pb shot density (Kruskal-Wallis test, $\chi^2_2 = 1.74$, $p = 0.419$) or grit availability (Kruskal-Wallis test, $\chi^2_2 = 0.71$, $p = 0.7$).

The faecal concentrations of Pb and Al did not differ between goose species (Table 2). Both elements in faeces showed a very significant linear relationship ($R^2 = 0.886$, $F_{1,98} = 763$, $p < 0.001$) that is consistent with the values found in the soil of winter cereal fields where geese were feeding and in the sediment from Eagle Marsh (Fig. 2). Only one faecal sample from a greater white-fronted goose with a higher Pb: Al ratio could be interpreted as

Table 1
Lead shot density and grit availability within three wintering sites of red-breasted geese in Bulgaria.

Wetland	Pb shot density (pellets/m^2)					Grit availability (kg/m^2)			
	N	Mean	SD	Min	Max	Mean	SD	Min	Max
Durankulak	15	23.6	74.0	ND[a]	283	1.98	2.22	0.14	6.02
Eagle Marsh	5	14.1	31.6	ND	71	2.96	4.82	0.07	11.39
Shabla Tuzla	10	ND	–	ND	–	0.97	0.98	0.05	3.03

[a] ND: Not detected (< 7 Pb shot pellets/m^2).

Table 2
Concentrations (d.w.) of lead and aluminium and the ratio between both elements in geese faeces and soil samples.

Samples	Pb (µg/g)					Al (mg/g)				Pb: Al ratio (× 1000)			
	N	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Bird faeces													
White-fronted goose	50	2.51^C	1.01	1.11	5.51	3.42^C	1.92	1.26	10.26	0.787^B	0.199	0.537	1.986
Red-breasted goose	50	3.12^C	1.00	1.23	5.59	4.40^C	1.76	1.59	9.14	0.733^B	0.106	0.503	0.963
Soils and *sediments*													
Durankulak	3	3.57^C	0.74	2.91	4.37	0.84^D	0.14	0.68	0.95	4.306^A	0.917	3.272	5.024
Eagle Marsh	3	11.22^B	0.74	10.60	12.04	16.47^B	1.77	15.23	18.51	0.687^B	0.098	0.596	0.790
Fields	16	19.96^A	2.65	16.01	25.70	47.04^A	5.42	35.32	57.31	0.430^C	0.075	0.289	0.591

^{A,B,C,D}Means sharing a letter do not differ significantly (P > 0.05).

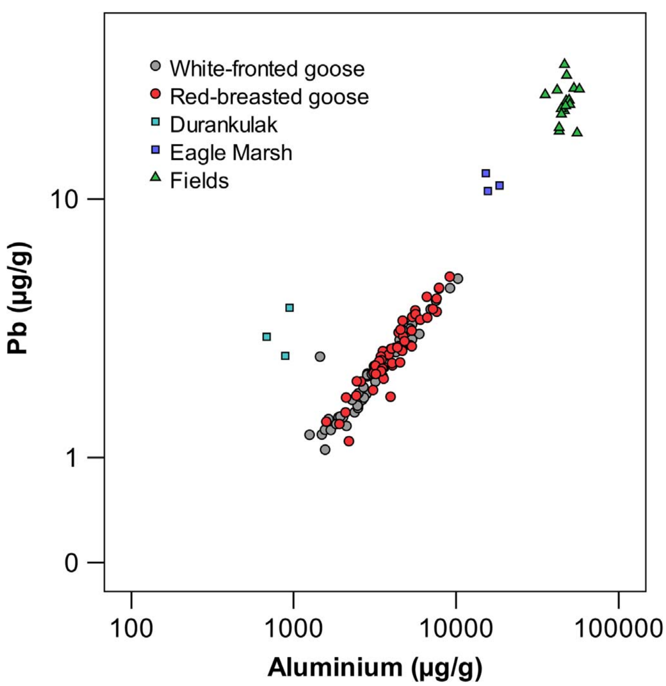

Fig. 2. The relationship between Pb and Al concentrations (d.w.) in samples of goose faeces, soil from fields and sediments from Durankulak and Eagle Marsh. The common regression line is: logPb =(0.757 × logAl) − 2.263.

having an abnormal Pb exposure (i.e. indicative of shot ingestion), but this sample was close to the values found at Durankulak Lake (Fig. 2). Significant differences existed among the levels of Pb and Al and the Pb: Al ratio in sediment/soil of the three studied areas (all $p < 0.001$). Cereal fields showed the highest levels of Pb and Al, and Durankulak the lowest (Table 2).

3.2. *Risk assessment of pesticide-treated seed and cereal shoot ingestion*

Four fungicides were detected in the field samples of germinated seeds: thiram, tebuconazole, difenoconazole and fludioxonil. The analysis of the pooled sample of germinated seeds revealed an overall concentration of 127.6 µg/g of thiram, 0.129 µg/g of tebuconazole, 1.95 µg/g of difenoconazole and 0.021 µg/g of fludioxonil. In the case of the ten samples of cereal shoots, tebuconazole was detected in two samples (0.032 and 0.044 µg/g) and fludioxonil in two samples (0.015 and 0.021 µg/g). One of these samples contained the two fungicides. Although the plant material found in faecal samples was almost completely composed of the remains of cereal leaves, the presence of cereal seed (i.e. small portions of the cuticle of the seed) was observed in seven samples (10.6%; Fig. 3). Three faecal samples contained residues of thiram (230, 241 and 261 ng/g) and another three showed residues of tebuconazole (50–68 ng/g), which indicates that geese can be exposed to pesticides used for seed treatment. Only one of the samples with visually detected seed remains contained a fungicide (thiram).

Two of the studied fungicides may represent a risk for geese wintering in Bulgaria (Table 3). During the sowing period in autumn, thiram shows an estimated daily intake in red-breasted goose and white-fronted goose that exceeds the acute HD_5 and the chronic NOEL values. Moreover, the risk of thiram persists in winter for both goose species because the estimated daily intake may exceed the chronic NOEL if birds were feeding only on germinated seeds. The second fungicide of concern in this risk assessment is tebuconazole, because the estimated daily intake for both geese species during the sowing period exceeds the chronic NOEL.

4. Discussion

Our study suggests that lead poisoning is probably not contributing to the recent declines in population size of red-breasted goose. However, pesticides used for seed treatment, especially thiram, could represent a risk for the migrating or wintering population in farmland areas.

Lead shot ingestion is a frequent cause of mortality in waterfowl species in heavily hunted areas, but not all species are equally vulnerable to lead poisoning. Diet, feeding methods, type of grit selected and grit availability are all important determinants for lead shot ingestion in waterfowl, in addition to the presence of

Fig. 3. Plant material observed in the geese faeces: remains of leaves (a) and seeds (b).

high lead shot densities in the wetlands (Figuerola et al., 2005). Geese are usually less prone to ingest lead shot than ducks, for several reasons. Firstly, they typically feed in grasslands or crops where lead shot density tends to be lower than in wetlands. Secondly, their mainly herbivorous diet makes the ingestion of lead shot mistaken as food items less likely than for granivorous ducks (Thomas et al., 1977). Thirdly, grit particles can readily be picked-up by geese from the surface of farmland areas (i.e. on paths or roadsides), where these particles tend to be abundant and the risk of confusion with lead shot is minimal (Mateo and Guitart, 2000). On the other hand, the lakes studied in Bulgaria have a depth of more than 50 cm over most of their surface, so the only areas where geese could pick-up lead shot are the shallow waters close to the shoreline. This shallow area is where we sampled to determine the lead shot density, since we had similar limitations to geese in accessing the lake bottom. The maximum lead shot density, found at Durankulak, was 23.6 shot/m^2, which is much lower than the maximum densities of 199–398 shot/m^2 found in several wetlands in Southern Europe (Mateo, 2009). Waterfowl hunting pressure has been higher at Durankulak in recent decades than in the rest of our study sites, so this seems to be the worst scenario in the study area (Petkov and Illiev, 2015; BSPB, unpublished data).

The analysis of faecal samples revealed that soil was the most probable source of lead in the bird excreta. Only one faecal sample from greater white-fronted goose with a higher Pb: Al ratio could be interpreted as a case of abnormal Pb exposure (i.e. shot ingestion), but the Pb: Al ratio of this sample was close to the values found at Durankulak Lake, so these lake sediments could in fact be the source of this higher lead level. Previous studies with greylag geese (*Anser anser*) in the Guadalquivir Marshes (Doñana, Southern Spain) carried out with faecal analysis have shown that soil

Table 3
Estimated daily intake doses of pesticides by red-breasted goose (RbG) and white-fronted goose (WfG) in Bulgaria during the sowing period (autumn) and later in winter. Estimates are made according to theoretical concentrations of pesticides based on application rates and on the concentrations measured in this study. The risk assessment is based on the comparison of the estimated daily intake and the toxicity thresholds (acute HD_5 and chronic NOEL). Estimated daily intakes above these toxicity thresholds may represent a risk for geese species.

Pesticide	Pesticide concentration in food (mg/kg$_{seed}$)		Estimated daily intake (mg/kg$_{bw}$)[*]				Toxicity thresholds[†]	
	Theoretical value in autumn (sowing)	Measured in winter	Autumn		Winter		Acute HD_5 (mg/kg$_{bw}$)	Chronic NOEL (mg/kg$_{bw}$/day)
			RbG	WfG	RbG	WfG		
Thiram	1750	128	**166**[ab]	**143**[ab]	**12.2**[b]	**10.5**[b]	36.8	0.8–37.5[‡]
Fludioxonil	20	0.021	1.90	1.64	0.002	0.002	208	11.1–62.8
Difenoconazole	60	1.95	5.70	4.92	0.185	0.160	207	9.7
Tebuconazole	375	0.129	**35.6**[b]	**30.8**[b]	0.012	0.011	347	5.8

[*] Estimated daily intake (EDI) based on food consumption of 95 g/kg b.w. by red-breasted goose and 82 g/kg b.w. by white-fronted goose.
[†] Acute hazardous dose HD_5 is the amount of pesticide estimated to cause 50% of mortality in those 5% of all bird species that are most sensitive. Data obtained from Mineau et al. (2001). NOEL is the No Observed Effect Level at long-term exposures (data obtained from EFSA scientific reports: EFSA, 2007, 2011, 2014).
[‡] Data calculated from No Observed Effect Concentration (NOEC) values in diet of 9.6 ppm for mallard and 500 ppm for Northern bobwhite quail (EPA, 2004). The NOEL value of 37.5 mg/kg$_{bw}$/day for Northern bobwhite quail was given by EC (2015). The NOEL value of 0.8 mg/kg$_{bw}$/day in mallard (1.35 kg bw) can be calculated from EFSA (2009) for cereal seed ingestion (80 g/kg$_{bw}$/day).
[a] EDI > acute HD_5.
[b] EDI > chronic NOEL.

contamination caused by a mine spillage was the main source of lead (Mateo et al., 2006). This occurred in a wetland where gizzard analysis showed that lead shot ingestion by geese had a relatively low prevalence of 0–10% in the general population (Mateo et al., 1998, 2006, 2007), although 27.7% of 101 greylag geese found dead had ingested Pb shot (Mateo et al., 2007). In Doñana, the study of the Pb isotope signature in faecal samples provided additional evidence of the relative contribution of different Pb sources, including ammunition (Martínez-Haro et al., 2013). Recently, faecal analysis has been applied to lesser white-fronted goose (*Anser erythropus*) and greater white-fronted goose in Greece, where soil was also identified as the main source of Pb in geese (Aloupi et al., 2015).

The second group of chemicals we studied were the pesticides used for cereal seed treatment. Bird poisoning due to the ingestion of seeds treated with pesticides has been recorded ever since their development for use in agriculture. Conceptually, the risk caused by adding a toxicant to a common food for birds is self-evident. As a result, the list of pesticides that have been implicated in bird poisoning through seed ingestion reflects the history of pesticide development and prohibition (Stanley and Bunyan, 1979). Initially, pesticides used for seed treatment were persistent and bioaccumulative, promoting toxic levels in tissues and producing adverse effects or even death. Alkyl mercury had its time as a fungicide for seed treatment between 1950 and 1970, with negative consequences for granivorous birds (Fimreite, 1970; Malmberg, 1973; Knight et al., 1974), and for raptors too (Henriksson and Karppanen, 1975). Organochlorine insecticides, especially the highly toxic cyclodienes (HD_5 values (mg/kg b.w.), endrin: 0.75, heptachlor: 3.47, lindane: 10.5), had devastating consequences in wild birds and this led to their substitution by less persistent compounds such as anticholinesterasic insecticides (i.e. organophosphates) (Stanley and Bunyan, 1979; Blus et al., 1984). But once again, poisoning of sensitive bird species was too frequent because of the high toxicity of organophosphates (HD_5 values (mg/kg b.w.), carbophenotion: 2, monocrotohos: 0.42) and, in some cases, their misuse (Hamilton et al., 1976; Flickinger et al., 1984; Pain et al., 2004). Several alternatives have been developed to replace the anticholinesterasic insecticides. In the case of cereal seed treatment, the neonicotinoids (i.e. imidacloprid) and fipronil have been among the most frequently used insecticides in the last decade. Although the persistence of such compounds is low, their toxicity is still elevated (HD_5 values (mg/kg b.w.) imidacloprid: 8.43, fipronil: 1.47). As a result, poisoning of birds feeding on seeds treated with these insecticides can easily occur (Berny et al., 1999; Gibbons et al., 2015) and several adverse effects on reproduction, immune function and thyroid homeostasis have been observed experimentally (Kitulagodage et al., 2011; Lopez-Antia et al., 2013, 2015a, 2015b; Johnston et al., 1994; Pandey and Mohanty, 2015).

Nevertheless, winter cereal seed treatment with insecticide is less frequent than their treatment with fungicides (Lopez-Antia et al., 2016). This was the case of the samples analysed in our study area, in which only fungicides were detected. Current fungicides are less acutely toxic for birds than insecticides (see Table 3 for HD_5 values of the fungicides detected in this study), because insecticides usually act on physiological mechanisms shared by insects and vertebrates (i.e. neuronal function). However, the risk of chronic exposure is still important for some fungicides that can act as endocrine disruptors or produce oxidative stress. Two of the four fungicides found in cereal samples of the study area could represent a risk for geese wintering in Bulgaria if cereal shoots are plucked out with the remaining seed. The presence of cereal seed remains and pesticide residues in geese faecal samples indicate that geese can be exposed to pesticide treated seeds throughout the winter, although the migratory phenology of the species can be a very important determinant of such exposure. The arrival of the first flocks of red-breasted geese to Bulgaria by mid-November usually occurs after the cereal sowing period, which depending on the weather conditions may expand from the second half of September to early November. Before their arrival to the NW Black Sea coast, the red-breasted geese have made several stops in arable lands of Kazakhstan, the Manych-Gudilo depression in Kalmykia (Russian Federation) and in Ukraine, where they may also get exposed to pesticides during the cereal sowing period (Dereliev et al., 2000; Cranswick et al., 2012; Petkov and Illiev, 2015). Moreover, geese can pluck out cereal shoots, and as we found in the second half of January, they can be exposed to low doses of pesticides during several months.

The estimated intake of thiram with treated cereal seed sown in autumn was 4.5 times the hazardous dose (HD_5) for the red-breasted goose and 3.9 times the dose for the greater white-fronted goose. In the case of chronic exposure, the estimated intakes were also higher than the no effect level (NOEL) values for both geese species (especially considering the NOEL value calculated for mallard; see Table 3). In the germinated seeds collected in winter (still red stained), the measured concentration yielded much lower estimated intakes. Even so, if birds were feeding only on germinated seeds that intake would still be one order of magnitude higher than the NOEL in mallard (EPA, 2004). Here we only found evidence of seed ingestion in 10.6% of the analysed faecal samples, so thiram intake in winter may be close to or below this NOEL.

The adverse effects of thiram on animals include the endocrine disruption of the hypothalamic-pituitary-gonadal axis, producing inhibition of ovulation and lower fertility, oxidative stress with effects on the antioxidant status of animals and their carotenoid-based teguments involved in sexual selection, and immune suppression in the offspring of exposed parents (see Lopez-Antia et al., 2015a). In previous studies, it has been observed that thiram acts as a repellent for birds, but the avoidance of the thiram-treated seeds tend to decrease over time of exposure (Lopez-Antia et al., 2014). Our results indicate that both goose species, especially the red-breasted, were at risk of acute and adverse effects of chronic exposure to thiram. Madsen (1996) also highlighted the potential risk of thiram for pink-footed geese feeding on peas sown in spring in Denmark and calculated that geese could ingest 100 g of thiram treated peas in 31 min of uninterrupted foraging, which may have sublethal effects on their reproduction.

Tebuconazole is less toxic than thiram, and it only represents a risk for geese during sowing and under chronic exposures. The estimated intake of tebuconazole in autumn was 6.1 and 5.3 times the chronic NOEL values in the red-breasted and greater white-fronted goose, respectively. Among the adverse effects of tebuconazole described in vertebrates are disturbance of the synthesis of steroid hormones, altered reproductive development, adrenal hypertrophy, hepatic toxicity and liver tumours (Taxvig et al., 2007; EFSA, 2014). In summary, geese feeding on recently sown cereal seed treated with thiram and tebuconazole may suffer adverse sublethal effects on the endocrine and immune systems in autumn, and in the case of thiram the risk of exposure may be extended through the winter. However, thiram is a non-systemic fungicide (i.e. not transferred to the aerial parts of the plant), so the risk of exposure to birds may be limited to the ingestion of the treated seeds or when birds pull up the shoots with the germinated seed. In contrast, tebuconazole is a systemic fungicide and the exposure in grazing birds may be low but more constant.

The interactions between pesticides is another issue frequently overlooked in field studies. We found that geese can be exposed to four fungicides, and Bro et al. (2016) detected residues of 15 plant protection products in eggs of grey partridge (*Perdix perdix*) from France. The monitoring of habitat use by these grey partridges showed that 71% of clutches were potentially exposed to ≥ 1

pesticide and 67% to ≥2 pesticides (Bro et al., 2015). These interactions can be especially relevant in the case of some ergosterol biosynthesis inhibiting fungicides (EBIFs), because of their capacity to induce or inhibit cytochrome P-450 enzymes (Ronis and Badger, 1995). This leads in some cases to the transformation of some pesticides (i.e. organophosphorous insecticides) into more toxic metabolites (Johnston et al., 1994, 1996). Triazoles, like tebuconazole and difenoconazole, are EBIFs but to our knowledge there is no information about their interactions with other pesticides in birds. Here we focused the analysis on some pesticides used for cereal seed treatment in the EU (Lopez-Antia et al., 2016), but other pesticides could be used as foliar applications during the plant development in the late winter. Moreover, pesticides formulations registered for seed treatment may differ among geographical areas within the EU due to agricultural, plant health and environmental conditions (Mandic-Rajcevic et al., 2013) and other pesticides could be on use along the migratory route of red-breasted goose outside the EU.

Some agri-environmental measures have recently been implemented in the study area, targeting the good management of suitable feeding habitat for the red-breasted goose. These include the rotation of maize and wheat and the avoidance of the use of rodenticides in winter (MoAF, 2015). However, there is a need to identify and regulate the pesticides used for seed treatments along the migratory route of red-breasted goose, and not only in their wintering grounds in EU countries.

5. Conclusions

Based on our study, we can rule out lead poisoning as a significant threat to the globally threatened red-breasted goose on its wintering grounds in Bulgaria. However, fungicides give cause for concern and may be contributing to observed population declines, but several other factors are likely to explain the decline of the red-breasted goose in addition to pesticides. The species faces serious threats along its almost 6000 km long journey from Siberia to its wintering habitats. Though legally protected in all countries, the redbreasts are shot at along the migration route by poachers, changes in land use practices have reduced food availability, and disturbance of feeding birds by hunters in vehicles is thought to impede foraging and prenuptial accumulation of fat reserves, therefore affecting survival during spring migration and breeding (Cranswick et al., 2012; Birdlife International, 2015). There are also indications that wind farm developments in Romania and Bulgaria have displaced foraging flocks (Petkov et al., 2012; Harrison and Hilton, 2014). Climate change and associated habitat shifts are also expected to have a negative impact, and modelling indicates that 67% of the breeding habitat for this species could be lost by 2070 due to climate change alone (Zöckler and Lysenko, 2000). In this scenario of multiple stressors, pesticides used for seed treatment may introduce sublethal effects with major consequences for the demography of the red-breasted goose.

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