Aflatoxin and Ochratoxin A residues in supplementary foods used for wild birds

Abstract

Provision of supplementary food for garden birds is practiced on a large scale in multiple countries. While this resource has benefits for wild bird populations, concern has been expressed regarding the potential for contamination of foodstuffs by mycotoxins, and the implications this might have for wildlife health. We investigated whether aflatoxin (AF) and ochratoxin A (OA) residues are present in foodstuffs sold for wild bird consumption at point of sale in Great Britain using high pressure liquid chromatography analyses. The hypothesis that production of these mycotoxins occurs in British climatic conditions, or under storage conditions after the point of sale, was tested under experimental conditions but was not proved by our study. While the majority of peanut samples were negative for AF residues, 10% (10/98) of samples at point of sale and 11% (13/119) of those across the storage and climate exposure treatment replicates contained AFB_1 that exceeded the maximum permitted limit of 20 µg/kg. No significant difference was found in the detection of either mycotoxin between branded and non-branded products. The clinical significance, if any, of exposure of wild birds to mycotoxins requires further investigation. Nevertheless, the precautionary principle should be adopted and best practice steps to reduce the likelihood of wild bird exposure to mycotoxins are recommended.

Highlights

- Provision of contaminated food risks exposure of wild birds to mycotoxins
- We tested samples of peanuts (whole and granule) and sunflower seed for mycotoxins
- 10% of peanut samples exceeded the Maximum Permitted Limit for aflatoxin B_1
- Storage and climate exposure treatments could lead to aflatoxin B_1 production

- Best feeding practice will reduce health risks to birds from mycotoxin exposure

Keywords

Mycotoxin, peanut, sunflower seed, wild bird health, garden birds, Great Britain

1. Introduction

Provision of supplementary food for wild birds at garden feeding stations is a common practice in Great Britain conducted throughout the calendar year (Davies et al., 2009). Given the scale of this anthropogenic activity, which is sufficiently large to alter the shape of bird communities (Plummer et al., 2019), there is a need to evaluate its impact on wild bird populations, both in terms of benefits and potential health hazards (Lawson et al., 2018). While supplementary food can provide valuable additional dietary resources for wild birds, concerns have been expressed regarding the quality of supplementary foodstuffs, for example their nutritional composition and the risk of mycotoxin contamination (Murray et al., 2016; Strandin et al., 2018).

Aflatoxins (AFs B_1, B_2, G_1 and G_2) and ochratoxin A (OA), secondary metabolites produced by *Aspergillus* and *Penicillium* fungi, are potent biological toxins that exert a range of adverse effects in birds. These range from acute toxicosis, with AFs principally targeting the liver and OA the kidney, to chronic subclinical impairment of growth, reproduction and immune function (O'Hara, 1996; Lawson et al., 2006; Heussner and Bingle, 2015).

Marked variation in the susceptibility of bird species to aflatoxicosis occurs, which has been demonstrated through experimental dietary challenge studies, primarily in galliform species (Ruff et al., 1990; Huff et al., 1992; Ruff et al., 1992). Whilst both AF and OA are known to be toxic in birds such as domestic poultry, there is currently little information available on the

susceptibility of garden bird species to AF or OA exposure. However, experimental exposure studies on the northern cardinal (*Cardinalis cardinalis*), a frequent visitor of backyard feeding stations in North America, suggests they are highly susceptible to aflatoxicosis (Moore et al., 2019). These authors estimated the lethal dose $(LD)_{50}$ of aflatoxin for adult cardinals ingesting aflatoxin-contaminated feed is about 500 ppb, compared with published LD50s of 360 ppb for 1-day old ducklings and 6000 ppb for adult chickens. Sublethal effects were noted with immunosuppression, evidenced by reduced white blood cell proliferation, occurring in cardinals fed AF levels as low as 25 ppb (µg/kg)(Moore et al., 2019). AF residues have been detected in the liver of two species, house sparrow (*Passer domesticus*) and greenfinch (*Chloris chloris*), found dead in gardens in Britain (Lawson et al., 2006), however, the pathological significance of these residues, if any, is unknown. Various pathogens are known to affect garden birds in Great Britain (Lawson et al., 2018) and the possibility that chronic AF exposure might impair the immune function of passerines and predispose them to infectious disease requires investigation (Lawson et al., 2006). No research into the presence of OA residues in wild bird tissues appears to have been performed.

A range of products sold as supplementary feed for garden birds are known to be vulnerable to AF and OA contamination (e.g. cereals, seeds, peanuts) (Pitt and Hocking, 1997), but there have been few studies quantifying levels present (e.g. Henke et al., 2001; Scudamore et al., 1997). Currently, the source of any AF exposure for garden birds in Great Britain is unknown, however, possible sources include supplementary food provided by homeowners, agricultural crops and wild seed. Our focus is on supplementary food, which might be contaminated with AF at the point of sale, contaminated after sale from production during storage, contaminated after sale from production whilst exposed to British climatic conditions at the feeding station

itself, or a combination of the above. Branded peanuts marketed for wild bird feed sometimes include information on the packaging to indicate that the contents have been screened for AF residues. However, peanuts for wild bird consumption are also sold through a wide variety of outlets, often unbranded, where labelling is not present and, consequently, the quality control (QC) procedures followed are unknown. All products sold in the UK, though, should have checks to ensure they comply with The Animal Feed (Composition, Marketing and Use) (England) Regulations (or the equivalent legislation in Scotland, Wales and Northern Ireland), which provide for enforcement and implementation of EU Regulations and Directives, and stipulate a maximum permitted level (MPL) of 20 µg/kg (ppb) AFB_1 in peanuts sold as food for animals, including wild birds (Anon. 2015). No MPL exists for OA, although European Union (EU) Commission Recommendation 2006/576/EC (Anon. 2006) provides a Guidance Limit of 100 µg/kg for poultry foodstuffs.

Optimal conditions for AF production occur at high temperature and relative humidity; however, Thompson et al. (2000) demonstrated AF production in corn (*Zea mays:* maize) under temperate climatic conditions (i.e. temperature 14-18 ^{o}C and relative humidity 35-40 %) that regularly occur in the UK. OA production also occurs in temperate conditions (Santin 2005). Historically, wild bird foods were provided mostly during the winter months in British gardens, but since the 1980s there has been a move to include summer feeding when climatic conditions appropriate for mycotoxin production occur (Jones 2018). Consequently, there is a potential risk for production of both AF and OA within feed products at garden bird feeding stations and a need to determine whether this occurs in the UK. Testing of food residues from hanging feeders in use at garden feeding stations in the UK detected AF residues in all seven samples tested, two of which were at particularly high values of 690 and 61,710 µg/kg (Lawson et al., 2018).

For the current study, we had four objectives: first, we investigated whether AF and OA residues are present in foodstuffs (peanuts, peanut granules and sunflower seed hearts) sold for garden bird consumption at the point of sale. Second, we compared the AF and OA residues in whole peanuts from branded versus non-branded sources. Third, we tested the hypothesis that AF or OA production occurs in peanuts in British climatic conditions at garden feeding stations and/or under storage conditions after the point of sale. Fourth, we conducted a questionnaire survey on garden bird feeding methods to help put the study findings in context.

2. Materials and methods

2.1 Sampling protocol - 2007

In early 2007, a total of 39 different whole peanut products marketed for wild bird consumption were purchased for mycotoxin screening; 19 were branded samples (with information on the packaging which indicated that the contents had been screened for AFs) and 20 were from non-branded sources with no packaging information. Peanuts were purchased by mail order from national suppliers (n=14) and from a range of supermarkets (n=5), home/DIY/garden centres (n=2), pet shops and pet market stalls (n=19) in England. The sampling protocol was designed to mimic, as far as possible, domestic conditions and of a typical bird feeder used in a garden setting in Great Britain. Either one kilogram of each food sample, or the smallest package size available in the commercial range for branded products, was purchased. The sample weight was selected to reflect the purchase size for small packets of wild bird food by a domestic consumer. The packet contents for branded peanuts, and the entire bag for non-branded peanuts, were mixed thoroughly by hand and four 250g aliquots were then taken at random: aliquot size was dictated by the volume of peanuts that would fit within the commercially

126 available bird feeders used in the trial. The remainder of the study was performed blind to the
127 details of the commercial source.
128 Point of sale samples ('Point of Sale' treatment), representing baseline mycotoxin levels, were
129 taken and individually stored in air- and water-tight plastic bags at -20 °C pending high pressure
130 liquid chromatography (HPLC) analysis. A second batch ('Spring Exposure' treatment) was
131 exposed to British climatic conditions for a period of 30 days in March-April 2007. Each
132 sample was transferred to a 'Discovery' hanging wire mesh plastic peanut feeder (CJ Wildlife,
133 UK). The feeders were suspended within a mock feeding station constructed from plywood
134 and wire mesh (c. 5 metres long by 1.5 metres wide by 1 metre high and raised 1 metre above
135 ground level – Supplementary Fig. S1) which was situated outdoors at the Institute of Zoology
136 (in Regent's Park, London, U.K.). Inside this structure, the contents of the feeders were
137 exposed to climatic conditions such as wind and rain, but access by birds and mammals was
138 prevented. A digital 'Memory Thermo-Hygrometer' (ATP Instrumentation Ltd.,
139 Leicestershire) was placed beneath the feeding station in a raised position and protected from
140 rain. Daily maximum and minimum relative humidity (%) and temperature readings (ºC) were
141 recorded to monitor the climatic conditions to which the bird food was exposed. The position
142 of each sample in the mock feeding station was randomly assigned through blind ballot. At the
143 completion of the exposure period, samples were transferred to air- and water-tight plastic bags
144 and stored at -20 °C pending HPLC analysis.
145
146 A third batch ('Summer Exposure' treatment) of each of the samples was exposed to British
147 climatic conditions for a period of 30 days in June-July 2007 using the same protocol as for the
148 Spring Exposure treatment. These samples had been stored at -20 °C for the period following
149 purchase before this component of the trial began and were frozen again at the end of the

150 treatment pending HPLC analysis. One sample was not tested under summer exposure
151 conditions because of insufficient sample volume.
152
153 A fourth batch ('Storage' treatment) of samples was placed in storage in a cool and dry indoor
154 room for a period of 90 days (March-June 2007). Each sample was stored in a non-sealed
155 plastic bag in a ventilated plastic crate. Samples were gently mixed on a weekly basis to
156 simulate peanuts being removed from a packet of food. A digital Memory Thermo-Hygrometer
157 was placed in the plastic crate and readings were taken on a weekly basis. At the completion
158 of the exposure period, samples were transferred to air- and water-tight plastic bags and stored
159 at -20 °C pending HPLC analysis.
160
161 A single sample of peanut granules was purchased from a branded source in 2007; four 250 g
162 aliquots were taken and processed in the same way as the whole peanut samples, although
163 results from these samples were not included in the analyses comparing climate exposure and
164 storage replicates.
165
166 **2.2 Sampling protocol - 2018**
167 Approximately a decade after the initial study, in January/February 2018, the point of sale
168 treatment was repeated for wild bird food products. A total of 45 different whole peanut
169 products marketed for wild bird consumption were purchased for mycotoxin screening; 25
170 were branded samples and 20 were from non-branded sources where no packaging information
171 was available. Whole peanuts were purchased from home/DIY/garden retail outlets (n=20), pet
172 shops (n=13), supermarkets/retailers (n=8), online mail order (n=3), and from a pet market stall
173 (n=1). In addition, 13 different peanut granule (also described as kibble or chips) products were
174 bought, 12 of which were from branded sources. A total of 20 different sunflower seed heart

175 products marketed for wild bird consumption were purchased: 19 were branded and one was
176 from a non-branded source.
177
178 Either one kilogram of each food sample, or the smallest package size available in the
179 commercial range for branded products, was purchased. Each sample was mixed thoroughly
180 and a 250g aliquot was taken at random and individually stored in air- and water-tight plastic
181 bags at -20 °C pending HPLC analysis. Analyses were performed blind to the details of the
182 commercial source.
183
184 **2.3 High Pressure Liquid Chromatography (HPLC)**
185 Samples were transported frozen to Fera Science Ltd. for HPLC analysis, stored at -20 °C and
186 allowed to thaw overnight before preparation for analysis. A homogenised slurry was created
187 from each sample by adding 200 ml of tap water per 250 g of food product and grinding the
188 sample in a clean food processor for twenty minutes.
189
190 Analysis of mycotoxins were carried out using fully validated and accredited (to ISO 17025 by
191 the United Kingdom Accreditation Scheme) methods based on those published for AF
192 (Sharman et al., 1991) and OA (Sharman et al, 1992) or for combined AF/OA methods (Chan
193 et al., 2004), depending on the availability of clean-up columns: validation and QC procedures
194 have shown that single or combined methods produce equivalent results (Chan et al., 2004).
195 To perform the extraction, an aliquot (36 g) of the slurried sample was homogenised at high
196 speed with extraction solution (acetonitrile/water, 60:24, volume/volume). The extract was
197 filtered, and 5 ml of the filtrate was added to 145 ml phosphate-buffered saline. For quality
198 control purposes, aliquots of known blank peanut slurry were spiked at a level equivalent to 5
199 µg /kg for each aflatoxin (20 µg/kg total aflatoxin) and 5 µg/kg ochratoxin A. These were

200 extracted and analysed in the same way as the study samples to determine the analytical
201 recovery of the analysis.
202
203 Fully automated immunoaffinity column clean-up and reverse phase HPLC with fluorescence
204 detection was carried out on the resulting aqueous solutions. Samples were cleaned up by
205 combined AF and OA immunoaffinity columns (AflaOchra) and either both toxins were
206 analysed together in one run (Chan et al., 2004) or the extracts were split and AFs and OA were
207 cleaned-up and analysed separately, for AFs using the conditions described by Sharman et al.
208 (1991) and for OA using the method of Sharman et al. (1992). Samples were run in batches of
209 twenty on consecutive days with the same equipment and operator to minimize laboratory
210 variability. In-house reference materials with known mycotoxin concentrations were used in
211 each batch run to ensure that variation between batches remained within acceptable limits. The
212 concentrations (µg/kg) of mycotoxins detected were corrected according to the percentage of
213 mycotoxin recovery achieved in each batch analysed, as determined from the results of spiked
214 samples.
215
216 **2.4 Statistical Analysis**
217 As the level of both AF (median = 0 µg/kg; maximum = 935 µg/kg) and OA (median = 0 µg/kg;
218 maximum = 18.4 µg/kg) recorded in the samples was highly skewed, we considered three levels
219 of contamination for AF: none - no detectable residue; low - detectable AF (of any type) but
220 ≤MPL of 20 µg/kg AFB_1; and high - >20 µg/kg AFB_1. There is no equivalent MPL for OA but,
221 for consistency, we also considered three levels of contamination; no detectable residue; ≤5
222 µg/kg; and >5 µg/kg. For all analyses we used ordinal regression to estimate the log-odds of a
223 sample being classified in a particular category (Venables and Ripley, 2003).
224

We first compared point of sale whole peanut, peanut granules and sunflower seed products purchased for 2007 and 2018 combined. We then compared whole peanut products purchased in 2007 and 2018 and assessed whether there was a difference between branded and non-branded samples. Finally, we tested for evidence for production of AF or OA under typical British climatic conditions or storage conditions by comparing the results of the Point of Sale treatment in 2007 with those for each of the other treatments in turn using the non-parametric Wilcoxon Signed-Rank Test for paired data with continuity correction for sample sizes. Because we were interested in whether exposure to climatic or storage conditions increased the level of contaminants, we used a one-tailed test.

2.5 Questionnaire on garden bird feeding practice

In order to determine how closely the conditions in this study simulated garden bird feeding practice in the UK, an anonymised questionnaire was distributed to employees of the Zoological Society of London (ZSL) and of the Royal Society for the Protection of Birds (RSPB), Bedfordshire, in August 2005. The target respondents were people who used hanging feeders to feed garden birds. Respondents were asked whether they purchased peanuts for wild birds and whether these were from branded or non-branded sources; the same question was asked regarding provision of wild bird seed. In addition, respondents were asked whether they provided supplementary food during winter only (November – February), year-round or on a variable basis; and whether they topped up their feeders with food, emptied them before re-filling, or a combination of the two.

3. RESULTS

3.1 Aflatoxin Residues

For the HPLC analyses, the recovery limits were within the acceptable range for quality control; the full set of corrected results obtained are presented in Supplementary Database S1. We considered the climatic variables measured in the spring, summer and storage treatments in 2007 (Supplementary Table S1) to be representative of the respective seasonal range for the British climate.

Point of Sale

In most (62%, 61/98) peanut (whole and granule) samples for 2007 and 2018 combined, no AF residues were recorded at point of sale while residues of AFB_1 exceeding the MPL were recorded in 10 (10%) of these samples (median of these 10 samples 56 µg/kg, range 23-800 µg/kg AFB_1, Fig. 1a). Recorded levels of AFB_2 (maximum = 150.0 µg/kg), AFG_1 (maximum = 7.3 µg/kg) and AFG_2 (maximum = 4.3 µg/kg) at point of sale were lower and only occurred in samples where AFB_1 was also present (Supplementary Database S1). No samples of sunflower seed at point of sale exceeded the MPL for AFB_1 (maximum AFB_1 = 5.4 µg/kg) and in 85% (17/20) no AF was detected (Fig. 1a). Relative to whole peanut products, peanut granule samples were much more likely to contain AF residues at point of sale (log-odds ratio = 5.0; 95% interval 1.5 – 17.4; $t_1 = 2.62$, $p = 0.009$) and sunflower seeds somewhat less likely to do so (log-odds ratio = 0.36; 95% interval 0.08 – 1.30, $t_1 = -1.50$, $p = 0.15$).

For whole peanut products, there was no difference in the frequency of detection of mycotoxins in point of sale samples in 2018 compared to 2007 (log-odds ratio = 0.97; 95% interval 0.39 – 2.46, $t_1 = -0.06$, $p = 0.95$), nor in branded compared to non-branded products (log-odds ratio = 1.39; 95% interval 0.56 – 3.56, $t_1 = 0.70$, $p = 0.48$, Fig. 1a).

Treatments

For the whole peanut products exposed to different treatments in 2007, there was no significant difference in AF levels in samples exposed to spring (mean 33.0 μg/kg, V = 117, p = 0.33, n=39), summer (mean = 89.0 μg/kg, V = 120, p = 0.30, n = 38) or storage (mean = 103 μg/kg, V = 125, p = 0.38, n = 39) conditions compared to the point of sale (mean = 59.8 μg/kg) (Fig. 2a). Similar results were obtained when branded and non-branded products were considered separately. Aflatoxin residues were not present evenly through each of the samples across these four treatment groups. Of the 40 products tested, 26% (10/39) were negative, and six (15%) were positive for AF across all four treatments; in 23 products (59%) AF was detected in some, but not all, treatments. A total of 17 samples across the treatment groups from eight (20%) products exceeded the MPL for AFB_1, although only one product exceeded the MPL in all four treatment groups (Supplementary Database S1).

The single source of peanut granules in the 2007 study had detectable levels of AF within each of the four treatment categories although all were below 5 μg/kg.

3.2 Ochratoxin A Residues

Point of Sale

OA residues were found in a similar number of peanut (whole and granule) samples at point of sale (36%, 35/98) as AF, but with lower absolute levels (range: 0 - 18 μg/kg) (Fig. 1b; Supplementary Database S1). OA was detected in more granule samples (6/14, 46%) at point of sale than whole nut samples (29/84, 34%), but at lower levels (max granule = 1.7 μg/kg, max whole nuts =18.5 μg/kg), although neither difference was significant (frequency: χ^2_1 = 0.28, p = 0.59; level: Wilcoxon W = 505, p = 0.57).

For whole peanut products, OA residues were more likely to be detected in 2018 than in 2007 (log-odds ratio = 8.28; 95% interval 2.92 – 27.7, $t_1 = 3.74$, $p < 0.001$). Also, OA was detected at significantly greater levels (maximum 18.5 µg/kg, W = 1317, $p < 0.001$) in 2018 than in 2007, when a maximum of 0.4 µg/kg was recorded in the five positive samples detected. There was no difference between branded and non-branded products for likelihood of detection (log-odds ratio = 0.86; 95% interval 0.32 – 2.28, $t_1 = -0.32$, $p = 0.75$) or levels of detection (Wilcoxon W=237, p_0.77).

Treatments

Across the four treatment groups in 2007, 63% (25/39) of the whole peanut products tested were negative for OA in all treatment categories and, of the positive products, OA was detected at 0.5 µg/kg or less in all treatment samples apart from one of the spring samples which contained 48.1 µg/kg. There was no significant difference in the levels of OA between any of the experimental treatments and the point-of-sale samples (all $p > 0.32$).

The single source of peanut granules in the 2007 study only had detectable levels of OA in the Summer Exposure treatment which contained the second highest level recorded in the study at 4.4 µg/kg.

3.4 Questionnaire of garden bird feeding practice

There were 251 respondents, of whom 78 (31%) fed non-branded peanuts and 70 (28%) fed non-branded seed. Amongst these, 55 (22%) of respondents fed both non-branded seed and non-branded peanuts. 130 (52%) of respondents fed branded peanuts and 168 (67%) fed branded seed, with 122 (49%) feeding both. . More people fed garden birds all year round

(154/251 respondents, 61%) than provided feed during the winter months only (November to March, 58/154, 23%). The remaining 37 respondents (15%) described their feeding patterns as variable (two did not answer this question). When asked whether they emptied and refilled feeders or just topped them up when necessary, 98 (39%) respondents said they topped feeders up, 61 (24%) said they emptied and refilled feeders each time, and the remaining 91 (36%) said they did a mixture of the two (one person did not answer this question).

4. Discussion

The results of this study indicate that supplementary food for wild birds in Great Britain can be a source of mycotoxins. While the majority of foodstuff samples had no detectable levels, a subset of both branded and non-branded products was found to contain AF and/or OA residues. AFB_1 exceeding the MPL of 20 μg/kg was detected in circa 10% of peanut samples at point of sale, and in circa 11% across the storage and climate exposure replicates. Although OA is considered a storage toxin produced under temperate climate conditions, no samples at point of sale or across the other treatment categories had levels that approached the poultry feedstuffs guidance limit of 100 μg/kg OA.

Mechanical damage to foodstuffs can increase the likelihood of mycotoxin production (Santin 2005). Milling of peanuts to produce granules may mimic this effect. Whilst only 14% (2/14) of peanut granule products exceeded the AFB_1 MPL, AF residues for granules were significantly more frequent at point of sale than for whole peanut products. In addition, the single peanut granule sample exposed to summer climate conditions (and which had OA levels of 4.4 μg/kg) formed a solid block of food with visible fungal contamination from which a

347 *Mucor* sp. was isolated (Supplementary Fig. S2). Particular care (see below) may be required
348 when providing granular foodstuffs.
349
350 Sunflower seeds were the least likely of the foodstuffs tested to have AF residues, and no
351 samples exceeded the MPL for AFB_1. Nevertheless, there are examples of sunflower seed
352 intended for animal feed consignments exceeding the MPL for AFs at importation into Europe
353 (RASFF, 2019), therefore seeds may also be affected by mycotoxin contamination. In recent
354 years, some consignments of peanuts imported to the EU and destined for use in supplementary
355 food products for garden birds also have failed to meet the AFB_1 MPL. Of 103 consignments
356 of feed materials rejected for mycotoxin contamination from 1st January 2017 to 6th September
357 2019, 32 were specified as groundnuts for birdfeed or wildlife feed (RASFF Portal,
358 https://webgate.ec.europa.eu/rasff-window/portal/).
359
360 Although AFs have previously been recorded in commercial pet food (Leung et al., 2006), only
361 a small number of published studies have tested for mycotoxin residues in foodstuffs sold or
362 used for wild animal consumption, each of which has detected AF and/or OA in a subset of
363 samples at variable levels. Most of these studies focus on food for game bird and cervid species
364 in North America (Schweitzer et al., 2001; Oberheu et al., 2001a,b; Fischer et al., 1995;
365 Dunham et al, 2017). In one study, 142 samples of wild bird seed marketed for use in backyard
366 feeders were tested from a variety of commercial sources in Texas, USA, and AF levels ranging
367 from non-detectable to 2780 µg/kg were found: 17% of the samples had AF>100 µg/kg, of
368 which 83% contained corn as an ingredient (Henke et al., 2001). A single study of pet foods in
369 the UK included testing of 15 wild bird food products that comprised eight peanut products,
370 three various seed products and four mixed products: 370 µg/kg AFB1 was detected in one of
371 the peanut samples and 6 µg/kg OA was detected in a wild bird mixed food (Scudamore et al.,

372 1997). Our results are consistent with this earlier work, but update findings over a 20-year
373 interval and comprise a larger sample size.
374
375 Whilst there was variation within the treatment groups, results were found to be relatively
376 consistent for many of the peanut samples across the four treatment categories. This indicates
377 that, although some heterogeneity among the samples was present, AF contaminated products
378 did not appear to be the result of an isolated pocket of mycotoxin production. While we found
379 no statistical evidence to support enhanced mycotoxin production in storage or exposure to
380 British climate conditions, the possibility that this may occur cannot be excluded. It is
381 interesting to note that the peak AF and OA values recorded were in samples from the climate
382 exposure and storage treatments and not in the point of sale samples. Oberheu et al. (2001a,b)
383 found an association between mean relative humidity and AF and OA concentrations in grain
384 samples within game bird feeders; evaluation of the relationship between relative humidity and
385 mycotoxin levels was not possible with the climatic data collected in this study.
386
387 There was no significant difference in the frequency of detection of aflatoxins in products
388 purchased in 2007 and 2018, however OA was detected more frequently whole peanut products
389 in 2018 than 2007. This could be due to a number of factors, including normal seasonal and
390 annual variation due to differing climatic conditions when the crops are produced and stored.
391 Indeed, whilst it is not possible to control for this annual variation in mycotoxin production, it
392 is plausible that the sampling year selection in this study could significantly influence our
393 findings. OA is produced by several different fungi, mainly various species of *Aspergillus* and
394 *Penicillium*. These contaminate a range of crops grown in different climates. For example,
395 species from the *Aspergillus ochraceus* group can infect cereals, coffee, and nuts, and
396 *Aspergillus carbonarius* has been associated with grapes, dried vine fruits and wine (Magan

and Aldred, 2005). *Penicillium verrucosum* is more common in temperate regions and usually associated with cereals, whereas *P. nordicum* generally contaminates protein rich food such as cheese and meat (Wang et al 2016). In general, OA formation on grains occurs mainly after harvesting on insufficiently dried cereal and cereal products. Factors influencing OA production include environmental conditions, such as temperature and water activity, but also the type and integrity of the seeds (Denli and Perez, 2010). Therefore, low moisture levels in commodities placed in storage or during transportation is key to preventing OA formation; if this is not well controlled then OA may be produced. Similarly, if there are fluctuations in temperature and humidity, such as can occur during transport, this can result in optimal conditions for OA formation.

In this study, we found no significant difference between AF or OA residues in branded versus non-branded foodstuffs. Some branded products state on their labels that they are tested for aflatoxins or tested as nil detectable aflatoxins. Whilst no QC information was available for the non-branded peanuts, it is important to note that the absence of information does not indicate that AF screening had not been performed.

Regulation (EC) No 767/2009 details current rules for labelling, packaging and presentation of food and defines 'non-food producing' animals as 'any animal that is fed, bred or kept but that is not used for human consumption, such as fur animals, pets and animals kept in laboratories, zoos or circuses': whilst free-living wildlife is not mentioned, products marketed as wild bird food are likely governed by this legislation since they are 'fed' by people. However, since wild bird foods are frequently whole feed materials without additives, as opposed to compound food, some specific mandatory labelling requirements, such as the indication of a minimum storage life, may not apply (Articles 15-17). A Best Before Date was printed on 80% (45/56) of the

422 branded wild bird food products purchased in 2018 in this study, where the labelling was
423 carefully checked for this information.
424
425 The importance of agricultural crops or wild seed as a source of AF or OA exposure for wild
426 birds requires evaluation to help put any risks from supplementary food in context. A study of
427 field corn in the Mississippi, U.S.A., found levels of AFB_1 ranging from 5-5000 ppb (μg/kg),
428 with greatest levels in corn found on the ground post-harvest, but no detectable OA (Couvillion
429 et al., 1991). An understanding of the proportion of wild bird diet that supplementary food
430 constitutes compared with agricultural crops or wild seed is required to assess the relative risk
431 of supplementary feed and to understand whether provisioning puts wild birds at an elevated
432 risk of mycotoxin exposure.
433
434 Whether birds have the ability to discriminate, and therefore avoid, mycotoxin contaminated
435 food remains uncertain. A study by Perez et al. (2001) of three species of wild-caught birds
436 found mixed evidence of avoidance of AF-contaminated food in an experimental trial.
437
438 Peanuts are a common supplementary food product for garden birds, although, with the
439 growing diversity of foodstuffs for wild birds that are now commercially available, their
440 relative use has reduced in recent years. Whilst 86% of participants in the British Trust for
441 Ornithology (BTO) Garden BirdWatch scheme offered peanuts to wild birds in their garden in
442 the winter of 2007, this figure reduced to 66% in 2017 (BTO, *unpublished data*); nevertheless,
443 peanuts will be available in the majority of gardens where supplementary food is provided. The
444 results of our questionnaire survey found that a greater percentage of respondents provided
445 branded peanuts and branded seed than non-branded products of either food type. It is
446 important to note, however, that ZSL and RSPB staff might not be representative of the public

447 in general in this regard. The majority of questionnaire respondents provided food for garden
448 birds year-round. Sweeney and Dobson (1998) summarized data on the range of ambient
449 temperature for *Aspergillus* and *Penicillium* sp. growth and mycotoxin production, and optimal
450 conditions for these to occur: these data indicate AF production may occur under ambient
451 temperature conditions similar to those likely to occur during the warmer summer, but not the
452 cooler winter, months in Great Britain. It is possible, therefore, that the move to summer-
453 feeding of garden birds, might be associated with an increased risk of mycotoxin exposure from
454 supplementary feed. In this questionnaire survey, only one quarter of respondents emptied bird
455 feeders before refilling them. Topping up the contents of a feeder may lead to residues at the
456 base of the feeder remaining in situ for some time. Whether cleaning was practiced in between
457 refilling feeders was not assessed in the questionnaire design. However, it should be noted that,
458 if feeders are not cleaned before replenishing with fresh food, there is a risk that contaminated
459 food residues remain that could promote subsequent fungal growth. Lawson et al. (2018) found
460 detectable, and sometimes high, levels of AF in feeder residues: consequently, feeders should
461 be emptied and cleaned before being refilled.
462
463 In conclusion, the findings of this study indicate that current practice at feeding stations
464 presents a potential risk of mycotoxin, in particular AF, exposure to garden birds from
465 supplementary feed. Whilst the significance, if any, of mycotoxin exposure to wild bird health
466 is currently unknown, the adoption of sensible precautions to minimize potential risks is
467 recommended. Foodstuffs should be purchased in volumes that will be used within a reasonable
468 period (e.g. quarterly period) or, by the Best Before Date if available. Buyers may wish to
469 enquire on the source of the product and its storage history. Foodstuffs should be stored in
470 sealed containers in a cool, dry environment with minimal temperature variation to avoid
471 condensation. Food should be offered in feeders in moderate volumes that are used within a

short period (e.g. 1-2 days). Feeders and tables should be regularly cleaned and disinfected to avoid build-up of waste material and potential fungal contamination; feeders should not be repeatedly topped up but left until empty so that the food is not allowed to become stale. Food residues at the base of feeders that are not eaten should be disposed of as waste to avoid them being consumed by wildlife or other animals at a later stage. A variety of foodstuffs should be offered as a supplement, rather than as a replacement, for the natural diet; therefore, any mycotoxin contaminated product is only likely to constitute a small proportion of the diet. Our survey responses indicate that those provisioning wild birds could further minimize the risk of consumption of mycotoxin-contaminated food by birds, so an education program could be beneficial. Companies and ornithological non-governmental organisations who market wild bird food can also assist by promoting best practice guidance.

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Competing interests

We declare no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Credit author statements

Becki Lawson: Conceptualization, Methodology, Investigation **Robert A. Robinson**: Formal analysis **Mita Parmar**: Investigation **Rowena Killick**: Investigation **Andrew A. Cunningham**: Supervision, Funding acquisition **Susan J. MacDonald**: Conceptualization Methodology, Investigation. **All authors**: Writing - Original Draft

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Figure Captions

Figure 1a Levels of Aflatoxin B_1 in wild bird supplementary food products purchased in 2007 and 2018 at point of sale. Bars indicate number of samples containing no detectable residues (white), detectable residues ≤MPL of 20 µg/kg (light blue), and above the MPL (dark blue).

Figure 1b Levels of Ochratoxin A in wild bird supplementary food products purchased in 2007 and 2018 at point of sale. Bars indicate number of samples containing no detectable residues (white), detectable residues ≤5 µg/kg (light blue), and greater than 5 µg/kg (dark blue). Note there is no MPL for Ochratoxin A so these are arbitrary ranges to visualize the data.

Figure 2a Levels of Aflatoxin B_1 in peanut products purchased in 2007. Bars indicate number of samples containing no detectable residues (white), detectable residues ≤ MPL of 20 µg/kg (light blue), and above the MPL (dark blue).

Figure 2b Levels of Ochratoxin A in peanut products purchased in 2007. Bars indicate number of samples containing no detectable residues (white), detectable residues ≤5 µg/kg (light blue), and greater than 5 µg/kg (dark blue). Note there is no MPL for Ochratoxin A so these are arbitrary ranges to visualize the data.

692

693 **Supplementary File Captions**

694 **Supplementary Table S1**: Environmental Temperature and Relative Humidity Range in the
695 Treatment Categories.

696

697 **Supplementary Figure S1**: Peanut samples in hanging feeders within mock feeding station
698 used for Spring and Summer Exposure treatments

699

700 **Supplementary Figure S2**: Peanut granule sample following Summer Exposure treatment in
701 2007: (a) as removed from feeder and (b) on cut transverse section

702

703 **Supplementary Database S1**: Wild bird food sample metadata (e.g. food type, branded versus
704 non-branded product) and aflatoxin and ochratoxin A concentrations (µg/kg) detected for 2007
705 Point of Sale (worksheet 1), 2007 Spring Exposure (worksheet 2), 2007 Storage (worksheet 3),
706 2007 Summer Exposure (worksheet 4) and 2018 Point of Sale (worksheet 5).

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