Environmental Toxicology

Proposed Indoor Test Procedure to Quantify Pesticide Treatment Effects on Seed Consumption by Birds

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Abstract: Pesticides used in seed coatings can influence seed consumption by birds and, therefore, actual exposure risk for them. A quantification of such effects on consumption is currently not regarded as a refinement factor in environmental risk assessments, although it is a possible option and should be considered, for example, for comparing exposure risk of different pesticides. It can highlight avoidance behavior, preventing birds from taking up lethal or sublethal pesticide doses. To formulate a standard, we developed an indoor test procedure based on established pen test methods, including 2- and no-choice phases with hunger periods. During testing, the highest standards of animal welfare were applied. Statistical approaches were used to determine the most appropriate number of replicates and for analysis. The effect on consumption of seeds is expressed as the ratio of consumed treated to untreated seeds. This consumption factor can be applied in avian risk assessments for seed treatments equivalent to an avoidance factor. We present, as an example, an application of the procedure to obtain a seed- and species-specific consumption factor for oilseed rape seeds (*Brassica napus*) provided untreated and treated with fungicides to greenfinches (*Carduelis chloris*) and Japanese quail (*Coturnix japonica*). Overall, bird constitution was not negatively affected by the test procedure in either species. The test procedure was suitable for showing differences in expected consumption patterns, such as greater avoidance of treated seeds in 2-choice than in no-choice tests. However, the consumption differed between species and fungicide treatments, allowing us to rank avoidance effects of different fungicides. Using the presented standard procedure to generate comparable pesticide- and species-specific consumption factors for more species and seed treatments may result in refinement of default values and reduce animal trials in different designs in the future. *Environ Toxicol Chem* 2020;39:359–370. © 2019 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

In the natural environment, a huge range of different food items are available to free-living birds. Food items are not consumed in equal amounts, and some are avoided (e.g., Kelrick et al. 1986; Prosser and Hart 2005; Cueto et al. 2006). Past studies have highlighted the avoidance of seeds of plant species caused by phytochemicals such as tannins or alkaloids (Díaz 1996;

Cueto et al. 2006), and this avoidance effect is also known from pesticide-treated crop seeds (Prosser and Hart 2005).

Besides the natural food selection, consumption can therefore be influenced by treatment of seeds with chemical substances. Seed treatments in agriculture are applied to act against pest organisms like insects and fungal infestation (Pascual et al. 1999a, 1999b; Lopez-Antia et al. 2014); if the consumption of such treated seeds is reduced, this effect is described as "avoidance," reducing chemical exposure to birds (McKay et al. 1999; Organisation for Economic Co-operation and Development 2011). Other seed treatments are directly used to reduce damage to sown seeds by birds and are considered to represent direct repellents (e.g., anthraquinone: DeLiberto and Werner 2016; methyl anthranilate: Werner and Avery 2017).

The avoidance effect was considered in the past and used as a common refinement factor (covering the reduction of consumption rate) for birds or mammals in the risk-assessment scheme under the guidance for authorizing plant protection products (PPPs) based on single trials (with 1 or 2 species only) per active substance in a PPP (European Commission 2002). The factor is still mentioned as an option for risk refinement of birds and mammals in the current version of the European Food Safety Authority (2009) guidance "Risk Assessment for Birds and Mammals". However, it is mostly ignored or even not applied during evaluation processes and common assessment by regulatory authorities. The main argument for not including such a factor in environmental risk assessments is that avoidance cannot be represented appropriately by a single factor because it is not constant over time for all chemical substances and it differs between species. In addition, the database for active substances under evaluation is often limited. Only single tests, with different designs, involving one or 2 species, are available; and these are considered not to be representative or to cover all variation of avoidance in the field across different species. Thus, an avoidance factor from the laboratory cannot be directly translated to the protection in the wild for all exposed bird species. However, following standardized procedures, the avoidance factor can be relevant particularly in a comparative assessment within the authorization procedure for PPPs. Avoidance as actually recommended in European Food Safety Authority (2009) can be a useful tool for decision-making, especially when 2 or more active substances, which act against the same pest, have similar toxicity levels. Results from the laboratory can help to compare between substances and hence save time and effort.

There are no internationally accepted guidelines for test procedures to measure consumption effects of birds to make results comparable. Besides studies presenting repellent and avoidance test results (e.g., Pascual et al. 1999a; Lopez-Antia et al. 2014), 4 national test concepts (France: Institut National de la Recherche Agronomique 1990; Germany: Biologische Bundesanstalt für Land- und Forstwirtschaft 1985, 1993; The Netherlands: Luttik 1993; United Kingdom: Fryday et al. 1999) and the preliminary Organisation for Economic Co-operation and Development guideline (2011, updated 2016) suggest general study requirements. These documents differ regarding the endpoint investigated (e.g., consumption or mortality) and with regard to the test design, including differences in test species, reproductive state, age of tested animals, number of test animals, housing conditions, duration, feeding conditions, administration of the substance, fasting period, exposure duration of test item, observation period, and examined parameters. They reflect a heterogeneity of options instead of providing guidance for standard procedures.

We used the existing documents and considered the ethical and welfare standards (Bundesministerium für Ernährung und Landwirtschaft 1996; Bundesministerium der Justiz und für Verbraucherschutz 2006, 2013; European Union 2010, 2013) and ecological traits of the species (Bezzel 1982; Prosser and Hart 2005; Holland et al. 2006) when examining the most relevant criteria for consumption rates in birds to develop a

procedure to quantify consumption effects attributable to chemical treatment of the seeds offered. Our approach allows the calculation of a consumption factor by measuring treatment-specific differences in birds foraging on seeds in pens with 2-choice and no-choice tests. Within the test procedure, statistical routines are suggested to calculate iteration numbers, minimizing the number of animals; this was one of the most important concerns for us following the legal framework in Germany with regard to animal welfare (Bundesministerium der Justiz und für Verbraucherschutz 2006, 2013). Subsequently, we used the procedure for a comparative exposure assessment of different treatments with fungicides on oilseed rape seeds (*Brassica napus*).

MATERIAL AND METHODS

Ethical note

Pen experiments were conducted with the permission of the animal ethics committee of LANUV North Rhine-Westphalia (file number 84-02.04.2015.A521). The experiments are used for the present study to demonstrate the practical use of the procedure. The fungicides used were not candidates for substitution in the European Union's renewal process of active substances (Mattaar 2017); thus, we do not name the products. All efforts were made to minimize the number of animals used and to avoid any suffering within the developed test procedure.

Test design proposal

The "design" refers to pen tests and can be conducted with different bird species.

Test seeds. Four fungicide treatments of oilseed rape (*Brassica napus*) seeds were used (including the control); we refer to these as c, f1, f2, and f3. The treatment rates covered the maximum levels expected in the field (Organisation for Economic Co-operation and Development 2011, 2016), as can be found in each respective analytical certificate of analyses. As treatments, only product formulations that could be expected in the field situation were used (Organisation for Economic Co-operation and Development 2011, 2016).

Test species. Following the available recommendations relating to avoidance tests with respect to animal welfare (Organisation for Economic Co-operation and Development 2011, 2016), we used only domestic stock birds for the pen trials (Bundesministerium der Justiz und für Verbraucherschutz 2006, 2013). Domestic male and female (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993) greenfinches (*Carduelis chloris*) and Japanese quail (*Coturnix japonica*) were used. The greenfinches were kept in pairs and the Japanese quail in groups of 3 (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993; Bundesministerium für Ernährung und Landwirtschaft 1996; Organisation for Economic Co-operation and Development 2011). The greenfinch is a representative small granivorous farmland bird in Germany and known to feed on oilseed rape seeds (Bezzel 1982; Prosser and Hart 2005;

Holland et al. 2006). Quail represent Galliformes like pheasants and partridges, which inhabit agricultural landscapes and feed on seeds (Bezzel 1982; Holland et al. 2006). They are well established as a bird species for food choice experiments (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993; European Union 2013; Organisation for Economic Co-operation and Development 2016). All birds were adults and in a good state of health.

Alternative food. Exclusive consumption of test seeds was avoided because test seeds would not constitute a sufficient and well-balanced diet for the birds (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993), and an appropriate standard aviary diet was chosen (Organisation for Economic Co-operation and Development 2011). As the alternative food, we used a mixture of corn and fruit/seeds (e.g., sunflower seeds, sorghum, peanut cores, hemp, oatmeal, raisins; Raiffeisen Wildvogelfutter) for greenfinches. For Japanese quail we used pellets (Deuka All-mash A) consisting mainly of wheat (35.7%), maize (28%), soy extract (17.7%), and wheat bran (5.0%). Separate consumption measurements were possible because we were able to distinguish between treated and untreated material (Organisation for Economic Co-operation and Development 2011).

Housing conditions. Pen sizes were based on species, animal numbers, and welfare considerations (Bundesministerium für Ernährung und Landwirtschaft 1996; European Union 2010; Organisation for Economic Co-operation and Development 2011). Each greenfinch pair was kept indoors in a plastic cage of 1.0 × 0.4 × 0.5 m (length × width × height), which is based on the recommendation for Fringillidae species from Bundesministerium für Ernährung und Landwirtschaft (1996). The cages had 3 opaque sides (Bundesministerium für Ernährung und Landwirtschaft 1996) throughout the experiment, except during the last test phase for follow-up observation where they were kept free-range in the pens used for the quail experiments. The cage included 2 bars and several twigs to perch on, as well as 2 food pots and water ad libitum.

For the quail groups, 4 tiled indoor pens were available, each measuring 2.6 × 3.0 × 2 m (length × width × height), which is larger than the minimum each individual of a group has to have (European Union 2010). Each pen had 2 or 3 opaque sides according to Bundesministerium für Ernährung und Landwirtschaft (1996). Between the choice-test phases, additional outdoor aviaries of 15 to 20 m², opaque on one side (Bundesministerium für Ernährung und Landwirtschaft 1996) with sandy ground, were used for housing. Both indoor and outdoor pens were provided with small plastic shelters. Water was available ad libitum in a bowl indoors. Food was offered in each corner of the indoor pen in 4 ceramic food racks with a diameter of 14.5 cm, which exceeds the minimum length of 4 cm per individual (Bundesministerium für Ernährung und Landwirtschaft 1996).

Procedure. The procedure developed consists of 7 phases (Table 1; for details, see Supplemental Data, Appendices A1

TABLE 1: Test procedure with 2 sequences: 2-choice prior no-choice test and no-choice prior 2-choice test[a]

2- prior no-choice test	Day	No- prior 2-choice test
Acclimatization	1–5	Acclimatization
2-choice test	5–9	No-choice test (pretreatment phase)
Recreation	9–12	Recreation
No-choice test (pretreatment phase)	12–16	No-choice test (treatment phase)
Recreation	16–19	Recreation
No-choice test (treatment phase)	19–23	2-choice test
Follow-up observation	23–27	Follow-up observation

[a]Both 2-choice and no-choice tests produce data, which can be used for exposure comparisons. Phases of acclimation and recuperation can take longer if required to ensure that birds are in suitable states for testing.

and A2). There are 2 sequences of choice-test phases; one with a no-choice prior to a 2-choice test (NpT) and the other with a 2-choice prior to a no-choice test (TpN). Thus, the importance of choice- and no-choice tests is considered (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993; Organisation for Economic Co-operation and Development 2011, 2016). The different sequences allow for the inclusion of individuals not familiar with the treatment (first choice phase) and individuals familiar with the treatment (second choice phase) in both 2- and no-choice tests; this addresses the possible influence of food neophobia and conditioning to food on consumption rates (Organisation for Economic Co-operation and Development 2011, 2016).

In general, animals always received water ad libitum. Greenfinches received 2 bowls with food on the left and right sides of the cage, whereas for quail 4 bowls were placed, one in each corner of the indoor pen. Position of the food bowls was switched each day during the test phase (greenfinches) and rotated clockwise day by day (quail), to avoid conditioning on a particular bowl. Additional bowls of food were always present, out of reach, and protected from consumption beside the cage of the greenfinches and within the indoor pen of the quail, to measure food-specific weight changes as a result of changing moisture content. Food spillage was always collected and added to the leftover food.

At the beginning of each phase, animal body weights were measured to calculate the endpoint parameter, which is the food consumption expressed as the amount of food eaten per gram of body weight. A health check of the animals was carried out at least once a day (Bundesministerium der Justiz und für Verbraucherschutz 2013). In the case of anomalies, such as behavioral changes, animals were substituted with new individuals.

The phase of acclimation was limited to 4 d (Organisation for Economic Co-operation and Development 2011) because we used domestic birds with alternative food ad libitum offered in all feeding bowls (Table 1). On the first day, all animal groups received alternative food for 1 d, 60 and 25 g per bowl per quail group and greenfinch pair, respectively. On the second day, food was refreshed, and all groups received alternative food for 3 d; consumption was measured on the last day, and

the value was used to calculate the minimum amount of alternative food that should be offered during no-choice tests.

During the 2-choice tests, which lasted 5 d (Table 1), the food was restocked and consumption was measured once per day. Alternative food and untreated rape seeds were given the control group in equal and more than sufficient amounts, with each food variety in one (greenfinches) and 2 (quail) feeding bowls, respectively. The same applied for the other test groups, which, however, received treated rape seeds instead of the untreated rape seeds.

During recreation phases, all groups received alternative food for 3 d; consumption was measured on the last day.

The no-choice test represents a worst-case scenario. It is divided into 3 periods over 24 h: first, a fasting period without food for 16 h overnight to simulate food deprivation and normal diurnal restrictions on consumption activity (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993; Organisation for Economic Co-operation and Development 2011); second, a test period of 4 h; and third, a resting period with alternative food ad libitum for another 4 h. During the test period, test material (rape seeds) was offered in weighed quantities ad libitum. The no-choice test is further partitioned into a pretreatment and a treatment phase (Table 1). In the 4 d of the pretreatment phase, birds are prepared to the fasting and consumption periods prior to the exposure period (Organisation for Economic Co-operation and Development 2011) but with untreated rape seeds being offered only. During the treatment phase, used for statistical analysis, control groups were offered untreated rape seeds. The other groups received treated rape seeds. In all treatments, an amount of alternative food comprising 10% of the average amount of food eaten within the 4 h of acclimation was offered during the test period to avoid starvation, to ensure animal welfare after the fasting period, and to encourage feeding (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993).

Follow-up observations were made for a further 14 d to detect delayed symptoms (Biologische Bundesanstalt für Land- und Forstwirtschaft 1993); during this time, alternative food was restocked, if required, and consumption was not measured.

Groups and replication. Each group of greenfinches consisted of one pair (1 female and 1 male). Groups of quail consisted of 3 individuals, either 2 females and 1 male or 2 males and 1 female, resulting in an overall sex ratio of 1:1. Four groups were tested at the same time, one for each rape seed treatment: the control (i.e., untreated rape seeds) and f1, f2, and f3 (i.e., the 3 different fungicide treatments). A control group was involved to assist in the interpretation of mortality and/or sublethal effects seen in the other treatments and to determine whether the test conditions biased the test by deterring consumption even in the absence of the test material (Organisation for Economic Co-operation and Development 2016).

To obtain an estimate of the required number of replicates (Bundesministerium der Justiz und für Verbraucherschutz 2013), we used the SIMR package (Green and MacLeod 2016), which calculates power curves for simulated data and mixed model analysis based on Monte Carlo simulations. For the simulations, we defined a setup with a comparison of one treatment versus control with repeated measurements over 4 d. To run simulations, we assumed the following input variables (which varied accordingly): the fixed effect for average consumption of food in the control group ($b_o = 100$, was held constant) and reduction of consumption in the treatment groups (f; $b_1 = -25$ and -50, i.e., 25 and 50% reductions of the control group), the random effect variance parameters for the pens or cages where the birds were held ($V = 100$, 50, or 10), and the residual variance ($s = 50$, 25, or 10). From the results (Supplemental Data, Appendix A3), we could see that 5 runs (temporal replicates) would provide sufficient power (i.e., 80%) to detect a 25% reduction in food consumption when residual variance is <25 and to detect a reduction in food consumption by 50% when residual variance is <50. The lower the variance of the random effect, the higher the power; but this effect was rather small. Based on these results along with practical considerations, we selected 6 replicates for the no- and for the 2-choice tests, acquiring 96 measurements per bird species—that is, 4 treatments × (3 NpT + 3 TpN) × 4 d = 96. The treatments must always be distributed randomly across the pens or cages for each replicate.

Statistical analysis

All analyses for greenfinches and quail were performed separately. In the text, arithmetic means ± 1 standard deviation are given. Statistical analysis was conducted using R (R Development Core Team 2017) and the packages lme4 (Bates et al. 2015) and nlme (Pinheiro et al. 2018) for linear mixed models, the package glmmTMB (Magnusson et al. 2017) for beta and tweedie mixed models, and the package emmeans (Lenth 2017) for obtaining confidence intervals and post hoc tests. The significance level was set to 0.05. The R code and the data are provided in Supplemental Data, Appendix A4.

Effects on state of health. Individuals' weights were recorded at the beginning of the experiment and after each test phase. If individuals had to be substituted because of anomalies or death, seed treatment and phase were recorded to exclude the possibility of general negative effects on bird conditions. To analyze body weight changes as a factor for negative effects of the test procedure on animals, a linear mixed effect model was fitted with body weight as the dependent variable and the fixed effects' time of measurement (before the experiment, after the experiment), rape seed treatment (control, f1, f2, f3), sequence (NpT, TpN), and sex (female, male), all 2-way interactions with phase, and the random effect of bird identity (greenfinches, 1–46; quail, 1–71). A significance test was conducted using the analysis of variance function in the package lmerTest (Kuznetsova et al. 2017) with the Kenward-Roger option for denominator degrees of freedom and restricted maximum likelihood (REML) fit. A significant main effect of phase or a significant interaction with phase would indicate body weight changes during the course of the experiment.

Effects on consumption. Feeding behavior of birds (i.e., the total consumption of food and the percentage consumption of the alternative food in the 2-choice test and the consumption of rape seeds in both the 2- and no-choice tests) was analyzed using general and generalized linear mixed effect models. These models account for the repeated measurements over days by using pens or cages (1–4) nested within temporal replicate (1–6) as random effects. Separate models for greenfinches and quail were fitted for the rape seed treatment (control, f1, f2, f3) and sequence (NpT, TpN), and the 2-way interactions were treated as the fixed effects. A model selection process based on the Akaike information criterion (AIC) was used to select the fixed effects (Burnham and Anderson 2002). Thus, the small sample size corrected version of the AIC (AICc) was used because sample size n was small relative to the number of model parameters K (i.e., $n/K < 40$). The model with the lowest AICc was considered best and used for model interpretation. The AICc values were also used to calculate the Akaike weights w_i, which can be interpreted as the probability that the selected model is the best model of those considered (Burnham and Anderson 2002). For model comparisons, models were fitted with maximum likelihood; and for post hoc tests and interpretation, models were refitted with REML (applies only to linear mixed effect models). Model assumptions were checked by plotting residuals against fitted values and against explanatory variables.

In the 2-choice experiment, the total consumption of food (i.e., the sum of treated rape seeds and of the alternative food) was modeled using a linear mixed effect model.

The percentage consumption of the alternative food (i.e., the alternative food divided by the sum of treated rape seed and alternative food) was modeled using a beta regression mixed model with logit-link, which can fit data that are restricted to a standard unit interval between >0 and <100% (Cribari-Neto and Zeileis 2010). Observed values of 100% were substituted with 99.9999%. In this modeling approach, we used the fixed effects but also included those terms to model the precision parameter (i.e., dispersion). Model selection was undertaken first by fitting models with all fixed and random effects but different sets of terms to model dispersion. Having selected the best set of terms for dispersion, models were then compared with different fixed effects.

The consumption of rape seeds was measured on a daily basis in the 2-choice test and after 4 h in the no-choice test. Hence, separate mixed models were fitted for the 2 tests. Because data on consumption were continuous, strictly positive, but also contained zeros, we fitted either generalized

linear mixed models assuming the tweedie family or general linear mixed models using the LN(X + 0.001) transformation, when models with the tweedie family did not converge or residuals did not meet model assumptions. The percentage reduction in consumption in comparison to the control was calculated for each treatment using the conditional means of the model.

Consumption factor. Risk of exposure for birds is lower the fewer birds feed on seeds. We calculated a treatment-specific ratio (R_T; based on European Commission 2002) for each species and choice test by

$$R_T = \frac{\bar{C}_T}{\bar{C}_A}$$

with $\bar{C}_T$ being the average consumption of the control target seeds and $\bar{C}_A$ the average consumption of pesticide-treated target seeds. An R_T value between 0 and 1 indicates an effect from complete to no avoidance attributable to the treatment; an $R_T > 1$ indicates preference, which increases with the value.

To report a conservative measure, we used the highest R_T to express the consumption factor for each treatment (CF_T), making sure that it reflects the highest risk for birds by

$$CF_T = \text{Max}\,[R_T...]$$

RESULTS

Effects on state of health

During the procedure, 4 individuals, 3 greenfinches and 1 quail, had to be replaced because of critical body conditions or death; 3 of them had no fungicide contact before (Table 2). The 3 greenfinches (1 male and 2 females) belonged to groups for testing f3. The male was replaced during the acclimation. One female that replaced another one in the pretreatment no-choice phase had to be substituted in the no-choice phase. A male quail from a control group was replaced during the pretreatment no-choice phase.

On average, body weight of greenfinches tested over the whole procedure was 28.8 ± 3.5 g and changed by –0.7 ± 3.2 g during the course of the experiment (Figure 1). We found higher body weights in NpT (no-choice prior 2-choice test sequence) than in TpN (2-choice prior no-choice test sequence) without significant body weight changes before and after the experiment (Supplemental Data, Appendix A5). There were no significant differences in body weight change between rape

TABLE 2: List of individuals that had to be replaced during the trials

Species	Sex	Replication	Sequence	Phase	Treatment
Greenfinch	Male	1	TpN	Acclimatization	f3
Greenfinch	Female	4	NpT	No-choice test (pretreatment phase)	f3
Greenfinch	Female	4	NpT	No-choice test (treatment phase)	f3
Quail	Male	2	TpN	No-choice test (pretreatment phase)	Control

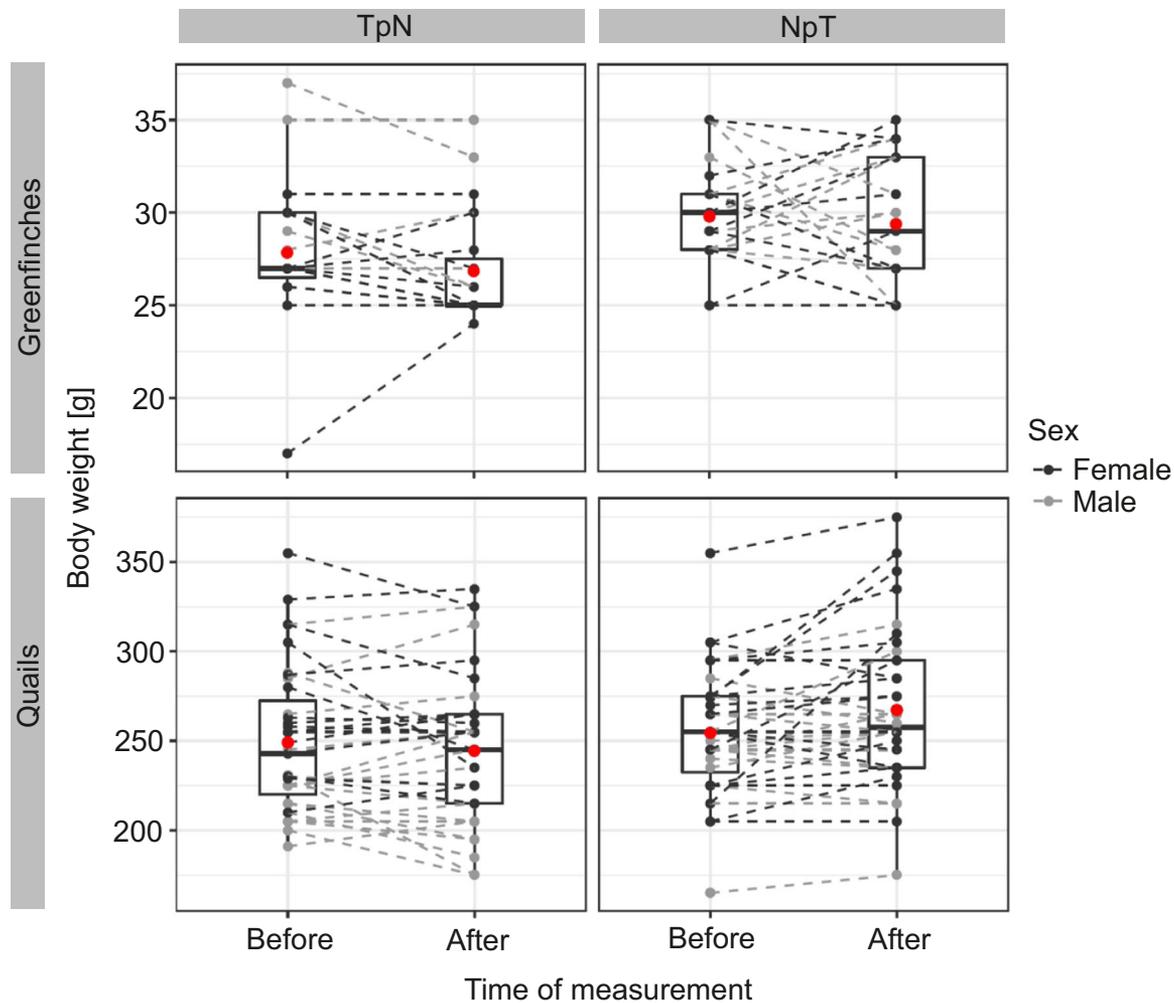NpT = no- prior 2-choice test; TpN = 2- prior no-choice test.

FIGURE 1: Boxplots of body weights before and after the test procedure in relation to sequence and sex for greenfinches ($n = 46$) and quail ($n = 71$). Observed values for individuals before and after the test procedure are displayed as points and linked with a dotted line. NpT = no- prior 2-choice test; TpN = 2- prior no-choice test.

seed treatments. Quail weighed 254 ± 4 g on average and changed in body weight approximately 4 ± 26 g. A significant interaction between phase and sequence indicated a 12.2 g higher body weight after the experiment in the NpT sequence (Supplemental Data, Appendix A5). Treatment had no effect on body weight change.

Consumption of untreated rape seeds versus alternative food (2-choice test)

In the 2-choice test, the greenfinches' total daily consumption (i.e., sum of alternative food and rape seeds per day in g/g body wt) was on average 0.162 ± 0.04 g/g body weight, with a maximum of 0.328 and a minimum of 0.072 g/g body weight (Figure 2). The model with treatment as the fixed effect gave the best fit with the lowest AICc and a w_i of 0.33 (Supplemental Data, Appendix A6), indicating a higher daily consumption by greenfinches in f1, f2, and f3 compared to the control. However, the AICc of the model was only 0.7 lower

than the null model, and hence the post hoc test did not confirm significant differences. For quail, the total daily consumption was on average 0.114 ± 0.027 g/g body weight ranging between 0.057 and 0.165 g/g body weight. The model with the fixed effect sequence gave the best fit with a w_i of 0.57 (Supplemental Data, Appendix A6), indicating a higher daily consumption in NpT than in TpN; but again, the post hoc test did not confirm significant differences (Figure 2).

The main part of the total consumption was the alternative food, with 87.1% on average for greenfinches and 95.6% for quail. For both greenfinches and quail, the average consumption of alternative food was >99% in f2 and f3 in both sequences. For greenfinches, the model with treatment as a fixed effect gave the best fit with a w_i of 0.49 (Supplemental Data, Appendix A6). Consumption of the alternative food was lowest but similar in the control as well as in f1 and differed from f2 and f3 (Figure 2). For quail, the model with the main effects treatment and sequence gave the best fit with a w_i of 0.49 (Supplemental Data, Appendix A6), with the lowest consumption of the alternative food in the control (Figure 2). The
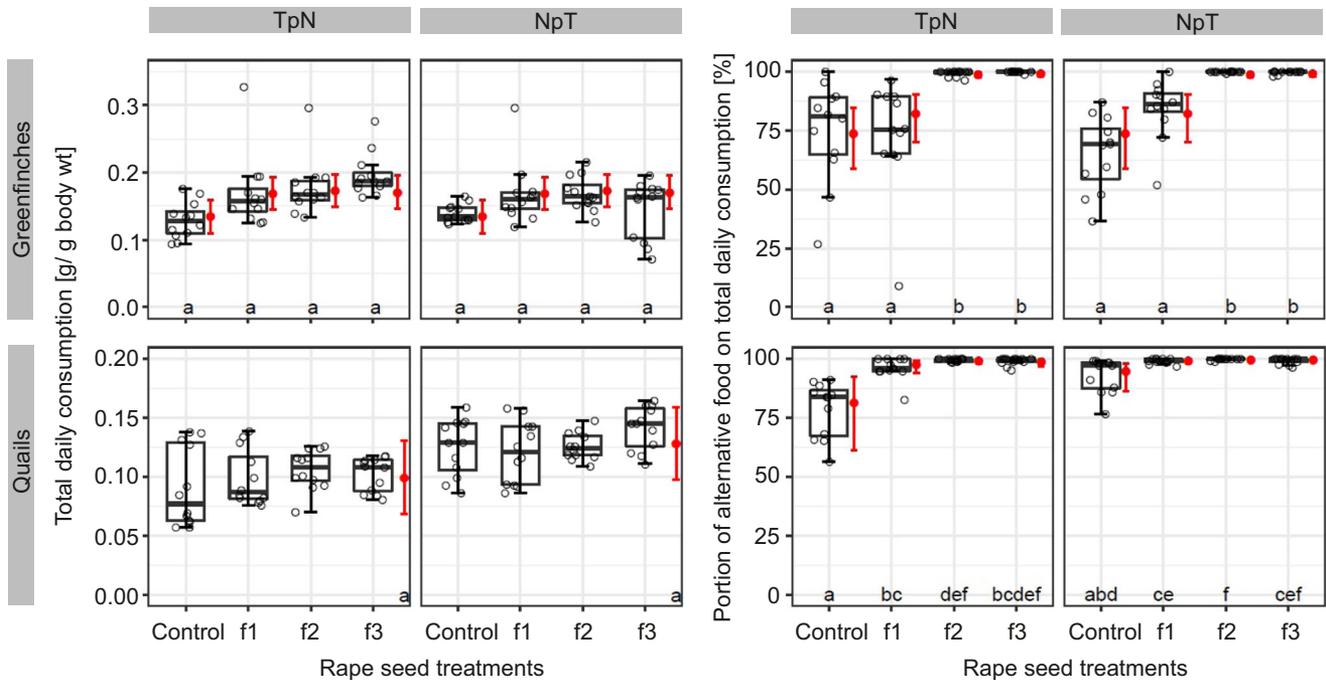
FIGURE 2: Total daily consumption and percentage of consumed alternative food per pen in relation to rape seed treatment (control, f1, f2, f3) and sequence for greenfinches and quail during the choice test. Boxplots are shown with observed values overlaid as jittered points. Conditional means and 95% confidence intervals obtained from mixed models are shown in red. Different letters indicate significant differences obtained from post hoc tests. NpT = no- prior 2-choice test; TpN = 2- prior no-choice test.

treatment had a higher effect on alternative consumption than the sequence, as indicated by the ΔAICc of the models with treatment or sequence as the sole main effect (Supplemental Data, Appendix A6; ΔAICc 0.8 vs 29.0).

Treatment effects on consumption

Greenfinches consumed on average 0.019 g/g body weight of rape seeds during 24 h in the 2-choice tests and 0.028 g/g body weight during 4 h in the no-choice test. In both tests, the model with treatment as the main effect had the lowest AICc (Figure 3; Supplemental Data, Appendix A7), and consumption was highest in the control and f1 and lowest in f2 and f3 (Figure 3). The percentage reduction in consumption in comparison to the control was on average 21 and 17% in f1 in the 2-choice and no-choice tests, respectively, and 99 and 80 to 84% in f2 and f3 in the 2- and the no-choice tests, respectively.

Quail consumed on average 0.004 g/g body weight rape seeds in the 2-choice test and 0.007 g/g body weight rape seeds in the no-choice test. Like greenfinches, the models with treatment as the main effect had the lowest AICc (Figure 3; Supplemental Data, Appendix A7). Rape seed consumption was highest in the control in both tests (Figure 3) and substantially lower in f1, f2, and f3 in the 2-choice test and in f1 and f2 in the no-choice test. The percentage reduction in consumption in comparison to the control was on average 92 to 98% in f1, f2, and f3 in the 2-choice test and 79 to 82% in f1 and f2 and 54% in f3 in the no-choice test.

Consumption values indicated that treatment led to avoidance of rape seeds because the ratio values R_T were always

lower than 1 (Table 3). The ratio was lowest for f2 and highest for f1. The consumption factor CF_T reflected the maximum values of R_T and was 0.80 for f1, 0.24 for f2, and 0.41 for f3, all originating from no-choice tests with greenfinches (f1) and quail (f2 and f3).

DISCUSSION

Test procedure

We developed a procedure to determine the consumption effect of PPPs used as seed treatment on birds; this procedure could be used for a comparative risk assessment within the PPP authorization procedure (Table 1). Using the procedure, we were able to show consumption differences in relation to different rape seed treatments. Such results could be used to identify PPP treatment with high avoidance effects in comparison to other PPPs of similar toxicity but exhibiting no avoidance in birds. However, adaptions and validations by field experiments are necessary before integrating results in the environmental risk assessment (see *State of affairs* section). All potential responses to the offered diet are covered through our procedure because individuals familiarized and not familiarized with the treatment were included. In addition, individuals were exposed to treated and untreated rape seeds over the course of 8 d during the procedure to calculate consumption rates.

The benefit of pen studies is that exact measurements of seed consumption are possible, whereas in field tests it is difficult to obtain precise data about seed consumption because of scatter (Organisation for Economic Co-operation and Development 2016), something that can be assessed in pen
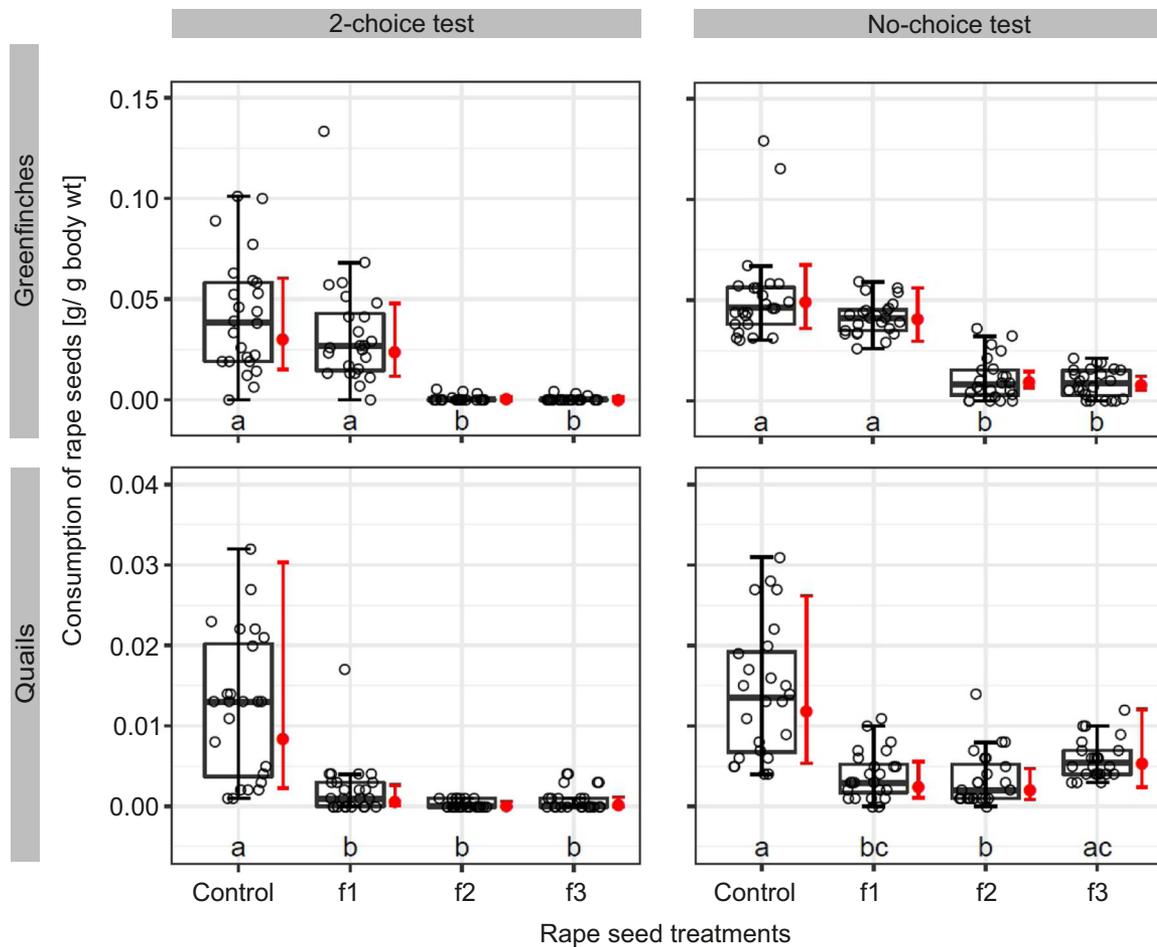
FIGURE 3: Daily consumption of rape seeds during the 2-choice and the no-choice tests for greenfinches and quail in relation to treatment (control, f1, f2, f3). Boxplots are shown with observed values overlaid as jittered points and conditional means and 95% confidence intervals from mixed models. Letters indicate significant differences obtained from post hoc tests and reflect comparisons within a test procedure (i.e., within the choice or no-choice test).

TABLE 3: Treatment-specific data in the 2- and no-choice experiments with greenfinches and quails, showing the arithmetic mean and 1 standard deviation of consumption of alternative food and consumption of rape seeds[a]

Species	Test	Treatment	C_A (g/kg body wt)	C_T (g/kg body wt)	R_T
Greenfinches	2-choice	Control	92.5 ± 23.7	42.1 ± 28.2	
		f1	135.6 ± 57.8	33.3 ± 27.1	0.79
		f2	172.5 ± 33.8	0.5 ± 1.4	0.01
		f3	170.8 ± 44.2	0.4 ± 1.0	0.01
	No-choice	Control	[b]	51.8 ± 23.4	
		f1	[b]	41.4 ± 8.5	**0.80**
		f2	[b]	10.7 ± 10.5	0.21
		f3	[b]	8.8 ± 6.8	0.17
Quails	2-choice	Control	95.3 ± 37.8	12.5 ± 8.8	
		f1	107.9 ± 27.9	2.2 ± 3.4	0.17
		f2	115.8 ± 17.3	0.3 ± 0.5	0.02
		f3	121.0 ± 25.8	0.9 ± 1.4	0.07
	No-choice	Control	[b]	14.3 ± 8.1	
		f1	[b]	3.9 ± 3.3	0.27
		f2	[b]	3.4 ± 3.3	**0.24**
		f3	[b]	5.9 ± 2.4	**0.41**

[a]Treatment-specific ratio (R_T) between 0 and 1 indicates an effect from complete to no avoidance. Bold values are the maximum values of R_T and the treatment-specific consumption factors (CF_T), respectively.
[b]Some alternative food was supplied (approximately 10% of the amount eaten during acclimation).
C_A = consumption of alternative food; C_T = consumption of target (rape) seeds.

studies. Two-choice tests have the potential to be representative of field situations because a lot of natural plant seeds and harvest leftovers are available as alternative food in agricultural landscapes over the year.

Using a validated standard procedure allows continuously updatable lists of PPP- and species-specific consumption factors to derive agreed minimum avoidance factors for an environmental and comparative risk assessment of PPPs.

Statistical methods

We provided a power analysis procedure to determine the number of replicates and to show how effect size and variability determine the statistical power to detect significant differences. However, from a biological perspective, the effect size (e.g., that food consumption in the treatment is reduced by a certain percentage over the control) may be of more interest than just statistical significance. This is why results of environmental risk assessment should be presented in great detail by providing raw data and summary statistics (e.g., as jittered points in boxplots) and reporting conditional means and confidence intervals of statistical models. In addition, the estimated model parameters and the R code to perform the analysis should be provided in the supplementary material.

We further used the framework of generalized linear mixed models (GLMMs), which properly account for repeated measurements using random effects and model the probability distribution of the response variable by defining the (error) family and a link function. For example, the response variable "percentage consumption of alternative food" should be analyzed using beta regression mixed models because percentage values are continuous and restricted to a range between 0 and 100% (Cribari-Neto and Zeileis 2010). Similarly, the response variable "consumption of food" should be analyzed using the tweedie distribution because those data are continuous, strictly positive, and may include zeros (Jorgensen 1992). The advantage of beta and tweedie regression models (and generalized linear models in general) over a transformation of the response lies in the direct interpretation of model coefficients and confidence intervals that are within the range of possible values. This is attributable to the use of a link function, namely the logit link in beta regression models and the log link in tweedie models. Hence, these models provide a better fit and more realistic confidence intervals than models with a transformed response. However, at the same time we experienced some restrictions caused by this method because tweedie models for food consumption by quail and gamma models for total daily consumption (which was strictly positive and >0) did not converge. More research and software

development for these models may help in the future. Hence, we advocate the GLMM framework together with inspecting residuals and confidence intervals from models to guide decisions about the statistical method.

Effects on state of health

Although some animals (<4%) had to be replaced during the test procedure, there were no obvious, negative effects of seed treatments and phases on the birds. An exchange of individuals was necessary in different phases and rape seed treatments. There were no general body weight changes during the procedure, but sequences (greenfinches) and sex (quail) had a significant effect. This highlights the fact that both sexes and animals familiarized with and not familiarized with the seed treatments should be included in tests to cover differences.

We found significantly higher body weight measurements in quail after the experiment in the NpT sequence (no-choice prior 2-choice test), probably because total daily consumption was higher in the NpT than the TpN sequence (2-choice prior no-choice test); but this corresponded to a <5% change in body weight given an average weight of 254 g in the NpT sequence.

Overall, the procedure had no negative effects on the health of animals. This might be attributable to the animals being in good health before the procedure started. The procedure can be stressful for some individuals, for example, by handling. However, the test could have negative effects on wild birds if they were captured shortly before starting the test. If such birds have low body reserves at the beginning of the experiment, there will be shorter periods of food avoidance (Organisation for Economic Co-operation and Development 2016). In addition, wild birds are not used to being handled by humans. Thus, for captured wild birds, the acclimation phase should be longer. The consumption factor may be the same for wild and domestic birds of the same species, but this needs to be verified. When after the beginning of the experiment behavioral changes related to the tested products are observed, we suggest termination of the experiments with a detailed description of why avoidance could not be determined.

Effects on consumption rates

The alternative food was clearly preferred over the rape seeds, showing that the exposure risk is less if other food is available (Table 4). If the target seeds had been preferred, a higher risk may be indicated for birds exposed to seed treatment PPPs. To achieve realistic food preference experiments, native available seeds should be used as the alternative food,

TABLE 4: Avoidance of untreated rape seeds in comparison to alternative food in the 2-choice test[a]

Species	Test	Treatment	C_A (g/kg body wt)	C_T (g/kg body wt)	R_T
Greenfinches	2-choice	Control	92.5 ± 23.7	42.1 ± 28.2	**0.45**
Quails	2-choice	Control	95.3 ± 37.8	12.5 ± 8.8	0.13

[a]Bold indicate maximum value.
C_A = consumption of alternative food; C_T = consumption of untreated rape seeds; R_T = ratio of C_A to C_T resulting in a consumption factor for untreated rape seeds of 0.45 for greenfinches.

being the baseline for risk assessments with crop seeds. Otherwise, worst-case scenarios (i.e., no-choice tests) should always be integrated to prevent negative impacts on farmland birds, as suggested by Lopez-Antia et al. (2014).

Consumption of treated seeds

The test procedure was suitable for highlighting known consumption patterns, such as higher avoidance effects of treated than of untreated seeds in 2-choice than in no-choice tests. However, not all seed treatments were avoided by birds equally (Lopez-Antia et al. 2014). The avoidance effect differed between species and fungicide treatments, resulting in the highest avoidance factor for f2, suggesting that not all seed treatments are avoided by birds.

This test design is able to detect reduced food consumption attributable to avoidance, and a consumption factor for an environmental comparative risk assessment for birds can be generated. When birds manipulate seeds in their beaks to remove the husk, the surface of the seed is brought into contact with their tongues and other tissues for some time (Ziswiler 1965; van der Meij 2004), allowing them to detect the PPPs, and several pesticides have been shown to be avoided as a result (e.g., Pascual and Hart 1997; Lopez-Antia et al. 2014). But even when a seed treatment is avoided, this may still not effectively prevent poisoning and delayed effects (Pascual et al. 1999a, 1999b; Lopez-Antia et al. 2014, 2018).

Besides use in environmental risk assessments, the consumption factor can be implemented as a factor in body burden approaches, which model the uptake of a toxic chemical and determine the critical dose (e.g., median lethal dose) or if metabolism and excretion are faster than uptake. Such body burden modeling approaches consider commonly the speed of uptake, toxicity, and degradation of the active substance, making sure that the rate of intake is often more important than the total quantity of food taken, especially in case of more toxic substances (Ducrot et al. 2016).

State of affairs

Alternative food, test species, and transferability of the data to the field are consistently the most crucial aspects of avoidance tests (European Commission 2002; European Food Safety Authority 2009; Organisation for Economic Co-operation and Development 2016).

In the wild, the availability of alternative foods varies spatially and temporally. It can be assumed that, most of the time, alternatives to contaminated food are available. Therefore, no-choice phases ideally combined with hunger phases before are recommended as a worst-case scenario in avoidance experiments (Organisation for Economic Co-operation and Development 2016). The duration of both phases should be realistic and adapted to the metabolic requirements of the test species (e.g., shorter for small birds) to guarantee animal welfare (Organisation for Economic Co-operation and Development 2011). We combined a 16-h hunger phase (according to Biologische Bundesanstalt für Land- und Forstwirtschaft 1993)

with a 4-h no-choice phase and a recovery phase without negative effects on the animals' health in general. Reducing the 10% of alternative food during the no-choice feeding test and longer no-choice phases could clearly improve the regulatory acceptance of the test as a worst-case scenario. However, it needs to be shown to prevent negative effects on health. Species react differently to food shortages, and this also depends on body condition and metabolic requirements, as mentioned in Organisation for Economic Co-operation and Development (2011). Thus, we suggest a sensitivity analysis to assess the influence of alternative food on animal health and consumption rates during the test. The test species should be representative of the agricultural landscape where PPPs are used and relevant to the environmental risk assessment. The selected species should be ecologically and metabolically relevant and representative of other species from the same feeding guild, as listed in European Food Safety Authority (2009). Details of how to determine a possible species for a specific crop scenario are presented in the avian and mammal guidance of, for instance, European Food Safety Authority (2009) and Dietzen et al. (2014). In the present study, quail and greenfinches were chosen because they were considered to be representative of granivorous birds occurring in agricultural landscapes, as well as because of their availability as bred and domesticated animals.

In the context of the environmental risk assessment, wild captured birds are more representative; however, with respect to wild animal welfare, domestic animals which are used to being caged and handled by humans have their benefits. Wild birds usually have lower weights and higher energy requirements than captive birds and may exhibit higher consumption rates (Mineau et al. 1994; Organisation for Economic Co-operation and Development 2016). In the case of using wild animals, it should be considered that the risk of ingesting a lethal dose before developing an avoidance response can be increased (Mineau et al. 1994). Wild animals should be allowed to acclimate to captive conditions, and they could need longer periods of acclimatization and shorter phases of hunger.

There is no agreement on the relevance of pen results to the field situation (Pascual et al. 1999a; Lopez-Antia et al. 2014; Organisation for Economic Co-operation and Development 2016). Provision of food (amount, spread, in pots, etc.), preparation of the animals (hunger, feeding time, training, start weight), group size, flocking effects, body condition depending on the time of the year, and prior experience of the target PPPs and untreated food can be different in field compared to pen studies (Organisation for Economic Co-operation and Development 2016). Although consumption rates often differ between laboratory and field studies, it can be assumed that the avoidance differences between the tested fungicides are similar. Avoidance studies from a laboratory can provide data about the avoidance potential of a substance (e.g., no–low–mean–high–very high) to be defined, even if they do not ensure a save use in the field. They can be particularly helpful for decision-making about possible substitution of substances in comparison to other substances. In addition, in case of no avoidance in the laboratory, field avoidance experiments would not be necessary, which saves time and effort and prevents potential environmental side effects caused by the

product freely available to all animals at the study site. Thus, a laboratory approach may be appropriate for comparisons and general predictions for avoidance. However, the results of our approach should be validated in field study setups before used in environmental risk assessments. If avoidance of a certain seed treatment is a general characteristic of an active substance, the test should be repeated with different granivorous species (e.g., wild captured species). The avoidance effect should be consistent for different species and confirmed in field before avoidance can be used in a quantitatively environmental risk assessment.

CONCLUSION

Our test procedure allows us to determine the consumption effects of PPPs applicable in comparative risk assessments. The pen trials concentrate on treated seeds and seed-feeding bird species as a first approach to defining a standard test procedure. Uncertainties in relation to transferability of the results in the field, availability of alternative food sources, and target species should be systematically investigated for an optimized standard procedure to minimize the number of tested animals in future trials. In addition, the proposed approach can be modified further in the future (e.g., by considering other aspects such as competition). Using a standard test increases the acceptance of tests, avoids tests without significant relevance, and continuously updates lists of PPP- and species-specific consumption factors, to create agreed minimum avoidance factors also for environmental risk assessments of PPPs.

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