Health & Ecological Risk Assessment

Utility of the avian sub-acute dietary toxicity test in ecological risk assessment and a path forward to reduce animal use

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Abstract

The United States Environmental Protection Agency (USEPA) has long required both avian sub-acute dietary and acute oral studies to inform risk assessments for pesticides. Recently, the USEPA collaborated with People for the Ethical Treatment of Animals to determine whether the results of the acute oral avian toxicity test or the sub-acute dietary toxicity test consistently generated the greatest risk predictions in USEPA tier 1 assessments for pesticides first registered between 1998 and 2017. Their study concluded that in 99% of the cases, risk conclusions were driven by the acute oral study (OPPTS 850.2100, OCSPP 850.2100, or similar) because using these data results in higher risk quotients than sub-acute dietary data. Shortly after publishing these results, the USEPA released a formal memorandum providing guidance for waiving the sub-acute dietary study for most pesticides. The USEPA will, however, retain the option to require sub-acute dietary studies for pesticides with certain chemical properties. However, as the avian sub-acute dietary study has an exposure regimen that is often more representative of how birds are exposed to pesticides under actual use conditions than does the acute oral study (i.e., as part of a dietary item eaten over the course of a day and not a bolus dose), this study can provide useful context for risk assessment on a case-by-case basis. Decision criteria are needed to determine a path forward that both minimizes vertebrate animal testing and positions the avian sub-acute dietary data as an option for risk refinement. Decision criteria are proposed here with recommendations for refining the design of avian sub-acute dietary studies to ensure that the data generated are optimized to support a science-based acute avian risk assessment, supported by a case study demonstrating when and how sub-acute dietary studies may be used in a higher-tier risk assessment. *Integr Environ Assess Manag* 2022;18:1629–1638. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

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INTRODUCTION

The goal of an ecological risk assessment (ERA) for a pesticide is to determine the magnitude and likelihood of adverse effects for potentially exposed aquatic and terrestrial receptors. To estimate risk, an appropriate predicted hazard endpoint must be selected and compared to estimated exposure concentrations or doses. Currently, United States' Environmental Protection Agency (USEPA) guidelines require two acute avian study types, the acute oral (OCSPP 850.2100/OECD 223) and sub-acute dietary (OCSPP 850.2200/OECD 205) studies (40 CFR Part 158-Data Requirements for Pesticides, 2007), to assess the risk of pesticides. An acute avian oral study involves administering a single bolus dose to fasted, young adult birds, followed by a 14-day observation period that includes measurements of mortality, feed consumption, and body weight, along with observations of clinical signs of toxicity (e.g., lethargy, hyperactivity, ptiloerection, moribundity, etc.). Most avian exposures to pesticides in the environment occur via feeding throughout the day rather than via a large single bolus dose (Best, 1977; Fautin, 1941; Kessel, 1957; Kluijver, 1950; Pinkowski, 1978), although there are some exceptions such as gorging on treated seed spills that more than approximate a bolus dose (Botha et al., 2018; Millot et al., 2017; Roy et al., 2019). The USEPA has historically required a sub-acute dietary study with chicks to evaluate this more realistic exposure route. The existing guideline for the sub-acute dietary study includes five days of continuous exposure to

chicks via treated diet, followed by a three-day observation period with untreated diet, with an option to extend the observation period if necessary. In an avian sub-acute dietary study, mortality, body weight, feed consumption, and clinical signs of toxicity are recorded.

The USEPA evaluates risk to birds using the terrestrial residue exposure model (T-REX) (USEPA, 2012b). The T-REX dietary-based acute risk assessment compares the LC50 endpoint (i.e., median lethal concentration) from the sub-acute dietary study to estimated pesticide residues on food items. The T-REX dose-based acute risk assessment adjusts the LD50 endpoint from the acute oral study for bird size class and compares this value to the estimated pesticide concentration on food items multiplied by an estimated food intake rate for that bird size class. A recent retrospective analysis of USEPA's pesticide avian acute risk assessments was conducted by the USEPA and People for the Ethical Treatment of Animals (PETA) for new pesticides registered between 1998 and 2017 (Hilton et al., 2019). The focus of this analysis was to determine which study type drives the USEPA's acute pesticide risk conclusions for birds: the acute oral study or the sub-acute dietary study. The analysis found that in 99% of the cases, acute risk estimates were more conservative when using the hazard endpoint from the acute oral study than when using the hazard endpoint from the sub-acute dietary study. The authors thus concluded that conducting only an acute oral study often suffices to produce the most conservative risk estimate. As a second outcome, the authors concluded that the sub-acute dietary study need not be performed and could be waived for pesticides that do not meet certain exemption criteria. Those exemption criteria include pesticides with delayed toxicity, a high potential to bioaccumulate, a high molecular weight, and/or that result in regurgitation in acute oral testing. The USEPA estimates that implementation of this guidance would reduce the number of birds used in pesticide toxicity testing by approximately 720 animals per year (USEPA, 2020). This number could be larger if chemicals that are not submitted to regulatory entities are taken into consideration. In 2020, the USEPA released final guidance for when the avian sub-acute dietary study can be waived for pesticides.

However, the ecological basis for this approach should be further considered as wild birds are most likely to be exposed to pesticide residues on dietary items as they feed throughout the day, making the dietary exposure route in the sub-acute dietary study more environmentally relevant than the single bolus dose exposure in the acute oral study (ECOFRAM Terrestrial Workgroup, 1999; Mitra et al., 2021; D. R. J. Moore et al., 2014; Stafford et al., 2003; USEPA, 2017). This is because the birds in the sub-acute dietary study are allowed to feed ad libitum over the course of the day, mimicking the likely exposure timing of wild birds. Because the sub-acute dietary study mimics both the matrix and the time over which wild birds are likely to be exposed to pesticide residues better than the acute oral study, it provides the more environmentally relevant characterization

of potential effects and a more realistic risk prediction under conditions of pesticide use. Therefore, the sub-acute dietary study can provide relevant information to refine the predicted risk to wild bird populations better than the dose-based risk assessment calculated using data from the acute oral study in some circumstances.

Reducing most data packages submitted for a new pesticide registration to only include the endpoint from the acute oral toxicity test and to waive out of the sub-acute dietary test will reduce animal use, which is an important goal. Data from the sub-acute dietary test can, however, provide highly relevant information that is required for further risk characterization and refinement for most exposure scenarios. However, this situation is complicated by the fact that the sub-acute dietary study design in its current form suffers from a number of methodological flaws that limit its useability in quantitative risk assessment. Thus, the purpose of this paper is twofold: (1) propose refinements to the sub-acute dietary study design to optimize its utility to support a science-based acute risk assessment compared to the current study design and (2) propose a decision tree with a tiered risk assessment scheme for acute avian risk, which positions the acute oral study as a first-tier screening test and the sub-acute dietary study as a risk refinement option. While avian toxicity studies can also be performed and are relevant for other chemical classes and regulatory needs, their primary regulatory utility in North America is for pesticide assessments. Therefore, the focus of this article will be on this area.

AVIAN DIETARY TOXICITY TEST: CURRENT GUIDELINE STUDY DESIGN AND PROCEDURAL REQUIREMENTS

The avian sub-acute dietary study is typically conducted according to the guideline OCSPP 850.2200 or OECD 205 (OECD, 1984; USEPA, 2012a). The goal of the study is to characterize the concentration–response for avian mortality after dietary exposure, and to either establish the sub-acute LC50 value and its 95% confidence limits or to determine that the LC50 is above the limit concentration (5000 mg/kg diet; ppm). Mortality, food consumption, body weight, overt signs of toxicity, and possibly gross necropsy findings and histopathological changes are recorded. The standard definitive test consists of a minimum of five dietary treatments of the test substance, plus appropriate controls, with 10 birds per group, unless preliminary testing clearly indicates that a limit test (single maximum concentration) is appropriate. A limit test evaluates a sufficiently high concentration for the risk assessment (usually 5000 mg/kg diet [ppm]); if no effects are observed, then no additional testing is required. An acceptable study requires that dietary concentrations, including test substance stability and homogeneity in the diets, must be confirmed by chemical analysis under test conditions.

Studies conducted with both an upland game bird (e.g., northern bobwhite quail [*Colinus virginianus*]) and a waterfowl species (e.g., mallard duck [*Anas platyrhynchos*]) are

TABLE 1 Currently required test conditions for the avian sub-acute dietary test

Test duration	Minimum eight days. Five days of exposure via treated diet, followed by minimum three days on untreated diet
Temperature range	22 °C to 38 °C (a gradient based on the age of young birds)
Light	14 h light/10 h dark, incandescent, or fluorescent
Pens	One or two per treatment level, >300 cm^2/bird for northern bobwhite and >600 cm^2/bird for mallards
Age at study initiation	10–14 days (bobwhite quail) or five days (mallard duck)
Sex	Sex cannot be phenotypically determined in chicks this young
Replication	Minimum 10 birds/concentration level. Minimum of 5 concentration levels plus a negative control
Endpoints	1) Percent mortality
	2) LC50 and 95% confidence interval
	3) Slope of the dose–response curve with a 95% confidence interval
	4) Body weight (Days 1, 5, and 8)
	5) Food consumption (Days 1–5; 3–8)
	6) Behavior and appearance

needed to meet the requirements of 40 CFR Part 158 (40 CFR Part 158-Data Requirements for Pesticides, 2007). The USEPA will use these and other data to assess acute hazards and risks to birds. See Table 1 for a description of the required test conditions.

AVIAN DIETARY TESTING: OPTIONS FOR IMPROVING THE TEST DESIGN

Several experimental design issues limit the utility of the current dietary study design and can be improved. Options for improving the avian dietary test design primarily focus on improving the accuracy of measured endpoints (e.g., food consumption and spillage) and statistical analysis of data from the study without increasing the number of birds used for testing as described below and summarized in Table 2.

Currently, the standard test design involves testing 10 birds per treatment or control group that are group-housed, either in two groups of five or single groups of 10. Group housing is a requirement in both the OECD and OCSPP guidelines for young chicks as their development requires social interaction (Joint Working Group on Refinement, 2001; OECD, 1984; USEPA, 2012a). However, this design reduces the number of replicates available for statistical analysis. It also leads to inconsistent study methods. Birds are weighed either individually or in groups, depending on version of the test design. Even with a test design in which the birds are individually weighed, housing the entire group together results in individuals that are not independent replicates, and individual food consumption cannot be measured.

Using older juveniles or young adults rather than young chicks provides several advantages. First, the animals can be housed individually. For species like quail that are covey species, individual housing of chicks may result in animal welfare issues (i.e., stress, behavioral problems, and potentially death [Joint Working Group on Refinement, 2001]; J. Beavers, Wildlife International Ltd., personal

TABLE 2 Proposed improvements to the guideline avian dietary study design

Study design improvement	Rationale
Use older birds (≥16 weeks old, same age as the avian reproduction study)	• Older birds can be housed individually (see below for benefits) • Older birds are more able to withstand periods of low food consumption than younger birds if the test compound has low palatability • Sex identification to test for male vs. female sensitivity
Study duration flexibility	• The duration of the exposure and observation periods can be shortened or extended to best approximate the relevant environmental exposure scenario
House birds individually	• Allows individual body weight and food consumption data to be collected
Weigh individual birds	• Increases statistical power
Increase # treatments, decrease # reps	• Increases statistical power
Record daily food wastage (uneaten food and spillage)	• Allows for the daily dose to be quantitatively measured

communication, 2012). Individual housing has the advantage that reliable individual body weight and food consumption measurements can be collected (food waste for individual birds can also be measured in the study, providing a more accurate determination of dose) and statistically analyzed since the birds would be independent sampling units (pen is the sampling unit for group-housed birds). Keeping the lighting low, as is done in the acute oral test, would minimize the possibility of the older birds entering a reproduction phase (egg-laying) during the dietary test. Second, older birds are better able to withstand periods of low food consumption than younger birds. If the test compound reduces the palatability of the diet or causes intoxication in the sub-acute dietary study, food consumption can become very low. Low food consumption can cause decreases in the daily dose (mg/kg body weight [bw]/day) consumed by birds at higher dietary concentrations compared to lower dietary concentrations. It can also be problematic for young chicks that are not able to withstand not eating for very long and can result in animal welfare issues, as well as difficulty differentiating between effects resulting from pesticide toxicity or those resulting from the palatability of food treated with the pesticide, leading to inanition and even outright starvation. Finally, young chicks without mature plumage cannot be easily sexed morphologically and thus differential sensitivities between sexes cannot be readily detected by the current sub-acute dietary study design without PCR-facilitated sex determination. The use of older birds that can be easily sexed eliminates this issue.

In the current test design, dietary exposure lasts five days, although the duration of sub-acute dietary studies can deviate from the guideline requirements to address different exposure scenarios or modes of action (USEPA, 2009b, 2016). However, caution must be exercised while increasing the exposure period if food avoidance occurs. A prolonged hunger phase, followed by a return to treated food consumption, may produce mortality that is a result of an altered physiological state caused by starvation in combination with the test substance exposure, particularly in passerine species. This scenario would not occur in a natural environment with varied food sources and the ability of free-ranging birds to forage in other locations.

Finally, a study design with more dose groups with fewer replicates per treatment is a more suitable design for the regression-based analysis required to generate a reliable exposure–response relationship (D. R. J. Moore & Caux, 1997; Sebaugh et al., 1991).

Together, these recommended design changes would considerably strengthen the scientific utility, statistical power, and confidence in the data produced by an avian sub-acute dietary study and can be customized to specifically address a given exposure scenario. The changes would not increase the number of animals used to conduct the sub-acute dietary study. This list of possible changes is not exhaustive, and as with any study design, there are many variables that can be adjusted to better address a specific hypothesis or scientific question. Ideally, if a sub-acute dietary study is performed, the study design should be modified to address a specific risk scenario. The proposed changes presented in this article are widely applicable across the majority of chemical classes and risk scenarios. All design changes should be discussed with regulatory authorities before conducting a sub-acute dietary study as a refinement for an avian risk assessment (Levine et al., 2019).

ENVIRONMENTAL RELEVANCE OF A DIETARY STUDY

The sub-acute dietary study can provide information on toxicity to birds over a period of days to weeks and provide a more environmentally realistic exposure scenario than the acute oral gavage study. Pesticides often are applied at times of the year when birds are nesting in or near treated fields. The time and energy demands on birds are greatest during nesting and are related to the requirements of the nestlings and the environmental factors affecting the adults (Best, 1977; Heagy & Best, 1983). As a result, nesting birds in agroecosystems, particularly brooding small passerines, typically forage for food for their nestlings and themselves persistently throughout the daylight period. For example, Best (1977) observed that male and female field sparrows (*Spizella pusilla*) make approximately 3–6 feeding trips per hour. Eastern bluebirds (*Sialia sialis*) average 4.8–6.5 feeding trips per hour, depending on brood size and the sex of the parent (Pinkowski, 1978). Gray catbirds (*Dumetella carolinensis*) make approximately 10–13 feeding trips per hour (Johnson & Best, 1982). The number of feeding trips per nest (males and females combined) by great tits (*Parus major*) varies from about 10 to 40 per hour (Kluijver, 1950). The combined number of feeding trips by male and female brown thrashers (*Toxostoma rufum*) varied from 6 to 10 per hour (Heagy & Best, 1983). The average number of yellow-headed blackbird (*Xanthocephalus xanthocephalus*) feedings per hour during care of nestlings was 9.6 (Fautin, 1941).

Feeding begins at about sunrise and continues to sunset. The most active feeding by nonbrooding passerine birds may occur during the early morning and early evening hours for a variety of passerine species, including European starling (*Sturnus vulgaris*) (Kessel, 1957), field sparrow (Best, 1977), gray catbird (Johnson & Best, 1982), yellow-headed blackbird (Fautin, 1941), and great tit (Kluijver, 1950). However, feeding hours are influenced by several variables, such as temperature, precipitation, wind, and food availability; thus, feeding continues at varying intensities throughout the daylight hours and it may vary between days. The feeding rate of eastern bluebirds is relatively constant throughout the day, with females having a peak rate early in the day and males having a peak rate later in the day (Pinkowski, 1978). Brown thrashers tend to have a relatively constant feeding rate throughout the day, but with a peak in the evening hours (Heagy & Best, 1983). In sub-acute dietary studies, food is available ad libitum and birds have the opportunity to feed throughout the daylight period.

Of the two standard avian toxicity studies used currently, the sub-acute dietary study more realistically simulates the typical foraging pattern of birds, particularly nesting birds, than does the acute oral study. This is important from a risk assessment perspective for pesticides because many pesticides are rapidly metabolized and excreted by birds, particularly those developed in more recent times. For example, Brewer et al. (2007) determined the time course of brain cholinesterase (ChE) activity depression and recovery in northern bobwhite quail (*Colinus virginianus*) following exposure to carbofuran. Recovery of ChE activity reflects the rate of carbofuran metabolism. The results of the study indicated that recovery of ChE activity was relatively fast, with half-life estimates for ChE recovery of 1.13, 2.92, and 4.36 h for the low (0.75 mg a.i./kg b.w.), medium (1.5 mg a.i./kg b.w.), and high (3.0 mg a.i./kg b.w.) oral dose treatments, respectively. Many other pesticides are similarly rapidly metabolized and excreted. When pesticides are mixed with food or consumed when the gastrointestinal (GI) tract has other food items present, they are absorbed less efficiently than when dosed in pure form into an empty GI tract (Lehman-McKeeman, 2008). The GI transit times in birds are quite rapid and can be on the order of minutes, depending on the species (Clench & Mathias, 1992; Levey & Karasov, 1989). Thus, a bird that forages throughout the day can metabolize and excrete the pesticide, likely before it reaches internal concentrations that would cause serious adverse effects. In acute oral studies, the bird receives the entire dose instantaneously, making it much more likely that the internal concentration associated with serious adverse effects will be exceeded (Hannas et al., 2016).

While toxicokinetic evaluations of pesticides in avian species are limited, reports of avian liver concentrations of imidacloprid in putative poisonings from seed treatment exposures varied significantly in two reported cases. South African spurfowl had residues of 16–29 ng/g, while pigeons and gray partridge in France had median liver residues of 1400 and 3000 ng/g (Botha et al., 2018; Millot et al., 2017). A toxicokinetic experiment that dosed Japanese quail with imidacloprid-treated seed as a one bolus dose at approximately 9% of the LD50 found a median liver value of 72 ng/g at 1 h post dosing, with lower concentrations at subsequent timepoints (Bean et al., 2019). No overt toxicity was observed in this study. This variability in liver concentrations and responses has been attributed to possible species differences in toxicokinetics (Bean et al., 2019). This scenario, as previously discussed, does not mimic the general feeding patterns of birds, with exceptions for gorge feeding on seeds and granular formulations of pesticides (Lehman-McKeeman, 2008).

Avian sub-acute dietary studies can also help determine if birds are likely to avoid exposure to a pesticide in the field, whereas acute oral studies cannot. Birds often reduce their feeding rate when exposed to acutely toxic pesticides in their food (Bennett, 1989; European Food Safety Authority, 2005; Fischer et al., 2005; Grue et al., 1997; Kononen et al., 1987; Stafford, 2007a, 2007b). The degree to which this occurs may vary by species. The reduction in feeding rate may be due to

(i) repellent taste or odor (e.g., methiocarb [Kononen et al., 1986]) or (ii) postingestional toxicity, which is a common mechanism for carbamate pesticides such as carbofuran (Fischer et al., 2005; Grue et al., 1997). Reductions in the consumption of treated diets would be expected to occur above a threshold concentration for pesticides that are repellent or above a threshold dose for pesticides that induce post-ingestional toxicity. Beyond the threshold concentration or dose, consumption of treated diet declines as the dietary concentration or dose increases (Bennett, 1989; Grue et al., 1997). Studies of birds given a choice between carbamate-treated and untreated diets suggest that birds can maintain normal or close to normal rates of overall food consumption if they can discriminate among the diets or associate the source of the diet with the toxicity symptoms (Bennett, 1989). This information is valuable in understanding the risks of pesticides to birds, and thus the avian sub-acute dietary study remains a valuable tool for refined avian risk assessments.

For some situations, the acute oral study provides relevant toxicity information. Although most nesting bird species have dispersed feeding activity throughout the day, there are situations where gorge feeding occurs in birds. For example, waterfowl species often show gorge feeding in the morning and evening, primarily in the fall and during migration (Bell, 1970; McWilliams & Raveling, 1998; Reed et al., 1977). Other migrating birds, such as the Swainson's hawk (*Buteo swainsoni*), may gorge-feed during stopovers, and some pigeons, such as the woodpigeon (*Columba palumbus*), may gorge-feed on seed if it is plentiful. In addition, for some granular or seed treatment formulations, a large bolus dose can be achieved by ingestion of a few granules or seeds (Botha et al., 2018).

CASE STUDY: COMPARING AVIAN ACUTE GAVAGE AND ACUTE DIETARY STUDIES FOR FOUR SURFACTANTS

Surfactants, as indicated by their name, are surface-active molecules and are added to pesticidal formulations to facilitate the active ingredient remaining in solution, to ensure that the active ingredient spreads and sticks on the target, and to allow the active ingredient to penetrate the target and/or maintain product stability during storage. Surfactants are classified as inert ingredients in pesticidal formulations and can be eligible for exemption of the requirement by USEPA to establish maximum legally permissible levels of residues in food products, known as a tolerance exemption. To gain tolerance exemption for an inert in a pesticidal formulation, environmental data are generated to enable the USEPA to evaluate if the use of this inert will affect the environment (USEPA, 2011, 2014). These data can also inform formulation assessments (USEPA, 2015).

Allowing for the assessment to be based on the endpoint from a dietary exposure study, particularly for some classes of substances, builds more realism into a higher-tier assessment and will not confound the results with supraphysiological doses by gavage. It is well established in the scientific literature that surfactants can elicit cytotoxic effects at threshold

concentrations. Disruption of membrane integrity is at the center of many of the observed biological effects of surfactants (Levine et al., 2007). The disturbance of normal cellular function results from changes in the permeability of cell membranes, solubilizing components of cell membranes, and fusion of membranes (Dimitreijevic et al., 2000; Lucy, 1970). Effects observed in mammalian toxicology studies after oral exposure by gavage often are through irritation of tissue (Cann & Verhulst, 1960; Daher et al., 2003; HERA, 2009; Martens et al., 2019; Potokar, 1992). Therefore, when surfactants are administered by gavage at high doses, they can cause severe gastrointestinal irritation that would not occur under a dietary feeding scenario (Martens et al., 2019). For this reason, the USEPA allowed the option of continuous dietary feeding exposures with OECD 422 guideline studies versus conducting the study by daily dosing by gavage (USEPA, 2009a).

Four model surfactants have been used as a case study to highlight the relevance of dietary over gavage acute toxicity studies for different surfactants. Two of the surfactants are nonionic etheramine ethoxylates: one with a straight-chain alkyl group and the other with a branched alkyl group; the third is a nonionic alkyl amidodimethylpropyl amine ethoxylate, and the fourth is an anionic alkyl sulfate. As a result of the nonspecific mode of action of surfactants on cellular membranes, dose–response or concentration–response curves have very steep slopes. Therefore, it is not uncommon to fully characterize the dose–effect or concentration–effect relationship over a twofold dose or concentration range in in vitro, aquatic, and avian studies (Levine et al., 2007; L. J. Moore et al., 2012).

Figure 1 compares the dose–response relationships for the four surfactants, which includes the three nonionic surfactants and one anionic surfactant. All studies were conducted in the avian toxicology facility at Wildlife International Ltd. (Easton, MD) following USEPA's test guidelines for acute oral and acute dietary studies at the time of the study. For each of these studies, an analysis was performed to confirm that the measured concentration of the surfactant in the treated diet on Day 0 was comparable to the nominal dose and was generally stable over the period of administration. Measured concentrations for the four surfactants on Day 0 ranged from 93% to 109% of nominal concentrations, and on Day 5, recovery ranged from 73% to 94% of nominal for three of the
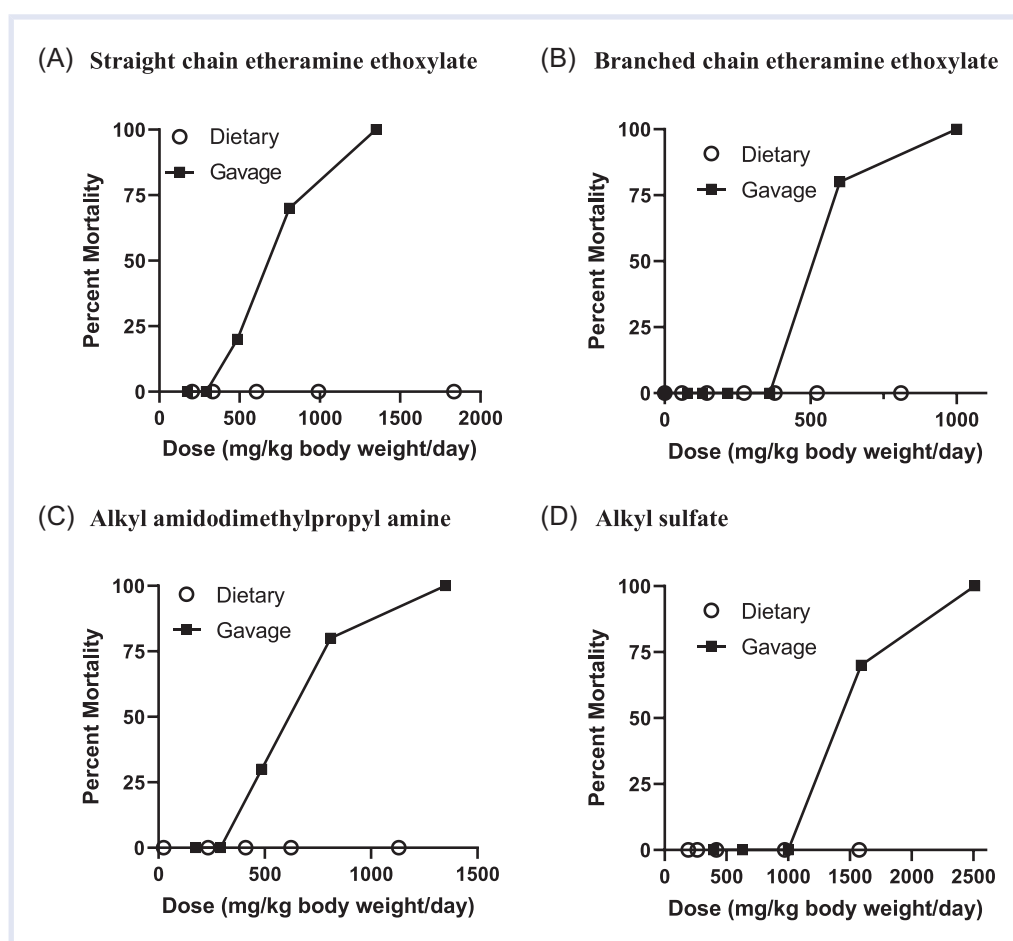
FIGURE 1 Comparison of dose–response curves for northern bobwhite following acute oral exposure by gavage or by acute dietary exposure for four different surfactants. The dose is presented as mg/kg body weight/day, and the response is presented as percent mortality. (A) Comparison of dose–response curves for acute oral exposure and acute dietary exposure to a straight-chain etheramine ethoxylate. (B) Comparison of dose–response curves for acute oral exposure and acute dietary exposure to a branched-chain etheramine ethoxylate. (C) Comparison of dose–response curves for acute oral exposure and acute dietary exposure to alkyl amidodimethylpropyl amine. (D) Comparison of dose–response curves for acute oral exposure and acute dietary exposure to alkyl sulfate

four surfactants. Lower recovery on Day 5 was observed for the branched-chain etheramine surfactant, which likely reflects the poor recovery of this surfactant from diet after five days in the feeder. Comparison between avian acute oral studies and acute dietary studies shows a huge difference in observed toxicity. This difference is likely due to the effects of administering a single bolus oral dose of the surfactant compared to continuous dietary surfactant exposure. Daily dietary doses were calculated considering the mean body weight (g), the mean daily feed consumption (mg/kg bw/day), and the test concentration. For example, for chicks with a mean weight of 30 g, consuming 6 g feed/bird/day at a dietary concentration of 1000 mg surfactant/kg diet would have an estimated daily dose of 200 mg surfactant/kg bw/day.

For the study with the straight-chain etheramine ethoxylate, there was a dose-dependent increase in mortality in the gavage study, with an LD50 value of 598 mg/kg; however, no mortality was observed in the dietary study at the highest mean dose of 1835 mg/kg bw/day (Figure 1A). For the study with a branched-chain etheramine ethoxylate, there was a dose-dependent increase in mortality in the gavage study, with an LD50 value of 511 mg/kg; however, no mortality was observed in the dietary study at the highest mean dietary dose of 809 mg/kg bw/day (Figure 1B). For the alkyl amidodimethylpropyl amine ethoxylate, there was a dose-dependent increase in mortality in the gavage study, with an LD50 value of 561 mg/kg; however, no mortality was observed in the dietary study at the highest mean dietary dose of 1130 mg/kg bw/day (Figure 1C). In addition, for the alkyl sulfate, there was a dose-dependent increase in mortality in the gavage study, with an LD50 value of 1612 mg/kg; however, no mortality was observed in the dietary study at the comparable and highest mean dose of 1574 mg/kg bw/day (Figure 1D). Taken together, these four examples with surfactants show an important difference in the toxicity profile between gavage and dietary studies, and therefore, results from the dietary study could be used for risk refinement to further inform regulatory decision-making (Levine et al., 2019).

PATH FORWARD

Tiered risk assessment is not a new concept, and is particularly well illustrated by the USEPA's "Guidance for Assessing Pesticide Risks to Bees" for pollinator risk assessment, where additional exposure information and more complex study designs can be used to refine risk conclusions made using more simple study designs (USEPA & CDPR, 2014). For the assessment of acute avian risk, the acute oral and sub-acute dietary studies have usually been used in tandem, and not in a tiered fashion. However, given the guidance from USEPA on waiving the dietary study in many cases, there is an opportunity to rethink the approach to designing an acute avian testing program. There remains a need for the avian sub-acute dietary study as a higher-tier option for the avian risk assessment for pesticides when warranted. In 2020, the USEPA released final guidance recommending that the dietary study be waived, in many cases, based on the results of the Hilton et al. (2019) historical

analysis (USEPA, 2020). The guidance listed a number of exceptions in which a waiver for the dietary study would not be granted. Briefly, these scenarios include the following:

- Modes-of-actions (MOAs) that are unique, unspecified, or were not evaluated in the Hilton et al. (2019) retrospective analysis.
- MOAs that suggest a mechanism for accumulative damage such as when effects increase with repeated exposure. High potential for bioaccumulation (high lipophilicity, low metabolism rate, low excretion rate) or a saturable facilitated mechanism of adsorption. An example of a chemical class where this would be applicable is the anti-coagulant rodenticides for which acute toxicity is underestimated by the acute oral study (Vyas & Rattner, 2012).
- Avian acute oral study cannot be conducted (e.g., birds regurgitate the test substance, preventing an accurate dose estimation).

The USEPA guidance describes these scenarios in greater detail and provides guidance on determining whether they are applicable to a particular pesticide. There are other scenarios and circumstances where a dietary study may be scientifically appropriate.

Avian dietary studies also have value as part of a refined avian risk assessment or when an acute oral study does not provide a feasible exposure scenario, such as with surfactants as described above. The USEPA guidance (USEPA, 2020) states:

> "*This document and its finding do not necessarily de sub-acute dietary testing for birds. Despite the protection [sic] nature of risk assessments relying on the single oral dose acute endpoint, avian sub-acute dietary testing may bring perspective to a risk assessment and improve the knowledge base supporting a regulatory decision.*"

In screening-level risk assessments, highly conservative assumptions and models are used to determine if the upper bound risk estimate exceeds a defined threshold. If a potential risk is identified, more complex models and effects studies can be used to develop more realistic estimates of risk, although there are challenges to the implementation of higher-tier data use (Levine et al., 2019). For many exposure scenarios involving pesticides, it is likely that the avian sub-acute dietary study will continue to provide a more realistic hazard endpoint than does the oral study. In the field, most birds forage throughout the day. As a result, birds may metabolize and eliminate a pesticide throughout the day, an opportunity that does not exist when birds are exposed to a pesticide in a bolus dose. The closest approximation to the bolus dose used in the acute oral test is when birds gorge-feed, an uncommon feeding strategy. Thus, the avian dietary test is useful for further characterizing the risk to birds

from pesticides that do not pass the conservative, screening-level dose-based risk assessment calculated using data from the acute oral study.

Although sole reliance on acute oral endpoints reduces animal use and testing costs, the lack of a realistic hazard endpoint produces overly conservative risk estimates. This limitation could lead to denial of a registration for one or more uses of a pesticide or mitigations that might not have been required with the use of a more realistic hazard endpoint.

Thus, we recommend that a framework be developed that gives clear guidance as to when and how to run an avian dietary study as a risk refinement option (see Figure 2). The goal of this framework is to prevent unnecessary animal use, in accordance with the goals stated by USEPA (USEPA, 2020), while also maintaining a path forward for the targeted and scientifically robust use of this study design and addressing potential avian risk issues. The first point in the decision tree in Figure 2 references the USEPA memo exceptions for waivers. If a potential risk is identified in the screening-level risk assessment, one should determine whether other alternatives, such as refining the exposure part of the risk equation, are appropriate. If refining exposure does not result in an acceptable risk estimate, the sub-acute dietary study should be considered as a second-tier option for refining the hazard endpoint. As discussed

previously, the OCSPP 850.2200 and OECD 205 guidelines have several deficiencies in experimental design that limit the accuracy and interpretation of the results. If data from a sub-acute dietary exposure are determined to have value for an avian risk assessment, experimental changes to the protocol could be made to customize the study for a specific chemical, use pattern, and exposure scenario. We have suggested some protocol changes that could be implemented to increase the utility and reliability of the data derived from a sub-acute dietary study (see Table 2). These protocol changes, when made in consultation with the appropriate regulatory agency, will ideally improve the useability of the data and produce a hazard endpoint that is more suitable for a second-tier acute avian risk assessment.

CONCLUSIONS

Reduction and refinement of vertebrate use in toxicity testing for pesticide risk assessment is an important goal shared by many stakeholders, including the USEPA, crop protection industry, nongovernment organizations, and the public. This article suggests criteria for when to conduct an avian sub-acute dietary study in the context of pesticide risk assessment as a risk refinement option. For many exposure scenarios involving pesticides, it is likely that the avian sub-acute dietary study will continue to provide a hazard
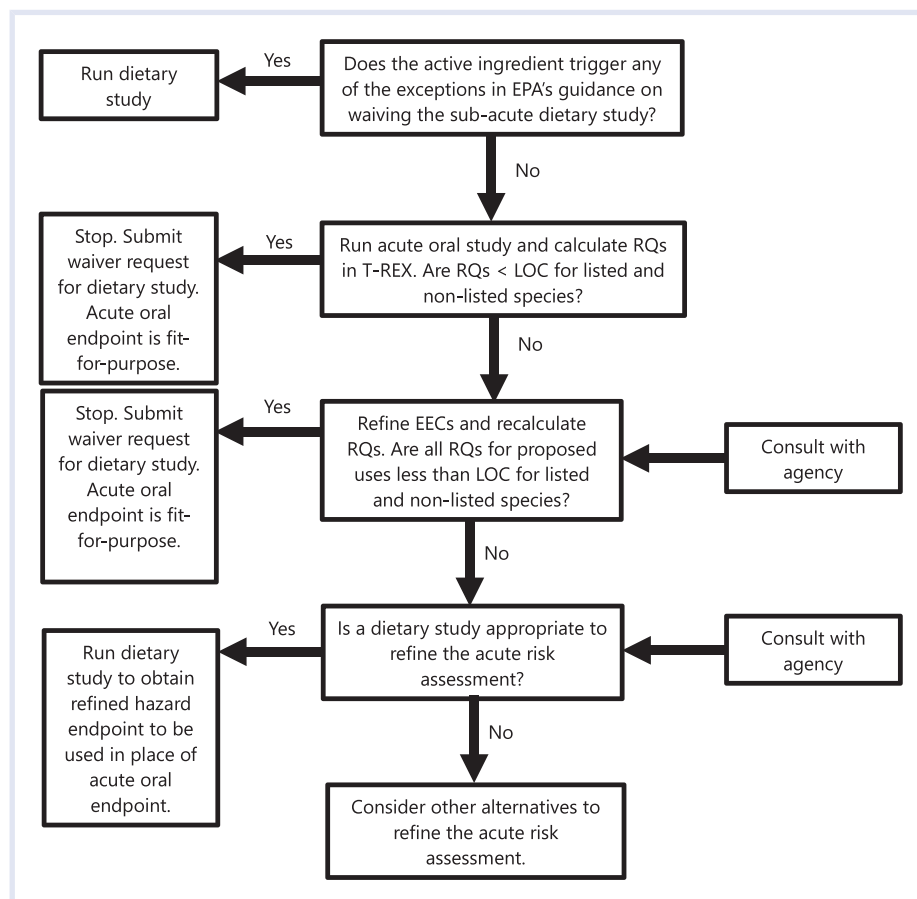

FIGURE 2 Decision tree for determining whether an avian dietary study should be performed to support an acute avian risk assessment for a plant protection product. EEC, estimated environmental concentration; LOC, limit of concern; RQ, risk quotient

endpoint more realistic and suited to approximating avian feeding habits compared to the acute oral study. Thus, the avian sub-acute dietary study may be useful in further characterizing acute risk to birds in certain exposure scenarios as a second-tier option when a screening-level assessment based on the acute avian oral study indicates a possible risk. The authors have also evaluated the limitations of the sub-acute dietary study and proposed possible improvements to its design to support the continued use of this study as a risk-refinement option on a case-by-case basis.

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CONFLICT OF INTEREST

S. L. L. and A. J. B. are employees of Bayer CropScience, a manufacturer of pest control technology and products. S. P. is an employee of BASF Corporation, a manufacturer of pest control technology and products.

DISCLAIMER

The peer review for this article was managed by the Editorial Board without the involvement of D. Moore.

DATA AVAILABILITY STATEMENT

Data for the surfactant case study can be accessed by contacting Steven Levine (steven.levine1@bayer.com). Data, associated metadata, and calculation tools are available from corresponding author Audrey Bone (audrey.bone@bayer.com).

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