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Critique on the Use of the Standardized Avian Acute Oral Toxicity Test for First Generation Anticoagulant Rodenticides

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ABSTRACT

Avian risk assessments for rodenticides are often driven by the results of standardized acute oral toxicity tests without regards to a toxicant's mode of action and time course of adverse effects. First generation anticoagulant rodenticides (FGARs) generally require multiple feedings over several days to achieve a threshold concentration in tissue and cause adverse effects. This exposure regimen is much different than that used in the standardized acute oral toxicity test methodology. Median lethal dose values derived from standardized acute oral toxicity tests underestimate the environmental hazard and risk of FGARs. Caution is warranted when FGAR toxicity, physiological effects, and pharmacokinetics derived from standardized acute oral toxicity testing are used for forensic confirmation of the cause of death in avian mortality incidents and when characterizing FGARs' risks to free-ranging birds.

Key Words: birds, first generation anticoagulant rodenticide, hazard, risk, standardized acute oral toxicity test.

INTRODUCTION

In the United States, rodenticides have been registered for use at residential, industrial, agricultural, and field sites to control a variety of mammals from the Orders Rodentia, Insectivora, Lagomorpha, and Carnivora (USEPA 1998). Three classes of rodenticides are registered: first generation anticoagulants, second generation anticoagulants, and acute or non-anticoagulants. The first generation and the second generation anticoagulants both inhibit Vitamin K epoxide reductase that ultimately results in the disruption of blood clotting and damage to capillary permeability. Whereas both classes of anticoagulants lead to hemorrhaging and death, under

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operational conditions they usually require different exposure scenarios. Mortality from the first generation anticoagulant rodenticides (FGARs) occurs following multiple feedings on the bait while the second generation anticoagulant rodenticides are acutely toxic and provide a toxic dose in a single feeding (see Clark 1978 for review; Lund 1981; Dubock 1982; Jackson *et al.* 1982; Ashton *et al.* 1986; Jackson and Ashton 1992; Ramey *et al.* 2007). For both classes of anticoagulant rodenticides, sublethal adverse effects (*e.g.*, prolonged clotting time) can arise within 12–48 h of exposure (Mount and Feldman 1983; Shulman *et al.* 1986), however mortality may occur ≥ 1 wk post-exposure (see Clark 1978 for review; Lund 1981; Dubock 1982; Jackson *et al.* 1982; Ashton *et al.* 1986; Jackson and Ashton 1992; Ramey *et al.* 2007; Swift 1998; Woody *et al.* 1992). Finally, rodenticides in the acute class vary in their modes of action but result in death shortly after exposure (MacNicoll 2007).

STANDARDIZED TESTS, THEIR LIMITATIONS, AND IMPLICATIONS

Birds are poisoned by rodenticides either by directly consuming the bait or via secondary poisoning (Newton *et al.* 1990; Stone *et al.* 1999, 2003; USEPA 2006; Lambert *et al.* 2007; Albert *et al.* 2009; The Ornithological Council 2010; Walker *et al.* 2010). Avian risk assessments for rodenticides mainly rely on results of standardized laboratory toxicity tests to predict hazards to birds in the field. The standardized testing protocol allows regulators to compare the toxicities of various chemicals to an avian species and to compare the sensitivities of different species to a particular chemical. In the wildlife-pesticide regulatory arena, the most commonly used endpoint for toxicity is mortality because of its definitive nature. The two commonly conducted standardized tests for lethality are the single-dose acute oral toxicity test and the 5-day subacute dietary toxicity test. The single-dose acute oral toxicity test, in general, is performed with a single dosage administration but it can also involve repeated exposures within a 24-h period in order to achieve sufficiently high dosage levels of compounds with low acute toxicity.

The 5-d subacute dietary toxicity test exposes the animals to contaminated feed for 5 d followed by an uncontaminated diet for 3 d. The function of the standardized 5-d subacute dietary toxicity test is to augment the standardized single-dose acute toxicity test (Hill 1994). The objective of both of these tests is to generate estimates of the dose–response curve and its midpoint, that is, the median lethal dosage (LD50) for the single-dose acute oral toxicity test and the median lethal concentration (LC50) for the 5-d subacute dietary toxicity test. The LD50 and LC50 values, their 95% confidence intervals, and slopes are used as indices for comparing different toxicants and species (Klaassen 1986; Hill 1994). Whereas these standardized test methodologies are followed in research and are routinely required by regulatory agencies for preparing risk assessments, their use without regard to the toxicant's mode of action can generate misleading results (http://www.pcrm.org/resch/PDFs/ae_ld50.pdf; Smith 1998).

Herein we address the limitations of using the standardized acute oral toxicity test for evaluating the avian toxicities to the three FGARs registered for use in United States: chlorphacinone (2-[(4-chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione), diphacinone (2-(diphenylacetyl)-1H-indene-1,3(2H)-dione), and

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warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl-2H-1-benzopyran-2-one). The standardized 5-day subacute dietary toxicity test design does provide multiple exposures over several days, which is more consistent with the FGARs' mode of action compared to the single-dose acute oral toxicity test, but even the 5-d test has notable shortcomings (*e.g.*, difficulty in measurement of the dosage received by each test animal).

Hill (1995) cautioned that the LC50 test is more properly considered as a measure of vulnerability to a potentially toxic food source (*i.e.*, "the product of the willingness to eat contaminated food, rate of feeding, sensitivity to the contaminant, duration of the contaminants availability in toxic amounts") rather than as a measure of inherent toxicity, and that it has limited value as a quantitative descriptor of toxicity in risk assessment because of the many chemical and biological factors that cannot be accommodated in a standard laboratory test. Pitt *et al.* (2011) showed that in laboratory dietary toxicity tests, bait palatability affected the efficacy of rodenticides. Mineau *et al.* (1994) also questioned the adequacy of the LC50 as a measure of toxicity and its use in risk assessments.

The rationale underlying the development of a testing methodology is to elicit a response that has resulted because the toxicant has engaged its target organ or system for sufficient time (Hill 1994, www.alttox.org/ttrc/tox-test-overview/). In other words, the methodology and duration of the test has to be in agreement with the time course of adverse effects of the toxicant. The toxicity of FGARs generally requires a different exposure regimen than acutely toxic rodenticides (*e.g.*, second generation anticoagulant rodenticides and acute or non-anticoagulant rodenticides); yet FGARs and the acutely toxic rodenticides are subjected to the same standardized testing methodologies (USEPA 2004). The acutely toxic rodenticides conform to the design of the standardized avian acute oral toxicity test because one or more dosage levels that produce mortality are environmentally relevant to free-ranging birds. Hence, the standardized avian acute oral toxicity test generates meaningful toxicity indices for these rodenticides. However, the fact that FGARs generally require multiple feedings over several days to achieve a threshold concentration in tissue and cause an adverse effect, suggests that a departure from the standardized avian acute oral toxicity test testing methodology is in order. Nonetheless, the U.S. Environmental Protection Agency (USEPA) has based much of its avian risk assessments for anticoagulant rodents on acute toxicity tests.

The limitations of the standardized avian acute oral toxicity test (single-dose or multiple-doses in a 24-h period) for FGARs are illustrated by recent diphacinone toxicity studies conducted on Northern bobwhites (*Colinus virginianus*), American kestrels (*Falco sparverius*), and eastern screech-owls (*Megascops asio*). Acute oral toxicity testing revealed that diphacinone is about 20 times more toxic to American kestrels than to Northern bobwhites (LD50s = 96.8 mg a.i./kg and 2014 mg a.i./kg, respectively) (Rattner *et al.* 2010, 2011a). This comparative testing does identify a more sensitive species. However, when using field residue data from rodents poisoned by diphacinone (Spurr *et al.* USGS, unpublished data; extreme value = 3.8 ppm in liver), an adult kestrel would have to consume more than 2000 mouse livers within a 24-h period to receive a median lethal dose using a deterministic approach (USEPA 2008; Rattner *et al.* 2011a). During testing it was problematic to gavage the bobwhite and kestrels because the volume of diphacinone that was required to evoke mortality was large, and therefore required administration of up

to four partial doses over a 24-h period (Rattner *et al.* 2010, 2011a). Rattner and co-workers (2011b) also compared the sensitivity of eastern screech-owls that were exposed to diphacinone either via multiple oral doses within 24 h (prescribed by the avian single-dose acute oral toxicity test protocol) and by a 7-d dietary exposure. By measuring the diphacinone-treated diet that was consumed daily (thereby overcoming a significant hurdle of the standardized dietary test design), the dosage received by each owl over the 7-d period was calculated. Rattner *et al.* (2011b) showed that diphacinone was more toxic to screech-owls by over a magnitude in the low-dosage multiple day exposure than in the acute oral toxicity test. The inappropriateness of the standardized acute oral toxicity test for FGARs has also been addressed for mammals. Using laboratory rats, Ashton *et al.* (1986) determined the single-dose acute oral toxicity test LD50 values for chlorophacinone, diphacinone, and warfarin to be up to 22-, 41-, and 113-times greater than the respective LD50 values derived from 5-d oral dose toxicity tests.

Birds and mammals are more sensitive to FGARs in toxicity tests that provide low level exposures over several days than in the standardized acute oral toxicity test. Furthermore, the low-dose multiple day exposure testing allowed for environmentally relevant exposures and time course for the overt toxicity (death) of FGARs' to be expressed (Ashton *et al.* 1986; Jackson and Ashton 1992; Rattner *et al.* 2011b). The greater treatment dosage levels necessary to elicit mortality and the large LD50 values are artifacts associated with use of the acute toxicity protocol for compounds that can have profound effects when administered repeatedly at low dosages, which coincidentally mimic real world exposures. Since the standardized avian acute oral toxicity test is designed to elicit a response from a single-dose or divided multiple doses within 24 h, and since FGARs generally require small doses over several days, the results of standardized avian acute oral toxicity tests using FGARs do not seem to be valid indices for evaluating toxicity among the three rodenticide classes.

Caution is warranted when interpreting FGAR the standardized acute oral toxicity test results. Because of the FGARs' time course of action, the standardized acute oral toxicity test requires large dosage levels to elicit a lethal effect and the resulting LD50 value is high. The FGARs, therefore, artificially appear less toxic than they are and may not trigger the levels of concern for identifying risk (Primus *et al.* 2001; USEPA 2004; Eisemann and Swift 2006). Consequently, the hazards and risks previously reported may be underestimated.

Caution is also warranted when using the findings of standardized acute oral toxicity tests to assist in the diagnosis of the cause of death of an animal collected from the field. Some of the physiological responses produced by the disproportionately large exposure levels in the standardized acute oral toxicity testing may not be consistent with typical signs of FGAR poisonings seen in wild birds (Smith 1998). For example, quail and kestrels that died in the high treatment groups did not exhibit frank internal or external bleeding and displayed only some evidence of macroscopic hemorrhaging (Rattner *et al.* 2010, 2011a). Additionally, kestrels exposed to diphacinone consistently exhibited microscopic hemorrhaging in various tissues during histopathology examinations (Rattner *et al.* 2011a). Birds killed by operational applications of rodenticides routinely undergo pathological examinations for evidence of frank bleeding and macroscopic hemorrhaging, but their tissues are

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not always reviewed for evidence of microscopic hemorrhaging. It is not known at this time if the microscopic hemorrhaging may be a consequence of the unrealistically large treatment levels given within a 24-h period as necessitated by the acute toxicity test (Smith 1998).

During a field investigation, if resources were used for harvesting tissues because microscopic hemorrhaging was considered to be a biomarker of FGAR exposure based on the results of standardized acute oral toxicity testing, valuable resources may be unnecessarily diverted from conducting additional carcass searches. Field mortality incidents are difficult to detect, but when discovered they provide valuable evidence of a pesticide's hazards following operational applications. A reduced carcass search effort can underestimate the magnitude of the mortality incident (Vyas 1999; Vyas *et al.* 2003). Additionally, if the cause of the field mortality incident was a pesticide other than FGAR, and if the tissues were prepared for histopathological examination, valuable biomarkers necessary for determining the cause of death (*e.g.*, brain cholinesterase activity for anticholinesterase insecticides) may be lost. Histopathological examination of tissues from anticoagulant-exposed wild birds and from captive birds subjected to low-dose multiple day exposures are critically needed to determine the environmental relevancy of the microscopic hemorrhaging observed in kestrels.

A second consideration of using data from an acute toxicity test to evaluate the cause of death in the field is that high single-dose acute FGAR exposures result in mortality of captive birds that are directly related to the toxicant, while free-ranging birds may become debilitated from the hemorrhaging but may die from starvation, extreme weather, and even otherwise minor injuries (Savarie *et al.* 1979; USEPA 2008; Rattner *et al.* 2011a). Furthermore, given the metabolism and excretion of FGARs in animals and that mortality in the field may be protracted compared to captive birds subjected to the acute oral toxicity testing, the pharmacokinetics (absorption, distribution, metabolism, and elimination) of FGARs in free-ranging birds experiencing low-dose multiple day exposures are likely to differ from those of birds in the standardized acute toxicity test (Birkett 2002; Jusko 2006; Rattner *et al.* 2011a). Therefore, FGAR residue values in tissues derived from acute oral toxicity test may not be suitable benchmarks for confirming FGARs as the cause of death in wildlife and for predicting the risk of secondary poisoning. FGAR residue measurements in tissues from affected wild birds and from captive birds subjected to low-dose multiple day exposures need to be compared in order to better determine the environmental relevancy of FGAR residue concentrations following acute oral toxicity testing.

The results of the standardized avian acute oral toxicity tests are used in deterministic and probabilistic risk assessment models to characterize FGARs' risks to wild birds. Therefore it is important for conservationists, natural resource managers and environmental regulators that review ecological risk assessments to understand the consequences of the current standardized testing design. For example, deterministic risk assessments, based on the results of the standardized avian acute oral toxicity tests, have repeatedly identified FGARs as being of low risk to birds (USEPA 1998; Primus *et al.* 2001; USEPA 2004; Eisemann and Swift 2006), although some have acknowledged the limitations of acute oral toxicity data by inclusion of information on multiple-dose studies in their assessments (Eisemann and Swift 2006).

Furthermore, a deterministic risk assessment for diphacinone, based on the results of avian acute oral toxicity testing, did not trigger risk for the federally endangered Hawaiian hawk (*Buteo solitarius*) and the state endangered Hawaiian owl (*Asio flammeus sandwichensis*) (Eisemann and Swift 2006). By contrast, a recent probabilistic risk assessment utilizing the results of the acute oral toxicity test on kestrels by Rattner *et al.* (2011a) identified risk to the endangered Hawaiian hawk and the Hawaiian owl following operational diphacinone applications. However, because the model used the results of the acute oral toxicity test, it did not trigger risk to non-endangered raptors at environmentally relevant diphacinone exposures (Rattner *et al.* 2011a). We believe that the low-dose multiple day testing conducted by Ashton *et al.* (1986) and Rattner and co-workers (2011b) provide the platform for standardizing toxicity testing methodologies that will allow comparative hazard determination and refine risk characterization using the current ecological risk assessment methodologies.

CONCLUSION

There are many inherent differences between laboratory populations and wild birds. Free-ranging birds contend with the chemical interactions, multiple exposures, nutrition, health, injuries, and other stressors that, however, are of less concern for captive birds. The exposure scenarios and stressors in the field can increase a bird's sensitivity to a pesticide and therefore environmental exposures need not be as high as the dosages used in a laboratory study to elicit an effect (Vyas *et al.* 2006). Exposure regimens and doses that do not take into account the exposure scenario and time course of response of FGARs in the field further remove the relevancy of laboratory results to free-ranging birds. The USEPA recently placed greater restrictions on the sale, packaging, and use of second generation anticoagulant rodenticides, and an increase in the use of FGARs is anticipated (USEPA 2008; <http://www.epa.gov/pesticides/mice-and-rats/>). However, despite literature (as far back as 1986) documenting that the standardized acute oral toxicity test is not suited for FGARs, the USEPA, USGS, U.S. Fish and Wildlife Service, and the U.S. Department of Agriculture continue to conduct standardized acute oral toxicity testing for FGARs and continue to use the results in their risk characterizations, and for planning and operational rodenticide applications. We integrated the toxicology of FGARs and their testing methods with respect to applied field work because conservationists, natural resource managers and environmental regulators need to be aware of the acute oral toxicity test's limitations for FGARs and the ecological applicability of the test results. The FGARs' mode of action, the continued use of standardized acute oral toxicity testing method, and the growing use of FGARs necessitate the development of a standardized avian low-dosage multiple-day toxicity testing methodology in order to correctly assess their hazards and risks.

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