Is Sensitivity to Anticoagulant Rodenticides Affected by Repeated Exposure in Hawks?
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ABSTRACT: A seminal question in wildlife toxicology is whether exposure to an environmental contaminant, in particular a second-generation anticoagulant rodenticide, can evoke subtle long lasting effects on body condition, physiological function and survival. Many reports indicate that non-target predators often carry residues of several rodenticides, which is indicative of multiple exposures. An often-cited study in laboratory rats demonstrated that exposure to the second-generation anticoagulant rodenticide brodifacoum prolongs blood clotting time for a few days, but weeks later when rats were re-exposed to the first-generation anticoagulant rodenticide warfarin, coagulopathy was more pronounced in brodifacoum-treated rats than naïve rats exposed to warfarin. To further investigate this phenomenon, American kestrels were fed environmentally realistic doses of chlorophacinone or brodifacoum for a week, and following a week-long recovery period, birds were then challenged with a low-level dietary dose of chlorophacinone. In the present study, neither hematocrit nor clotting time (prothrombin time, Russell’s viper venom time) were differentially affected in sequentially exposed kestrels compared to naïve birds fed low-level dietary dose of chlorophacinone. While the present findings do not reveal lasting effects of anticoagulant exposure on blood clotting ability, findings in laboratory rats and other species have demonstrated such effects on blood clotting, and even other molecular pathways associated with immune function and xenobiotic metabolism. Additional studies using an environmentally realistic route of exposure and dose are underway to further test this hypothesis.

KEY WORDS: anticoagulant, birds, brodifacoum, clotting time, chlorophacinone, hazard, non-target hazard, risk, secondary poisoning

INTRODUCTION  
Over 25 years ago, Hadler and Buckle (1992) stated that few modern pesticide groups have had such a long history of successful use as the anticoagulant rodenticides (ARs), and they remain one of the principal chemical groups used in the near billion dollar global rodenticide industry (Zion Market Research 2016). These compounds are used worldwide for the control of vertebrate pests and for the eradication of invasive species in ecological restoration projects (Jacob and Buckle 2018). While their risks to children, companion animals, and non-target wildlife have resulted in some restrictions in application of the more hazardous second-generation ARs (SGARs) (Health Canada 2012, US EPA 2017), SGAR use is allowed in many circumstances in the United States and Canada (e.g., by permit, by professional applicator), and is extensive in other parts of the world (Jacob and Buckle 2018).

In the past three decades, over 40 reports have appeared in the scientific literature documenting the presence of ARs residues in tissues of non-target predatory birds and mammals. A summary of data from Canada, Denmark, France, New Zealand, Spain, the United Kingdom, and the United States indicated that over 58% of the 4,187 specimens tested contained residues of one or more AR, principally SGARs (López-Perea and Mateo 2018). A recent report described liver AR residues for four species of raptors admitted to a wildlife clinic in Massachusetts between 2012 and 2016 (Murray 2017). Ninety of the 94 liver samples contained AR residues; all 16 individuals diagnosed with AR toxicosis contained SGAR residues; and 74 of 78 asymptomatic birds contained SGAR residues. Notably, 60% of the birds contained residues of two or more SGARs, and two SGAR positive samples also contained the first-generation ARs (FGARs) diphacinone and chlorophacinone. Other wildlife studies have provided evidence of individuals carrying burdens of both FGARs and SGARs (e.g., Christensen et al. 2012, Sérieys et al. 2015).

The aforementioned AR residue data indicate that some predatory birds and mammals are exposed to ARs on multiple occasions, and that exposed individuals may carry AR burdens yet be seemingly asymptomatic. A longstanding question in the fields of wildlife toxicology and forensics is whether AR residue burdens evoke subtle effects on body condition or physiological function. An often cited study by Mosterd and Thijssen (1991) demonstrated such lasting effects of the SGAR brodifacoum. In this study, Wistar rats were orally administered 0.2 mg brodifacoum/kg body weight (BWt).
Prothrombin complex activity was depressed (i.e., clotting time prolonged) for several days following exposure, but after a week, prothrombin clotting time returned to normal. However, activity of hepatic microsomal vitamin K epoxide reductase (VKOR; key enzyme in vitamin K recycling antagonized by ARs) remained depressed from baseline activity for seemingly more than 30 days. On day 8 post-brodifacoum exposure, when clotting time was in the normal range, rats were administered a second AR dose (i.e., the FGAR warfarin, single subcutaneously dose at 0.1 mg/kg BW). While this warfarin dose caused modest anticoagulant effects in unexposed control rats, pronounced coagulopathy developed in brodifacoum exposed rats on day 8 post-brodifacoum exposure. On day 25 post-brodifacoum exposure, another group of brodifacoum–treated rats received a second AR dose (subcutaneous 0.1 mg warfarin/kg BW); a similar but less dramatic effect on clotting time was observed in these rats. These findings suggest that AR-exposed individuals, and possibly predatory wildlife, may more readily exhibit coagulopathy with subsequent and unintentional AR exposure events. Such repeated exposures and intoxications could potentially affect body condition, physiological function and possibly even survival.

Herein we describe a somewhat similar sequential AR exposure study using the American kestrel (Falco sparverius) as an avian wildlife model species (Bardo and Bird 2009) to further examine and test the hypothesis that multiple AR exposures might affect sensitivity and enhance intoxication caused by these compounds. The data from our study may have application for hazard and risk assessments of predatory birds that are repeatedly exposed to an AR or multiple ARs while foraging in urban, suburban and agricultural settings.

MATERIALS AND METHODS

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the Patuxent Wildlife Research Center, and similar to those of a previous study (Rattner et al. 2015). In the spring of 2017, adult one to two year old male kestrels, propagated from the colony at Patuxent, were transferred from flight pens to small outdoor cages (1.2 × 0.8 × 0.6 m) containing a shade roof, perches, food tray and water bowl. Individually housed kestrels were acclimated for three weeks, during which time they were fed dead mice. Kestrels were then weighed and a 0.9 ml jugular venipuncture sample was collected into 0.1 ml of 3.2% sodium citrate from each bird for subsequent determination of baseline clotting time. Microhematocrit was determined and then the remainder of each blood sample was centrifuged (2000 × g for 5 min), citrated plasma harvested, and various volumes pipetted into cryotubes that were frozen and then stored at -80°C for clotting time assays. Kestrels were then shifted to a diet of two ~35-g Classic Bird of Prey diet meatballs (Nebraska Brand; hereafter NBP) containing the vitamin supplement Vionate® (Gimborn, OH) for 10 days prior to sequential AR exposure trial.

Rationale for Study Design

Information from several toxicity studies was used to design the sequential exposure trial. Brodifacoum mortality data was obtained from a toxicity trial in which captive adult kestrels were fed tissue from meadow voles (Microtus spp.) that contained various quantities of brodifacoum (0, 0.3, 0.8, 1.6, 3.2 and 6.0 µg/g wet weight; hereafter ww) (LaVoie 1990). Mortality occurred in one of eight kestrels fed 0.8 µg/g ww diet, and four of eight kestrels that received 6.0 died, with two of these exhibiting hemorrhage. In other studies examining sublethal effects (Rattner et al. 2016, Rattner et al. 2018), kestrels consuming diets containing 0.3, 1.0 or 3.0 µg brodifacoum/g ww for seven days exhibited dose-dependent bruising, overt and microscopic evidence of hemorrhage, coagulopathy, anemia and the accumulation of hepatic brodifacoum residues (~0.5-1.4 µg/g ww), but not mortality. For kestrels receiving a diet containing 0.5 µg brodifacoum/g ww for one week, coagulopathy was apparent, but upon shifting birds to an untreated diet, clotting time was restored to baseline values within one week. There was no mortality, but hepatic brodifacoum residues exceeded 0.7 µg/g ww over the course of the 28 day trial. In a study examining sublethal chlorophacinone toxicity (Rattner et al. 2015), kestrels fed diets containing 0.15 to 1.5 µg/g ww for one week exhibited dose-dependent external signs of intoxication, microscopic evidence of hemorrhage, prolonged clotting time, and accumulation of chlorophacinone in liver (~0.05-0.20 µg/g ww), but not mortality. Diet- and tissue-based chlorophacinone toxicity reference values for coagulopathy were generated from this investigation. Using the aforementioned kestrel data, we selected dietary exposure doses for a sequential exposure trial predicted to cause coagulopathy, but not mortality. Furthermore, the dietary dose of brodifacoum chosen would likely result in liver residues (> 0.7 µg/g ww, well-above the 0.1-0.2 µg/g exposure threshold used in forensic investigations; e.g., Thomas et al. 2011) for an extended period of time. We hypothesized that such SGAR residues may affect VKOR in a manner that might affect sensitivity to subsequent AR exposure as observed in studies in laboratory rats (Mosterd and Thijssen 1991).

Diets Used in Exposure Trial

Several NBP diets were used in the exposure study. These included the following: (1) control diet containing 1% corn oil vehicle; (2) chlorophacinone (CPN) diet nominally targeted to contain 1.5 µg CPN/g ww; (3) brodifacoum (BROD) diet nominally targeted to contain 0.5 µg BROD/g ww; and (4) CPN diet used as a challenge exposure nominally targeted to contain 0.25 µg CPN/g ww. The diets were formed into 25 ± 0.1-g meat balls (overall mean ± SD moisture content for the four diets: 62.0 ± 1.1%, n = 5 meatballs/diet). The nominal 1.5 µg CPN/g ww diet, 0.5 µg BROD/g ww diet, and the 0.25 µg CPN/g ww challenge diet were analytically verified and found to be (mean ± SD, n = 5) 91.5 ± 3.4%, 80.4 ± 1.6%, and 93.5 ± 8.3% of target concentrations, respectively.

The concentrations of BROD and CPN in these test diets are believed to be environmentally realistic for rodent prey in proximity to AR control activities. For example, nominal concentration of brodifacoum in the kestrel test diet was 0.5 µg/g ww; average carcass concentrations in
target meadow voles (Microtus pinorum) and black rats (Rattus rattus) following field control and eradication activities were reported to be 0.35 and 3.75 µg/g ww, respectively (Merson et al. 1984, Pitt et al. 2015), and estimated whole body concentrations in several species non-target small mammals range from 0.3 to 3.6 µg/g ww (reviewed in Shore and Coeurdassier 2018). Likewise, nominal concentrations of CPN in kestrel test diets were 0.25 and 1.5 µg/g ww, and were within the range of concentrations detected in carcasses of Belding’s ground squirrels (Spermophilus beldingi), pocket gophers (Thomomys bottae), and voles (Microtus spp) following field baiting trials (0.131 to 1.59 µg/g ww; Primus et al. 2001, Ramey et al 2007). A complicating factor is that several of these values (Primus et al. 2001, Ramey et al. 2007, Pitt et al. 2015) were derived carcass samples from which liver was removed, and may underestimate potential exposure. Brodifacoum and chlorophacinone residue concentrations in prey liver can be much greater than in prey carcass (e.g., Vyas et al. 2012, Shore and Coeurdassier 2018). Hawk species, for which the kestrel may be the most appropriate model, do not ingest entire mammalian prey, but selectively feed on liver, muscle and some organs. Thus, for hawks there is uncertainty in AR exposure based on the prey tissue preferentially consumed, and for non-target raptors (e.g., hawks, owls) there uncertainty in both the frequency and duration that AR exposed prey would be consumed following a control or eradication activity.

**Sequential AR Exposure Trial**

During the initial exposure period, kestrels received either control diet, 1.5 µg CPN/g ww diet or 0.5 µg BROD/g ww diet provided daily as two 25 ± 0.1 g meatballs for a seven-day period (n = 6 birds/treatment). Our previous krestel studies have demonstrated that consumption of these concentrations of CPN or BROD for seven days result in pronounced coagulopathy (Rattner et al. 2015). These 18 kestrels were then shifted to control diet for a seven-day recovery period, followed by a seven day challenge period during which 0.25 µg CPN/g ww diet was provided to all 18 birds. This challenge CPN exposure was at a dietary dose level that causes prolonged clotting time in 10% of naïve birds (i.e., benchmark dose 10; Rattner et al. 2015), but potentially exhibits a more pronounced effect in animals previously exposed to SGARs (as demonstrated in rats by Mosterd and Thijsen 1991). Hereafter, AR treatment groups are designated as follows: control-chlorophacinone challenge (CON-CPN), chlorophacinone-chlorophacinone challenge (CPN-CPN), and brodifacoum-chlorophacinone challenge (BROD-CPN). A group of untreated kestrels (n = 6, untreated controls) were fed dead mice and monitored during the study.

In this trial, birds were fed daily between 1100 and 1200 hours, and uneaten food scraps were carefully removed from each kraft paper lined pen the following day between 0800 and 1100 hours during both the initial exposure and the challenge periods. The scraps were weighed, stored, dried, and converted back to weight wet as previously described (Rattner et al. 2015). Kestrels were weighed at the beginning of the initial exposure period (day 0), and both weighed and examined for signs of bruising or external bleeding after the seven day initial exposure period and the seven day recovery period. At the end of the challenge period, each bird was examined, weighed, bled (0.9 ml jugular venipuncture blood draw into 0.1 ml 3.2% sodium citrate), sacrificed using carbon dioxide, and then necropsied. Blood samples were also collected from the six untreated control kestrels.

**Clotting Time Assays**

Prothrombin time (PT) and Russell’s viper venom time (RVVT) of citrated kestrel plasma samples were used to evaluate AR effects on post-translational processing of clotting Factors II, VII, IX, and X. Thrombin clotting time was used as an indicator of fibrinogen concentration in plasma samples. Fibrinogen formation is insensitive to deficiency of vitamin K-dependent clotting factors, but its deficiency resulting from improper blood sample collection can prolong clotting time and confound rodenticide toxicity studies. Thus, it is important to verify that fibrinogen concentration is adequate to promote clot formation (>46 mg/dL in kestrels). The conduct, performance, and use of these assays in birds have been previously described (e.g., Rattner et al. 2010, Rattner et al. 2015). For this study the mechanical clot endpoint was determined using a START4 Fibrometer (Diagnostica Stago Inc. Parsippany, NJ). The coefficient of variation of duplicate determinations (mean ± SD) of study samples were 0.88 ± 0.81% for PT; 2.49 ± 2.56% for RVVT; and 2.48 ± 2.35% for fibrinogen.

**Statistical Methods**

All endpoints (body weight, weight change, CPN consumption during challenge period, hematocrit, fibrinogen concentration, PT, and RVVT) were tested for homogeneity of variances and normality, and then evaluated by one-way analysis of variance followed by Tukey’s HSD test. The number of individuals exceeding baseline clotting time by two standard deviations was compared among groups using Fisher’s Exact Test. Values presented in the text are mean ± standard deviation.

**RESULTS**

All kestrels (AR-exposed birds and untreated controls) survived the initial exposure, recovery and CPN challenge periods. At the end of the initial seven-day exposure period, a small drop of blood was observed on the foot of a BROD-exposed bird, and on day two of the recovery period, drops of blood were observed on the rope perch of another kestrel that had been exposed to BROD. There were no other overt signs of intoxication or bruising in any birds during the three study periods. At the end of the study, quality blood samples were obtained from nearly all birds in the trial. Collection of a jugular venipuncture sample from one kestrel in the BROD-CPN group was unsuccessful, and this bird succumbed moments before necropsy. At necropsy, internal examinations of all CON-CPN, CPN-CPN, and BROD-CPN birds were unremarkable.
Table 1. Anticoagulant rodenticide consumption by kestrels during initial and challenge exposure periods.

<table>
<thead>
<tr>
<th>Initial Exposure</th>
<th>Recovery</th>
<th>Challenge Exposure</th>
<th>CPN or BROD consumption during initial exposure period (µg/kg bird/day)a</th>
<th>CPN consumption during challenge exposure period (µg/kg bird/day)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 1-7</td>
<td>Days 8-14</td>
<td>Days 15-21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated Control</td>
<td>Untreated Control</td>
<td>Untreated Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control NBP diet</td>
<td>Control NBP diet</td>
<td>0.25 µg CPN/g NBP diet</td>
<td>0</td>
<td>59.1 ± 12.6</td>
</tr>
<tr>
<td>1.5 µg CPN/g NBP diet</td>
<td>Control NBP diet</td>
<td>0.25 µg CPN/g NBP diet</td>
<td>308.9 ± 46.0</td>
<td>54.7 ± 5.6</td>
</tr>
<tr>
<td>0.5 µg BROD/g NBP diet</td>
<td>Control NBP diet</td>
<td>0.25 µg CPN/g NBP diet</td>
<td>85.8 ± 7.6</td>
<td>51.9 ± 4.1</td>
</tr>
</tbody>
</table>

aCPN = chlorophacinone, BROD = Brodifacoum, NBP = Nebraska Bird of Prey; values are mean ± standard deviation, n=6.

At the initiation of the study, body weight (n = 24 kestrels, three AR treatment groups plus untreated controls) was 101.2 ± 4.5 g. Among these four groups of kestrels, there were no differences in weight change (g/100 g initial BW) on day seven (end of initial exposure, P = 0.34), day 14 (end of recovery period, P = 0.25), or day 21 (end of challenge exposure, P = 0.24). However, all groups did exhibit a slight weight loss compared to initial body weight over the course of the trial (CON-CPN: -4.2 ± 3.0 g/100 g; CPN-CPN: -5.5 ± 2.2 g/100 g; BROD-CPN: -3.2 ± 2.1 g/100 g; untreated controls: -2.1 ± 3.8 g/100 g). Estimated daily CPN consumption during the challenge exposure period was comparable among all three AR-exposed groups (P = 0.34, Table 1).

At the initiation of the study, baseline hematocrit averaged 47.4 ± 2.9 for the three AR treatment groups plus the untreated controls. When birds were bled after the 21 day trial, there was no difference in hematocrit among groups (P = 0.77; CON-CPN: 43.9 ± 2.9%; CPN-CPN: 44.8 ± 4.3%; BROD-CPN: 45.8 ± 2.0%; untreated controls: 45.0 ± 1.7%). Fibrinogen concentration in all but one sample was >46 mg/dL. One pre-study baseline sample with low fibrinogen concentration (45.3 mg/dL) had prothrombin time (23.05 sec) and Russell’s viper venom time (42.3 sec) that were very long (>10 standard deviations above other pre-exposure baseline samples), and another pre-exposure sample that contained a visible clot before assay; these samples were excluded from the data set. All other samples seemingly had sufficient fibrinogen to form a stable clot (discussed in Rattner et al. 2010, Rattner 2015). The average fibrinogen concentration in baseline pre-study samples was 71.1 ± 23.4 mg/dL; at the end of the trial 77.1 ± 21.7 mg/dL for untreated control birds; 73.8 ± 17.7 mg/dL for the CON-CPN group; 66.6 ± 18.3 mg/dL for the CPN-CPN group; and 60.4 ± 9.3 mg/dL for the BROD-CPN group.

For PT, the pre-exposure baseline of all birds was 12.8 ± 1.0 sec. At the end of the study, PT was similar (P = 0.70) for all groups (13.3 ± 0.8 sec untreated control birds, 13.5 ± 0.9 sec for CON-CPN, 13.0 ± 1.4 sec for CPN-CPN, and 13.8 ± 1.2 sec for BROD-CPN) (Figure 1). Using an increase in PT from pre-exposure baseline of the mean plus two standard deviations, potentially affected birds (i.e., respondents) had PT ≥ 14.8 sec. There were no respondents in the untreated control and CON-CPN groups, one of six in the CPN-CPN group, and one of five BROD-CPN group, with no difference in number of respondents among groups (Fishers Exact Test, P = 0.47).

For RVVT, the pre-exposure baseline for all birds was 17.1 ± 2.1 sec. At the end of the study, RVVT was similar (P = 0.45) for all groups (19.1 ± 1.1 sec untreated control birds, 20.1 ± 3.4 sec for CON-CPN, 18.7 ± 1.7 sec CPN-CPN, and 21.0 ± 3.4 sec BROD-CPN) (Figure 1). The pre-exposure baseline plus two standard deviations was 21.3 sec for RVVT. Using this threshold, zero of six untreated control birds were affected, but two of six CON-CPN, one of six CPN-CPN, and two of five BROD-CPN birds were affected, and there was no difference in number of respondents among groups (P = 0.57).

**DISCUSSION**

Using hematocrit and blood clotting times as endpoints, dietary exposure of captive kestrels to environmentally realistic concentrations (Merson et al. 1984, Primus et al. 2001, Ramey et al. 2007, Vyasa et al. 2012, Pitt et al. 2015, Shore and Coeurdassier 2015) of the FGAR chlorophacinone or the SGAR brodifacoum did not affect sensitivity to a subsequent low-level dietary dose of chlorophacinone. Based upon previous studies in kestrels (Rattner et al. 2015), a seven-day, no-choice dietary exposure to 1.5 µg chlorophacinone/g-ww or to 0.5 µg brodifacoum/g ww evokes coagulopathy and results in substantial hepatic residues of these ARs. Furthermore, hepatic residues of brodifacoum remain elevated in kestrels for a sustained period (months) (Rattner et al. unpubl.), as has been demonstrated in many species of mammals (reviewed in Horak et al. 2018). The apparent absence of an effect of the chlorophacinone challenge dose in kestrels was in contrast to that elicited by a warfarin challenge dose in brodifacoum treated rats (Mosterd and Thijssen 1991), and may be due to species difference in AR pharmacokinetics or VKOR activity and its inhibition. However, available tissue clearance and VKOR kinetic data comparing birds to laboratory rodents are too limited...
(Watanabe et al. 2010, Watanabe et al. 2015) to firmly draw such a conclusion. Alternatively, the chlorophacinone challenge dose used in the present study could have been too low to clearly detect such an effect. To test the latter hypothesis, we are currently repeating this kestrel exposure study using a greater chlorophacinone challenge dose (0.75 μg/g ww diet, benchmark dose 90; Rattner et al. 2015).

The results of the present study do not refute findings in the laboratory rat (Mosterd and Thijsen 1991) demonstrating that exposure to an SGAR can have lasting effects on VKOR activity and blood clotting function. Somewhat similar effects have also been observed in Japanese quail (Coturnix japonica) sequentially exposed to SGARs (Butler 2010). Quail administered brodifacoum as a single oral dose in corn oil (200 or 400 μg/kg BWt), and then 26 days later with a second gavage dose of brodifacoum (200 μg/kg BWt), exhibited longer clotting time and more protracted coagulopathy than quail receiving only a single brodifacoum dose. While the brodifacoum exposure doses in rat, quail, and the kestrel studies were comparable (200 or 400 μg/kg BWt versus cumulative seven-day dose of ~528.5 μg/kg BWt), the challenge compounds (i.e., FGAR warfarin, SGAR brodifacoum versus FGAR chlorophacinone), and exposures method differed (subcutaneous injection in rats, single oral dose in quail versus multi-day dietary exposure in kestrels), and could also account for disparate findings. Exposure via the diet is seemingly more environmentally realistic than single subcutaneous exposure in rats and single oral dose in quail. While we do not necessarily dispute the findings of coagulopathy in the quail, it is noteworthy that the clotting assay methodologies and their precision vastly differed between studies (i.e., single PT determinations, lengthy control clotting time, and precision not specified in Butler 2010, versus high precision in PT and RVVT assays, short clotting times, and fibrinogen determination to rule out nonspecific effects in present kestrel study). Clotting time assays and reagents for use in birds have yet to be standardized, which

Figure 1. Prothrombin time and Russell’s viper venom time in seconds. Bar is mean ± standard deviation, “o” are individual observations, …… (dotted line) = baseline mean and ------ (dashed line) = baseline mean plus two standard deviations.

One of the critical information gaps in assessing the risk of ARs to non-target species is the potential for long lasting effects that might alter body condition, survival and recruitment (Rattner et al. 2014). While coagulopathy could influence both individual- and population-level parameters, there is recent evidence that AR exposure can affect disease susceptibility, immune function, and a myriad of other effects in mammals independent of coagulopathy (Rattner et al. 2014, Fraser et al. 2018, Serieys et al. 2018). Because of the likelihood and environmental relevance of exposure to multiple FGARs and SGARs, additional investigation of potential sublethal effects is warranted.

DATA AVAILABILITY STATEMENT
Much of the data described in this manuscript is publicly available; Rattner, B. A., 2018. Is Sensitivity to Anticoagulant Rodenticides Affected by Repeated Exposure in Hawks?: U.S. Geological Survey data release, https://doi.org/10.5066/F7RB73XW.

ACKNOWLEDGEMENTS
The authors wish to acknowledge the assistance of W. C. Bauer, D. D. Day, and S. L. Schultz in the conduct of the study. We thank N. B. Vyas and R. F. Shore for reviewing a draft of this manuscript. Any use of trade, product or firm names is for descriptive purposes only.

LITERATURE CITED


