Acute and Subacute Toxicology in Evaluation of Pesticide Hazard to Avian Wildlife

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ABSTRACT

Single-dose acute oral and short-term subacute dietary toxicity tests with captive birds provide critical information on the potential hazard of pesticides to wild populations. The two tests have similar experimental designs and both generate a lethality curve and estimation of its midpoint, the median lethal dosage (LD_{50}) or concentration (LC_{50}). Although LD_{50}s and LC_{50}s are widely used to characterize pesticide toxicity, the lethality curve and critical observation of animal response to chemical challenge provide necessary insight for hazard evaluation. The highly controlled acute test is based on graded dosage by body mass and provides a sound method of comparing naive sensitivity to toxicant and a means of detecting pesticides that may cause large-scale field kills. In contrast, the subacute test presents graded concentrations of a chemical in the diet for a specified duration, usually 5 days. This feeding trial provides an evaluation of response to repeated chemical exposures as may be encountered in the field. This chapter is an appraisal of the two basic tests of lethality with an emphasis on factors that may affect interpretation of potential hazard.

KEY WORDS

birds, pesticides, lethal toxicity, hazard

INTRODUCTION

The single-dose acute oral toxicity test is used in preliminary evaluation of virtually all substances of suspected biological activity. The test is based on administration of graded dosage of chemical in relation to body mass. The primary objective is to generate estimates of the dose-response or lethality curve and its midpoint, the median lethal dosage or LD_{50}.[1] Once these statistical parameters and their associated errors are properly determined this test of lethality provides a proven means of quantifying chemical potency and comparing substances of different mechanisms and sites of action.[2] The value of an acute test is greatly enhanced by detailed observation of each animal from the time of dosage to its death or recovery. Too often, however, comparisons and interpretation of acute tests are focused on the LD_{50} exclusive of its statistical reliability and without reference to the lethality curve or other supplemental observations that provide important dues about acute toxicity and hazard evaluation. The LD_{50}, per se, is simply a convenient index of toxicity that is subject to error, and its indiscriminate use can be misleading.[3]

In wildlife toxicology, two tests of lethality are routinely required on birds for pesticide registration in the United States.[4] The first is a standardized acute test of captive reared adult mallards (*Anas platyrhynchos*) or northern bobwhites (*Colinus virginianus*).[5] The second test is similar to the acute test except graded concentrations of chemical are presented ad libitum in the

feed for 5 days to young mallards or northern bobwhites of specified ages, and the midpoint of the lethality curve is quantified as the median lethal concentration or LC_{50}.[6] This subacute feeding trial is intended to augment the acute test by measuring response to repeated exposures and accumulative effects. Whereas the acute test provides a measure of a species' naive sensitivity to a toxic substance and a convenient index for rating its potency, the subacute test provides a measure of the species' ability to cope with a contaminated diet for a specified duration, allowing for the metabolic changes that occur over time.[7] Careful observation for changes in behavior and rate of feeding and for onset and course of toxic signs is especially important during subacute tests because the subjects voluntarily eat the potentially lethal diets. These two tests of lethality must never be viewed casually because they are often the only required avian tests for pesticide registration.[4,8]

This chapter is an appraisal of avian single-dose acute oral and 5-day dietary subacute toxicity tests as they are used in the evaluation of pesticide hazard. The basic tests of lethality, their toxicologic rationale, and key statistical treatments are described. Data are presented to illustrate experimental factors that affect toxicologic interpretation. The focus of the examples is on contemporary pesticides, many of which work through the same toxic mechanisms but often yield profound differences in response and potential environmental hazard.

THE BASIC TESTS

Classical acute toxicity tests are designed to determine exposures that cause death under a prescribed protocol with treatment levels that are based on animal response rather than practical residues. When treatments are properly arranged, however, the resultant lethality curve provides estimates of the LD_{50} and other dose-response coordinates that may be used in hazard assessment. Once the basic lethality curve and response to a substance are determined for several appropriate species, determination of only the general order of the substance's toxicity by approximate tests[9,10] with alternative species or finished product formulations may then be adequate. The choice between use of a full-scale or an approximate test depends on the purpose of the study. Although one should always strive to use the smallest number of animals, good science that is supported by sound statistical analysis must never be compromised.

Toxicologic Rationale

Toxic response is graded by the concentration of the substance that penetrates the target and remains in contact for a sufficient time to elicit change. The concentration of substance that penetrates the target is usually correlated directly with the dosage that is received by the organism. However, various biological chemical, and physical factors influence translocation and penetration of substances, and individuals may not be equally sensitive to a chemical. Therefore, response will vary even within a homogeneous population.[11] This natural diversity is approximated by a normal Gaussian distribution with about one third of the population divided equally between hyper- and hyposensitive individuals. When individua1 responses are described quantitatively, the frequency-response curve tends to be skewed toward hypersensitive respondents because their arithmetic range of tolerance is smaller than that of hyposensitive individuals.[1] Because the representation of hyper- and hyposensitive individuals is assumed to be equal in a homogeneous population, a series of groups may be randomly selected from the population and gradation of dose-related responses between groups may be generated if dosages

of test substance are properly spaced. Responses can be quantified as qualitative changes by a preselected all or nothing (binary) endpoint. In acute testing of lethality, the endpoint is alive or dead, and the responses can be evaluated quantitatively because the percentage of respondents increases with dosage. This concept and the factors responsible for diversity of response among individuals are well documented.[1,2, 9-14]

Dose-Response or Lethality Curve

The percentage of respondents in a lethality test is related to the composite tolerances of the population.[1,13] The pattern of response to graded dosages of substance is analogous to the graded tolerances of individual specimens and gives a frequency distribution skewed toward hypersensitivity and an asymmetric sigmoid curve when percentage response is plotted against dosage. The resultant dose-response curve is quite steep from its origin to the inflection point (at about the 30% response level) and then becomes gradual until virtually asymptotic. Because skewed data are difficult to analyze statistically, test dosages are usually arranged logarithmically to normalize the distribution of responses.[1,12] Normalization gives a symmetric sigmoid dose-response curve with the inflection point at the exact midpoint, the 50% response level.

The symmetric dose-response curve represents a cumulative normal distribution of log-tolerances. Steepness of the curve is similar for many substances but may become significantly steeper or shallower depending on the substance's mechanism of action, route or method of exposure, or shift of tolerance in the population. Thus, the dose- curve has interpretive value in addition to determination of probable dose-response coordinates. However, the linear portion of the curve is limited to a range of only 30 to 35 percentage points on either side of the 50% response level. The entire curve can be made linear by transforming the percentage response for log-dosage to probits.[1,12] Responses can then be analyzed by probit analysis, a method of calculating maximum likelihood fit of a probit-log-dose line by an iterative weighted regression analysis. The analysis provides critical interpretive statistics such as the median response level and its 95% confidence interval, and the slope of the weighted linear regression of probits on log-dose and its error. A systematic probit analysis, including calculation of all relevant toxicity statistics, is presented by Finney.[1] Although probit analysis or shortcut procedures by probit analysis are traditionally used in statistical evaluation of acute-type lethality tests, the movement is toward use of logit analysis as a more convenient computational method.[12]

Toxicity Comparisons

Comparison of toxicity between chemicals is possible with data generated by probit analyses if the level of tolerance of test populations is the same and the probit regression lines are parallel.[1] The level of tolerance can be assumed comparable if the test subjects are selected randomly from a single population and are tested concurrently in a completely randomized experiment.[1] In hazard evaluation of pesticides, data sets from many laboratories usually provide the basis of comparison, and such restrictive criteria cannot often be met. Even when tests are conducted in one laboratory, problems as indicated by Finney,[13] may arise: "One feature possessed by all biological assays is the variability in the reaction of the test subjects and the consequent impossibility of reproducing at will the same results in successive trials, however carefully the experimental conditions are controlled." This variability can be corrected

statistically by concurrent testing of a standard preparation that has the same biologically active principle as the test preparation.[13] This too is impractical because ever' pesticides that act on the same physiologic system may do so in different ways; e.g., central nervous system (CNS) stimulation by chlorinated cyclodiene insecticides or cholinesterase (ChE) inhibition by organophosphorus (OP) insecticides. Nonetheless, the researchers who generated most of the early avian subacute lethality data on pesticides believed that the test of a general standard substance should accompany all tests irrespective of mechanism of action.[16,17] Dieldrin was used as the standard and results have been summarized.[17-19] Even though the basic data from these reports have been widely used in hazard evaluation, a literature search failed to reveal evidence that the dieldrin standard was ever used as suggested for correction of LC_{50}s. Such specific corrections may best not be made on the basis of the dieldrin standard because consensus presently favors use of a nonspecific standard primarily for intralaboratory quality control rather than routine adjustment of LD_{50}s or LC_{50}s.[19-21]

Statistical techniques for comparison of potency among chemicals, including median response levels and slope of the probit regression curves, have been described.[1] A simplified method for separation of LD_{50}s or LC_{50}s is to compare the 95% confidence intervals for overlap; if they do not overlap, the median response levels may be considered different at $p < 0.05$. Other methods such as the two-tailed *t* test and Bonferroni s *t* statistics[22] are also used for comparison of median response levels. Median response levels must be statistically separable ($p < 0.05$) before quantitative comparison is credible. Toxicologic literature is replete with conclusions from comparison of LD_{50}s that aye obviously not different or the data are inconclusive because of omission of the 95% confidence interval or other estimate of variation. Even when the median response levels are statistically different, the same relationship cannot be assumed at different response levels without testing the slopes of the dose-response curves for parallelism.[1,17] When the slope of the dose-response curve and the median (50%) response level are known, any derived response level can be estimated.[1,17-19] Although response levels other than the 50% response may be desired, estimates of this type must be used cautiously because extrapolation from a standard probit regression line can be misleading if the true regression equation has some curvature.[1] In wildlife toxicology, the historical focus of acute toxicity testing has been on estimation and general comparison of LD_{50}s with approximate statistical procedures that do not provide for statistical estimation of the dose-response curve.[23,24]

Test Protocols

Single-Dose Acute Oral Toxicity Test

Optimal use of the acute test in hazard evaluation requires statistical estimation of the lethality curve and its midpoint and descriptive information on toxic response. The test for birds is basically the same as that described for laboratory animals.[3,10] The test involves dosage of test substance as a proportion of body mass and detailed observation of response until death or recovery. Ideally, a statistically adequate number of adult nonbreeding birds are drawn from a homogeneous population, weighed, and randomly assigned to individual test pens in a controlled environment room about 2 weeks prior to testing. A few extra birds are provided in case substitution is necessary. Room temperature and photoperiod are maintained at about 24^{o} to 28^{o}C and 10L:14D. The short day ensures reproductive quiescence to minimize sex differences. After 1 week the birds are evaluated and any that appear obviously substandard are replaced. On the

morning of the day prior to testing, birds are weighed in order to calculate dosage and are given a general health check. That evening, feed is removed in preparation for dosing the next morning.

Overnight-fasted birds receive a single dose of the test substance at midmorning. Feed is provided immediately after dosing, and observations for signs of intoxication are continued throughout the day. Special attention is given to the time of first evidence of toxicity, recovery, or death. Observations are continued twice daily or more often as indicated for 2 weeks after treatment or as long as toxic signs persist. Excellent summaries of observed toxic signs in acute tests of birds are available.[3,25] Gross necropsy should be performed on all birds that die and on a subsample of survivors to document significant toxic lesions.

Test substance is usually administered to the proventriculus in gelatin capsule or by gavage in water or suitable organic solvent. About five birds per sex are tested at each of five or six geometrically arranged dosage levels spanning the expected 10 to 90% mortality levels. Dosage levels are determined from a preliminary study of three widely spaced dosages administered to three to five birds each. Three kinds of controls (negative or sham, vehicle, and positive) may accompany each test; negative and vehicle are mandatory. The size of negative and vehicle control groups must each be equal to at least one dosage level; e.g., five birds per sex, with individuals integrated into the initial experimental design ant treated exactly the same as those on test substance. Negative controls receive sham treatment - insertion of empty dosing apparatus. Vehicle controls receive vehicle minus test substance. Positive controls, if used, receive a standard substance of known potency with the same biological action as the test substance. Use of the standard substance requires a full test to compare the slope of the dose-response curve and LD_{50}.[13] The LD_{50} and its 95 % confidence interval, expressed as milligram of active ingredient per kilogram of body mass, and the slope and error of the dose-response curve are derived by probit,[1] logit,[12] or other appropriate analysis.[3,10,15]

When only the general order of acute toxicity is desired, (e.g., to compare many species or fin shed product formulations), an approximate test of lethality may be used.[9,10,25,26] The treatment of test animals and post-dosage observations in these studies are the same as described for the full-scale acute test. The difference is that as few as three groups of three to five subjects are tested against a series of prearranged dosages, with LD_{50} and its 95% confidence interval calculated from published tables.[9,24]

Five-Day Subacute Dietary Toxicity Test

The design of the subacute test is based on the single-dose acute oral test.8 The test was developed to quantify the toxicity of contaminants for which the diet was considered an important source of exposure.[16] The subacute test was optimized with young precocial birds, such as ducks and quail, but virtually any species can be tested under the protocol if it can be maintained in captivity in good health and cannot survive for 5 days without eating.[21,27,28] If a portion of the test population can fast for 5 days, the results are erratic and not easily reproduced. Thus, the species of choice must be susceptible to the test protocol. This condition of susceptibility has been questioned because death by starvation does not represent the direct toxicity of a chemical.[29] Others have demonstrated that susceptible birds eventually eat rather

than starve,[30] and even though death is undoubtedly influenced by nutritional status, it remains primarily a chemical effect.[28]

Like the acute test, the subacute test generates a lethality curve and its midpoint as well as descriptive information on toxic response. The basic design uses the same number of animals, treatment levels, and control groups as the full-scale acute test. However, when testing very young precocial species, birds must be maintained in groups in heated brooder units with at least 14 hours of light.[6,18] Therefore, only one pen of equal-aged birds is usually tested at each concentration of test substance. To ensure susceptibility to the 5-day test, the recommended test ages for the most common model species are 5 days for mallard, 10 days for ring-necked pheasant (*Phasianus colchicus*), and 14 days for northern bobwhite and Japanese quail (*Coturnix japonica*).[6,18,21] Because of the young age at start, randomization to test pen is usually 2 days prior to testing. Any apparently substandard birds are replaced by surplus hatchmates.

Test substance is presented midmorning in an ad libitum diet to birds of the prescribed age and is continued for 5 days. Mortality and signs of intoxication are monitored at least twice daily. Food consumption is measured at 24-hour intervals. Fresh feed is added to all pens each day. After the fifth day, all feed, including that of control groups, is replaced with untreated feed and the study is continued for at least 3 days. When toxic signs persist, observation is continued through complete remission. The LC_{50} and its 95% confidence interval, expressed as milligram of active ingredient per kilogram of feed (or parts per million) in a 5-day ad libitum diet, and the slope and error of the dose-response curve are derived by probit analysis or other suitable method exactly as acute tests.

COMPARATIVE TOXICOLOGY

Birds vs Laboratory Rats

Acute tests of laboratory rodents are the most readily available toxicologic data on vertebrates and often serve as the primary factor in decisions on pesticide hazard to wildlife. For example, a rat LD_{50} above 200 mg/kg is generally considered only moderately toxic; if the pesticide also has poor affinity for lipids and is therefore not likely to bioaccumulate, the pesticide use may be considered low risk for general purposes of environmental impact, and often no additional attention is paid to potential wildlife hazard. However, such a conclusion may be inappropriate because the pesticide may be applied many times during the year, with its fate influenced by widely diverse factors, and the sensitivity to acute exposure may be quite different in birds than in laboratory rats.

Acute sensitivity to pesticides is not the same in birds as in laboratory rats. In Table 1, LD_{50}s for ring-necked pheasants and red-winged blackbirds (*Agelaius phoeniccus*) are compared to LD_{50}s for laboratory rats for OP insecticides of widely variable toxicity. All tests of each species were conducted at a since laboratory. Pheasants and blackbirds are presented because both species have general feeding habits, but represent extreme body mass compared to rats. The pesticides are all anticholinesterases that require metabolic activation for maximum potency, but whose extreme mammalian toxicity (i.e., rat LD_{50} for phorate or temephos) varies over 4000-fold. By most criteria for ranking acute toxicity, phorate is classed highly or extremely toxic and

temephos is practically nontoxic.[2,10,18] Phorate is also highly toxic to ring-necked pheasants, but it is about three times more toxic to rats than pheasants whereas temephos is about 250 times more toxic to pheasants than rats. The blackbirds are consistently most sensitive to OP exposure, possibly because of influences of differential metabolic rate, but more likely because red-winged blackbirds are especially deficient in hepatic microsomal monooxygenase activity that is often essential for detoxication.[34,35]

Beyond phorate and disulfoton, the rank of the individual pesticides is quite variable among the species, but the real importance to acute hazard evaluation is in comparison of the compounds with rat LD_{50}s above 200 mg/kg. As mentioned, this level implies only moderate toxicity to rats and therefore little acute field hazard would be expected from dimethoate, fenitrothion, malathion, or temephos. However, of the four pesticides, only malathion is not classed as extremely toxic (i.e., LD_{50}<40 mg/kg to both pheasants and blackbirds, and field application of fenitrothion has killed wild birds.[36] All insecticides listed in Table l elicit primary toxicity through the same mechanism, yet produce marked differences in toxicologic relationships between birds and rats; birds are much more sensitive than rats to the less toxic anticholinesterase. The differential sensitivity of birds and mammals to anticholinesterases is reviewed elsewhere.[37] This remarkably different response by birds and rats in response to chemicals of like action suggests that equal or greater differences should be expected for dissimilar pesticides and therefore reliance on rat data for prediction of hazard to birds is not adequate.

Interspecies Sensitivity

LD_{50}

Avian species vary widely in sensitivity to acute pesticide exposure.[25,26,33 38] Table 2 presents LD_{50}s for ten anticholinesterase pesticides tested at a single laboratory on an array of species that weigh between 25 g (house sparrow, *Passer domesticus*) and 1.2 kg (ring-necked pheasant). Anticholinesterases are again presented because chemicals of the same toxic mechanism should yield the most conservative results. In contrast to OP compounds (Table 1), all of which require metabolic activation for maximum potency, examples (Table 2) include compounds that are direct ChE inhibitors; i.e., monocrotophos, dicrotophos, and the three carbamates. Monocrotophos and

Table 1. Avian Sensitivity to Organophosphorus Pesticides of Widely Variable Toxicity In Mammals

	Rat[a]		Pheasant[b]		Blackbird[c]	
	Rank	**LD_{50}[d,e]**	**Rank**	**LD_{50}[d]**	**Rank**	**LD_{50}[d]**
Phorate	1	2	1	7	1	1
Disulfoton	2	7	2	12	2	3
Azinophos methyl	3	13	7	75	5	8
EPN	4	36	6	53	2	3
Ethion	5	65	10	1297	9	45
Phosmet	6	113	9	237	6	18
Dimethoate	7	215	3	20	4	7
Fenitrothion	8	740	4	26	7	25
Malathion	9	1375	5	167	10	>100
Temephos	10	8600	8	35	8	42

[a]Sherman strain male laboratory rats, 3 months old, n = 5-60 per test; dosage by gavage in peanut oil.[31,32]

[b]Farm-reared male and female ring-necked pheasants, 3 to 4 months old, n - 8-28 per teat; dosage by gelatin capsule.[25]

[c]Wild-captured pen conditioned male and female red-winged blackbirds, adult, n = 8-28 per test: dosage by gavage in propylene glycol.[28,33]

[d]LD_{50} = mg active ingredient (technical grade) per kg of body mass calculated to kill 50% of test population.

[e]All rat LD_{50}s are statistically separable ($p < 0.05$).

Table 2 Sensitivity of Seven Avian Species to Diverse Anticholinesterase Pesticides[a,b]

	House Sparrow		Red-winged blackbird		European Sterling		Rock Dove		Chukar		Mallard		Ring-necked pheasant	
Pesticide	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**	**Rank**	**LD_{50}**
Monochrotophos	1	1.6	1	1.0	2	3.3	3	2.8	2	6.5	4	4.8	1	2.8
Dicrotophos	2	3.0	2	1.8	1	2.7	1	2.4	3	10	3	4.2	3	3.2
Parathion	3	3.4	4	2.4	5	5.6	2	2.5	5	24	1	2.1	6	12
EPN	4	13	5	3.2	6	7.5	5	5.9	4	14	8	53	2	3.1
Propoxur	4	13	6	3.8	7	15	9	60	5	24	6	12	8	20
Chlorpyritos	6	21	8	13	3	5.0	7	27	9	61	9	76	5	8.4
Fenthion	7	23	3	1.8	4	5.3	4	4.8	7	26	5	5.9	7	18
Temephos	8	35	9	42	9	> 100	8	50	10	270	10	79	9	32
Landrin	9	46	7	10	9	> 100	10	168	8	60	7	22	10	52
Mexacarbate	10	50	7	10	8	32	6	6.5	1	5.2	2	3.0	4	4.5
Sensitivity rank[c]	3		1		6		3		7		5		2	

[a]Toxicity as LD_{50} = mg active ingredient (technical grade) per kg of body mass calculated to kill 50% of test population.

[b]Table reconstructed from Tucker and Haegele[38] with red-winged blackbird and European starling data from Schafer[33] and Schafer et al.[26] All studies were conducted at the Denver Wildlife Research Center (Denver, CO) by the same protocol. Mallards and gallinaceous species were farm-reared males and females, 2 to 4 months old; rock doves and passerine species were wild-captured pen-conditioned male and female adults. Eight to 28 birds were dosed per test either by gavage in propylene glycol (blackbirds and starlings) or by gelatin capsule.

[c]Sensitivity rank is based on the mean of acoss-species order of sensitivity to each pesticide.

dicrotophos, whose primary structural difference is a single methyl group, rank as the most or second most toxic compound to all species except mallard, and both yield the most consistent results across the seven species. The extreme LD_{50}s differ by factors of about 6 to 7x for dicrotophos and monocrotophos with a median difference of 15x across species for all ten compounds. In contrast, the carbamates give highly variable results across species and among compounds. Extreme carbamate LD_{50}s differ across species by about 16 to 17x.

The red-winged blackbird is either the most or second most sensitive species to seven to ten compounds, whereas the chukar (*Alectoris chukar*) is either the most or second most tolerant species of eight of ten compounds (Table 2). The other five species are from four taxonomic orders and each species is either most or least sensitive of the seven species to at least one compound. When the seven species are compared in all possible combinations, LD_{50}s of the ten compounds correlated well between species in 18 of 21 comparisons ($r = 0.74$, $p < 0.05$ to $r = 0.99$, $p < 0.01$). The three exceptions ($0.05 < p < 0.1$) are mallard compared with chukar ($r = 0.68$), ring-necked pheasant ($r = 0.58$), and European starling (*Sturnus vulgaris*, $r = 0.59$). These data suggest any of the test species, except possibly mallard, represent the acute sensitivity of birds to anticholinesterase pesticides, but the response of one species cannot be used to predict the sensitivity of another species to a specific pesticide. The same conclusions are also reported for pesticides with other toxic mechanisms.[38]

Neither body mass nor close taxonomic relation can be consistently used to predict the sensitivity of birds to pesticides. A list of species in ascending size reveals no apparent trend in sensitivity (Table 2). The largest (ring-necked pheasant) and smallest (house sparrow) are ranked second and third in across-species sensitivity, whereas the chukar, a Phasianidae, is ranked seventh. LD_{50} is lower for pheasants than for chukars for listed pesticides, but the difference varies from 1.2 (NS) to 8.4x ($p < 0.05$). It may be significant that the pesticides yielding the least difference between chukar and pheasants are the three carbamates and the two yielding the largest difference of 7.3 and 8.4x are the least toxic OP pesticides, chlorpyrifos and temephos.

LC_{50}

Species response to the subacute protocol has been thoroughly studied only for young of the precocial northern bobwhite, Japanese quail, ring-necked pheasant, and mallard.[18,19,21,30] The differences in LC_{50}s usually are not as large among the young as among adults of the same species." When the subacute tests are conducted on birds of about the same level of susceptibility to the 5-day trial (i.e., recommended ages for regulatory purposes[6]), the order of response most often negatively correlates with body mass: bobwhite = Japanese quail > ring-necked pheasant > mallard.[18] This is probably an interactive function of differential maturation of detoxicating processes and rate of feeding and subsequent exposure in relation to body mass. Even though all combinations of species order of response occurred during tests of more than 100 pesticides, a typical species order tends to prevail within each class of chemicals and LC_{50}s for any two of the test species strongly correlate.[18] Nonetheless, tests of multiple species are always desirable.

LD_{50} vs LC_{50}

Acute and subacute tests yield different toxicologic relationships.[7,37] The differences are exemplified by listing a series of diverse pesticides in ascending order of LD_{50} for young adult

mallards and comparing to LC_{50}s for 5-day-old ducklings (Table 3). All studies of each type were conducted at a single laboraory[18,25] with birds of the preferred age for regulation purposes.[5,6] The pesticides represent a near continuum of acute toxicities by overlapping confidence intervals for successive LD_{50}s that result in clusters of several consecutive inseparable LD_{50}s. When the subacute toxicities are compared for pesticides within a cluster of LD_{50}s (e.g., parathion through endrin), the LC_{50}s are almost always statistically separable. The disparity of response to the two tests is indicated by the arithmetic difference between LD_{50}s of little more than 2x for parathion and endrin, monocrotophos and methyl parathion, and endrin and methiocarb In contrast, the difference in subacute toxicities within each of these LD_{50} clusters is about 60x between LC_{50}s for monocrotophos and aldicarb, 130x for monocrotophos and DDVP (dichlorvos), and 70x for endrin and DDVP. Each of the clusters of four or five pesticides contains both latent and direct ChE inhibiting OP compounds, a carbamate, and a chlorinated hydrocarbon. When the pesticides are ranked by ascending LC_{50}, no more than two successive compounds have overlapping confidence intervals. Overall, no statistically significant correlation exists between the paired LD_{50}s and LC_{50}s.

Some Factors Affecting Interpretation of LD_{50} and LC_{50}

LD_{50}s and LC_{50}s change significantly during growth and development of precocial birds.[21,30,39,40] The direction and amount of change often differ widely between the two tests of lethality. In the acute test, change is believed to be primarily influenced by developing metabolic processes that affect both toxication and detoxication of xenobiotics and an immature immune system. The subacute test is influenced by these same processes and by the highly individualistic response of the experimental animal to the ad libitum toxic diet. Changes in sensitivity as reflected by the oral LD_{50} often follow different patterns depending on the basic toxic mechanism of the pesticide (Table 4). For example, mallard LD_{50}s for anticholinesterases that require activation for maximum potency (i.e., latent cholinesterase inhibitors) tend to decrease between hatch and 7 days and then increase with maturation to adulthood, whereas the opposite pattern occurs for direct acting OP and carbamate anticholinesterases. LD_{50}s for both CNS stimulating chlorinated hydrocarbons follow the pattern of the latent ChE inhibitors. Significant change in LD_{50} occurs between successive ages at least once for each of the pesticides, but little change is evident in the overall order of toxicity among the compounds at the different test ages.

In contrast to the dichotomy of change between successive LD_{50}s during early avian maturation, LC_{50}s typically increase in variable degrees with age during early growth of precocial species.[21,30] The increase occurs across chemical class and is assumed to be primarily due to a change in the ability to cope with the toxic diet for the duration of the subacute protocol; i.e., larger (= older) chicks that eat less proportional to body mass are better able to survive a 5-day trial by reducing food consumption and, therefore, toxic exposure. This is demonstrated by a series of subacute tests with Japanese quail from a single hatch.[30] Food consumption of controls in proportion to body mass averaged 48 g/100 g at 3 days of age, 31 g at 10 days, 24 g at 17 days, and 19 g at 24 days, which is a reduction of about 35, 23, and 21%/week from hatch to 3 weeks of age. During this period, the average increase in LC_{50} for nine pesticides (three organophosphorus and two each of carbamate, chlorinated hydrocarbon, and methyl mercury) is 36% between l and 7 days, 43% between 7 and 14 days, and 28% between 14 and 21 days. In an acute study with mallards,[39] eight pesticides are compared and the LD_{50}s increase between 1 and

7 days for two compounds by an average of 70% decrease for three compounds by an average of 80% and are unchanged for three compounds (Table 4).

Table 3. Comparative Toxicity of Diverse Pesticides to Mallards Tested Acutely and Subacutely

		Acute[a]			Subacute[b]		
Pesticide	**Class[c]**	**Rank**	**LD_{50}**	**(95% CI[d])**	**Rank**	**LD_{50}**	**(95% Cl)**
Fensulfothion	OP-L	1	0.7	(0.6-0.9)	3	41	(32-55)
Parathion	OP-	2	2.4	(1 7-4.0)	5	76	(61-93)
Aldicarb	CB	3	3.4	(2 7 4.3)	10	594	(507-695)
Monocrotophos	OP D	4	4.8	(3.4-6.6)	1	10	(8-12)
Endrin	CH	5	5.6	(2.7-11.7)	2	18	(15-21)
DDVP	OP-D	6	7.8	(6.0-10.1)	12	1317	(1043-1674)
Methyl parathion	OP-L	7	10	(61-16.3)	8	336	(269 413)
Ethoprop	OP-D	8	13	(11-15)	7	287	(215-382)
Methiocarb	CB	8	13	(7-22)	11	1071	(808-1405)
Morsodren	Hg	10	53	(32-89)	4	51	(43-60)
Toxaphene	CH	11	71	(38-133)	9	538	(474 614)
Dieldrin	CH	12	381	(141-1030)	6	153	(123-196)

[a]Single-dose oral toxicity: LD_{50} as mg active Ingredient (technical grade) per kg of body mass calculated to kill 50% of test population. Farm-reared male and female, 3 to 7 months old, n = 8-28 per test; dosage by gelatin capsule.[25]
[b]Five-day dietary toxicity: LC_{50} as mg active ingredient (technical grade) per kg of feed in ad libitum diet calculated to kill 50% of test population. Five groups of 10 unsexed ducklings (5 days old) were tested per pesticide.[18]
[c]Pesticide class: CB, carbamate: CH, chlorinated hydrocarbon; Hg, organic mercury; OP-D, organophoaphorus-direct cholinesterase inhibitor; OP-L, organophosphorus-latent cholinesterase inhibitor.
[d]CI = confidence interval.

LC_{50}s must be used cautiously in comparison of pesticide toxicity among species because the species may not be equally challenged by the test protocol. However, as discussed previously, a reproducible LC_{50} can probably be obtained for any species that cannot survive for 5 days without eating.[27,28] When a portion of the population can survive severe food reductions for the duration of the test, responses tend to be erratic and produce an expanded 95% confidence interval for LC_{50} and a shallow lethality curve that may be a product of factors other than sensitivity. These relationships are demonstrated by subacute tests conducted at a single laboratory with 5- and 10-day-old mallards.[18,41] (*Note:* About 50% of 10-day-old mallards can fast for 5 days, whereas 5-day-old ducklings cannot.[21]) Comparable data sets for nine pesticides indicate variable degrees of increase between LC_{50}s at 5 and 10 days of age (Table 5). LC_{50}s for five of six anticholinesterases increase by an average of 180% while the sixth, fensulfothion, the two chlorinated hydrocarbons, and the methyl mercury are essentially unchanged. Overall, the proportional size of the 95% confidence interval (division of upper by lower bound) averages about 20% smaller and the slope of the lethality curve about 25% steeper for 5-day-old than 10-day-old ducklings. Methiocarb, the only carbamate, has the largest difference in LC_{50}s between

ages, extremely wide confidence intervals at both ages, and the steepest lethality curve at 10 days. Carbamates typically yield the most erratic response by birds to both acute (controlled dosage) and subacute (uncontrolled dosage) toxicity tests.[19,25,30,41]

Table 4. Acute Oral Toxicity of Anticholinesterase and CNS Stimulating Pesticides to Mallards from Hatch through Adulthood[39]

	LD_{50}[a](95% CI)			
Pesticide	**1.5 days**	**1 week**	**1month**	**6months**
Carbofuran[b]	0.4	0.6	0.6	0.4
	(0.3-0.5)	(0.5-0.7)	(0.4-0.6)	(0.3-0.5)
Aldicarb[b]	1.9	3.6	6.7	4.4
	(1.6-2.4	(2.9-4,5)	(5.3-8.6)	(3.5-5.6)
Monocrotophos[c]	5.9	7.2	5.1	3.4
	(4.7-7.3)	(5.8-9.0)	(4.4-5.9)	(2.8-4.1)
Demeton[c]	13	15	15	8.2
	(11 - 16)	(13-18)	(12-19)	(6.6-10.2)
Parathion[d]	1.6	1.4	1.6	2.3
	(1.4-2.0)	(1.1-1.8)	(1.4-2.0)	(2.0-2.8)
Chlorpyrifos[d]	145	29	50	83
	(56-377)	(19-47)	(32-78)	(44-158)
Endrin[e]	22	3.4	2.9	5.3
	(10-50)	(2.4-4.8)	(2.2-3 9)	(3.7-7 7)
Endosulfan[e]	28	6.5	7.9	34
	(23-34)	(5.2-8.1)	(5.8-10.8)	(26-45)

[a]Toxicity as LD_{50} = mg active ingredient (technical grade) per kg of body mass calculated to kill 50% of test population.
[b]Carbamate (direct ChE inhibitor).
[c]Organophosphorus (direct ChE inhibitor).
[d]Organophosphorus (latent ChE inhibitor).
[e]Chlorinated hydrocarbon (CNS simulator).

Table 5. Subacute Dietary Toxicity[a] of Widely Diverse Pesticides to 5- and 10-Day Old Mallards[18]

	5-day Old			10-day-old		
Pesticide	**LC_{50}**	**(95% Cl)**	**Slope[b]**	**LC_{50}**	**(95% Cl)**	**Slope[b]**
Monocrotophos[c]	10	(8-12)	5.4	32*	(19-57)	1.7
Endrin[d]	18	(15-21)	5.7	22	(17-31)	3.4
Fensulfothion[e]	41	(32-55)	5.1	43	(36-51)	4.4
Morsodren[f]	51	(43-60)	8.2	60	(47-76)	7.5
Parathion[e]	76	(61-93)	4.4	275*	(183-373)	9 7
Dicrotophos[c]	94	(80-111)	3.9	144*	(110-185)	3.3
Dieldrin[d]	153	(123-196)	5.4	169	(131-217)	4.9
Methyl parathion[c]	336	(269-413)	5.3	682*	(541-892)	3.2
Methiocarb[g]	1071	(808-1405)	2.5	4113*	(2817-7504)	5.1

[a]Five-day dietary toxicity: LC_{50} as mg active ingredient (technical grade) per kg of feed in ad libitum diet calculated to kill 50% of test population. Asterisk indicates paired LC_{50}s are statistically separable ($p < 0.05$).
[b]Slope probit on log concentration.
[c]Organophosphorus (direct cholinesterase inhibitor).
[d]Chlorinated hydrocarbon (CNS stimulator).
[e]Organophosphorus (latent cholinesterase inhibitor).).
[f]Organic mercury.
[g]Carbamate (direct cholinesterase Inhibitor).

Sex, reproductive condition, genetic lineage, nutritional status, and exogenous and endogenous stress may have variable effects on LD_{50} and LC_{50} determinations, but the importance of the factors is not well established for birds. Historically, most acute avian studies tested nonbreeding subadult game birds or adult passerines of both sexes.[25,26,33] This was done to reduce sex effect and thereby conserve the number of birds required for testing species sensitivity and ranking the acute toxicity of pesticides. The legitimacy of pooling sexes of reproductively quiescent birds has been validated for acute toxicity testing.[27,33,38,42] However, beyond general comparisons, this narrow focus may not be adequate for hazard assessment because pesticides are intensively applied in nature during avian breeding seasons and knowledge of sex differences in sensitivity is essential. The importance of this variable is indicated by an acute test of fenthion toxicity that showed female northern bobwhite to be 2.3 times ($p < 0.05$) as sensitive as males.[43]

Research on birds usually is with captive-reared specimens from haphazardly outbred stocks or wild-captured birds of unknown origin. Reproducibility of acute toxicity tests with birds of such vague genetic lineage is not known. However, in a study with equal-aged farm-reared northern bobwhites of both sexes from eight commercial breeders, extreme LD_{50}s for technical grade diazinon were 13 and 17 mg/kg body mass.[44] These two extremes are statistically inseparable, although the eight stocks differed in apparent vigor and body mass at dosing. Both factors are known to affect acute response,[45] but genetic variability from outbreeding could obscure detection of minor differences based on LD_{50} alone.

Adequate methods are not available to evaluate the suitability of a wild-captured individual or species for acute toxicity testing. Simple survival and weight maintenance for a few weeks in captivity may not reflect subtleties such as nutritional imbalance or stress response to confinement, isolation, or crowding. Whether captive specimens, either wild or farm hatched and reared, truly represent their free-living counterparts is not known. For example, DDT and several organophosphorus insecticides were tested subacutely on wild bluejays (*Cyanocitta cristata*), house sparrows, northern cardinals (*Cardinalis cardinalis*), and wild and farm northern bobwhites.[27] All birds were at their capture weight and believed to be adequately conditioned to captivity at the time of testing. Bluejays were the most sensitive species to all compounds and farm bobwhites the most tolerant. Bluejays are adaptable generalized feeders that are reputed to be quite resilient in contaminated environments[46] and are easily kept in captivity, yet based on LC_{50}s they are about 1.5 to 50 times as sensitive as the other species to the various insecticides. Wild bobwhites had much less subcutaneous and visceral fat than their farm counterparts, weighed about 25% less, and consistently gave lower LC_{50}s. The difference is attributed in large part to consumption of significantly more toxic feed proportional to body mass by the wild birds during the 5-day trial rather than to differential sensitivity. Neither body mass nor rate of feeding explains the unexpected bluejay sensitivity because they are nearly twice as heavy and eat proportionally less than either house sparrows or cardinals.

HAZARD EVALUATION

It is clear from the foregoing that the most often used criteria of toxicity, the single-dose acute oral LD_{50}, varies unpredictably among avian species, and responses by laboratory rats to acute tests do not adequately represent avian response. When feeding for 5 days is substituted for controlled dosage, the resultant subacute LC_{50} often produces relationships among species and chemicals that are quite different from those for LD_{50}s Acute and subacute tests provide complementary measures of relative potency for the identification of chemical substances of potential lethal toxicity to wildlife. Although neither the LD_{50} nor LC_{50} per se is more than a convenient statistical reference point, evaluation of associated dose-response curves and observations of toxic responses enhance the utility of acute-type lethality tests in hazard assessment. These tests are meager considering that avian habitat is routinely treated with a variety of formulations and combinations of pesticides and that many factors alter the chemical fate and availability of a pesticide. However, ingestion is believed to be the most common route of pesticidal exposure in birds,[46] and therefore these oral tests of lethality provide a sound basis for preliminary screening.

LD_{50} and LC_{50} provide a statistical measurement that can be used to classify pesticides by an established scale of toxicity.[5,6,18,36] This criterion provides simplistic guidance in first-line reviews of any array of pesticides for lethal hazard. Caution must be exercised to ensure that comparisons are based on test subjects that are equally susceptible to the experimental protocol (e.g., special attention to age, body mass, and feeding habits) and that the median response level is supported by its 95% confidence interval. LD_{50} is derived by controlled dosage and therefore provides a tangible measure of naive sensitivity to toxic challenge that can be used for direct comparison of species, life stages, and chemicals. Although the emphasis herein is on oral dosage, the basic acute test can also be used to evaluate percutaneous toxicity. In comparative

studies with mallards and several passerines, oral LD_{50}s were consistently lower ($p < 0.05$) than percutaneous LD_{50}s for an array of pesticides.[47,48] An LD_{50} is difficult to relate to a field application of pesticide because some combination of inhalation, percutaneous, and ingestive exposure is probably the rule.

LC_{50} provides a basis for comparison of the ability of the test population to cope with chemically contaminated feed for 5 days. This subacute test is believed by some to be more practical than its acute predecessor because the birds must voluntarily ingest the pesticide and are then subject to the effects of repeated dosage as might be experienced in nature. However, subacute studies usually use technical grade pesticide mixed into dry feed, whereas natural ingestion of the finished product formulation may be from varied sources such as water, seeds, foliage, invertebrates, vertebrates, and granular pesticides,[46] and the toxicity of the pesticide may be different in each matrix because of its form or availability. In a realistic sense, except for some carbamates, a field residue equivalent to an LC_{50} in a specific food matrix may not be especially hazardous to a mobile population if the birds choose to emigrate. Emigration is more likely due to food deprivation (i.e., reduced arthropod population) than toxicity.[49-51]

Some insight into potential hazard associated with a specific level of 5-day subacute toxicity is provided by comparison of cumulative mortality patterns during exposure to LC_{50} concentration of carbamate, OP, chlorinated hydrocarbon, and organic mercury (Figure 1). The response curves are based on studies of 14-day-old Japanese quail and are typical for most compounds in the represented pesticidal classes.[19,30] (Comparable mortality patterns occur for 5-day-old mallards and 10-day-old ring-necked pheasants.[55]) LC_{50} is presented because it is the focus of the experimental design, and therefore responses are least variable, but lower or higher response levels produce the same characteristic pattern, with the sigmoid response beginning about I day later at lower levels and I day earlier at higher levels.

The mortality pattern for dicrotophos is consistent with the cumulative response theoretically necessary to kill a portion of the test population during 5-day exposure to a nonaccumulative toxicant. Mortality from OP compounds is rare after withdrawal of

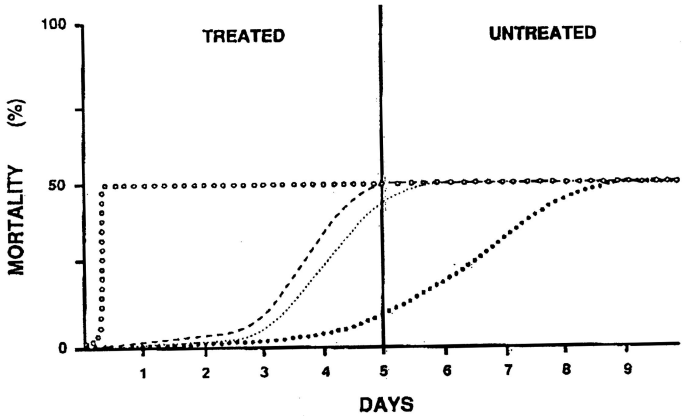

FIGURE 1. Cumulative mortality patterns for 14-day-old Japanese quail fed LC_{50} concentration of carbofuran (open circle), dicrotophos (dash), dieldrin (dot), and Ceresan M® (closed circle) for 5 days followed by untreated feed.

treated feed.[19] A typical response to OP exposure occurred with dicrotophos. Consumption decreased by 30% compared with controls during the first-day of exposure, by 55% during the second and third days, and by 60 during the fourth and fifth days.[55] Feeding at lower and higher response levels is described in detail elsewhere for many species.[19,27,28,30,41] Dieldrin produced essentially the same cumulative response pattern as dicrotophos but some mortality occurred during the first day on untreated feed. Although dieldrin is lipophilic and accumulative, latent mortality is not common, provided ad libitum untreated feed is available.[19,30] Consumption of dieldrin-treated feed decreased compared with controls by about 15, 30, 40, 45, and 45% during the first through fifth days.[55] Quail fed Ceresan M® showed little evidence of toxicity preceding the first death on the last day of exposure, then toxic signs began to intensify and deaths ensued through the fourth day of untreated feed; all toxic signs remised in survivors by day 13.[30] Consumption of Ceresan M®-treated feed was consistently about 5 to 15% less than control consumption, but daily differences were not significant. A detailed account of subacute response to mercury is presented elsewhere.[40] In contrast to each of the above patterns, all deaths from carbofuran occurred during the first few hours of feed presentation. After an initial decrease of about 60% feed consumption was reduced by only 25% on the second day and comparable to or in excess of controls thereafter.[55] This temporal pattern also occurs at higher and lower response levels and is generally representative of other carbamates.[19] The OP fensulfothion produced a carbamate-type response pattern with mallards,[17] but a typical OP pattern with Japanese quail.[30]

When the subacute response patterns depicted in Figure 1 are considered with their corresponding rates of consumed toxic feed, many different exposure scenarios can be developed to enhance the evaluation of the potential hazard. For example, potential effects on migrants can be compared to resident populations, and mobile residents to breeders, and so on. Certainly, from these patterns it would not have been difficult to predict that carbofuran poses an acute hazard to birds, which it does;[52,53] or that Ceresan M® is much more hazardous than indicated by its single-dose LD_{50} of 668 mg/ kg (95% confidence interval, 530 to 842 mg/kg) for adult Japanese quail.[25] Nonetheless, caution must be used when projecting results of subacute studies to the field because in the laboratory, reasonably consistent exposure can be provided over time, whereas field exposure is erratic because pesticide is naturally degraded and translocated. Care must also

be used in the interpretation of experimental feed consumption because subacute trials usually test technical grade chemical mixed into dry mash. Pesticide presented in this way may be easily sensed and consumption reduced; in the field, finished product formulation may be less easily detected when present in natural matrices including plant and animal tissues. Thus, different factors may render a pesticide either more or less toxic in the field than predicted from laboratory studies.

The dose-response or lethality curve calculated from acute and subacute toxicity tests is critical to the evaluation of potential pesticide hazard to wildlife. The curve is used in the same general way for both tests, but their interpretive implications are somewhat different because of the method of exposure. The most important concept applicable to both tests is that a steep lethality curve indicates increased hazard if for no reason other than proportionally less chemical increases effect; thus, applicator precision is essential. However, chemicals that produce shallow curves may be even more hazardous if the slope is not known. These somewhat contradictory notions are explained by comparison of hypothetical pesticides A and B with slopes (probit on log dose) of 8.0 and 2.0 and both with an arbitrary LD_{50} of 10 mg/kg (Figure 2). Assume the slope is known for pesticide A and the expected exposure is 6 mg/kg which may kill about 5% of the population; if treatment is accidentally doubted and results in exposure of 12 mg/kg it would kill about 75% of the population, a 15-fold increase. In contrast, assume the slope is not known for pesticide B. but its LD_{50} of 10 mg/kg is the same as for pesticide A, and this time the target exposure of 6 mg/kg is met. The shallow slope indicates that about 35% of the population would be killed. Pesticides such as carbofuran tend to yield shallow slopes[30,42] and have been implicated in numerous avian die-offs.[54]

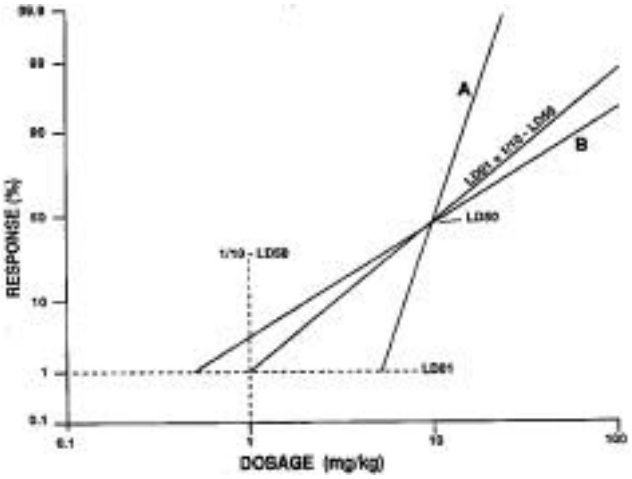

FIGURE 2. Dose-response curves of hypothetical pesticides A (slope 8.0) and B (slope 2.0) and a line (slope 2.3) intercepting the coordinates of the LD_{01} and 1/10 LD_{50}.

For regulatory purposes, a popular method is to use some fraction of the LD_{50} or LC_{50} to denote hazard and restrict use of treatments that probably yield an exposure potential to wildlife. Suppose the acceptable residue in the equivalent of one feeding bout is set at 1/10 of the LD_{50}, or 1 mg/kg. In this example, pesticide A would appear safe and pesticide B lethal to about 5% of the exposed population (Figure 2). In Figure 2 the 1/10 LD_{50} is arbitrarily intercepted with the calculated LD_{01} for reference. The resultant slope is about 2.5, which is much more shallow than

that calculated for most pesticides tested either acutely or subacutely with birds.[18,19,42] Therefore, the 1/10 LD_{50} or LC_{50} criterion appears to be a reasonably conservative parameter for most purposes when the slope of the dose-reponse curve is not known.[42] Even when the dose-response curve is known, use of coordinates outside the linear limits (i.e., $\pm$ 1 S.D. of the midpoint of the curve or the 16 and 84% response level) is discouraged.[1,17]

In a practical sense, the steepness of the dose-response curve can be reduced to a qualitative index based on the ratio between two constant response levels; e.g., LD_{10} and LD_{50}. The smaller the ratio, the more hazardous the substance because proportionally smaller amounts increase effect and thereby reduce the acceptable margin of error in a pesticidal application. In contrast, shallow slopes indicate greater inherent safety because it takes proportionally more chemical to increase effect; however, low levels may cause unacceptable effects.

CONCLUSIONS

Single-dose acute oral and 5-day subacute dietary toxicity studies are the preponderance of available data for preliminary assessment of pesticidal hazard to wildlife. Properly designed, these tests provide a method of comparing pesticides by lethality from one, (acute) or multiple (subacute) exposures that generate statistical estimates of the dose-response curve and its midpoint, LD_{50} or LC_{50}. When these tests are supplemented with detailed observations of individual responses and food consumption through remission of toxicity, a meaningful appraisal of potential lethal hazard is possible.

Historically, only LD_{50} or LC_{50} has received extensive use, and often without consideration of its statistical validity. This approach is inappropriate because both LD_{50}s and LC_{50}s vary widely in unpredictable ways between chemicals, species, and the life stage of the test subjects. Therefore, careful review of test compatibility is essential before any comparisons are attempted. However, once the credibility of the study is ascertained, LD_{50} and LC_{50} provide useful guides to chemical potency for comparing pesticides of different mechanisms of toxic action. Specifically, LD_{50} provides a direct measure of sensitivity, whereas LC_{50} yields information on sensitivity to the chemical and the ability of birds to cope with toxic feed for a specified duration. A review of the responses indicated from mortality patterns and slopes of dose-response curves gives insight into potential hazards of both an acute and chronic nature.

However, literal projection of either acute or subacute tests to nature is not possible. Most laboratory tests use a technical grade chemical, either administered directly to the bird or in a dry feed. Field application almost always uses a finished product formulation of pesticide, and formulations may vary in toxicity and availability depending on the use and factors of environmental degradation. Therefore, extreme care is recommended in the use of acute and subacute toxicity tests; when used in combination and judiciously, the two tests of lethality are invaluable tools for preliminary evaluation of potential hazard of pesticides to wild birds.

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