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Significance of Regurgitation in Avian Toxicity Tests

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Previous studies have commented on apparently wide variations in the toxicity of organophosphorus pesticides to starlings (Rattner and Grue 1990, Hart and Westlake 1986). The LD50 of chlorfenvinphos to the starling has been reported as ranging from 3.2 to 23.7 mg/kg (Schafer *et al.* 1983), and in our studies individual starlings have survived substantially higher doses than these (unpublished data). Observations of starlings dosed with chlorfenvinphos (Hart 1993, Fryday *et al.* in prep.) have suggested that the ability of these birds to regurgitate may be an important factor in determining the results of acute oral toxicity tests. A wide range of avian species, particularly insectivorous and omnivorous species, are reported to be capable of regurgitation to some extent, although this ability may be limited by the musculature of the gizzard (Tucker 1944, Prys-Jones *et al.* 1973). Vomiting has been reported as a toxic symptom for a number of pesticide active ingredients (Hudson *et al.* 1984). The effects of even partial regurgitation on pesticide toxicity evaluation has potentially significant implications for risk assessment procedures.

Volumes between 0.1 and 5 µl carrier/g body weight, as well as gelatin capsules, with and without pre-dose fasting, have been reported as routine dosing methods for pesticide toxicity evaluation (ASTM 1981, Fairbrother *et al.* 1988, Mineau *et al.* 1990, Rattner and Grue 1990). This variation in oral dosing procedures may contribute to the variability in toxicity estimates reported for pesticides. For example, it is conceivable that regurgitation is less likely if the volume of the dose solvent is low or if the bird has been fasted. This paper describes an experiment designed to measure the extent of regurgitation and test these hypotheses. The implications of the results for risk assessment procedures are discussed.

MATERIALS AND METHODS

Starlings (*Sturnus vulgaris*) were wild-caught in Lincolnshire, UK, and held in captivity for at least six months prior to use. Chlorfenvinphos (Birlane) was a gift from Shell UK, Sittingbourne, Kent. All other chemicals were obtained from Sigma Chemical Company, Poole, Dorset.

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Twelve female birds were weighed, assigned to groups of 3 by matching for weight and transferred to individual cages indoors at 1700h on day 1. The day-night light cycle was set to simulate the current natural timing on day 1 (19 March). The birds were allowed to acclimatise for 40h prior to dosing.

Random number tables were used to assign the 3 birds in each group to 3 separate treatments, giving a total of 4 birds per treatment. Birds in treatments 1 and 2 were allowed food *ad libitum* prior to dosing (unfasted), while birds in treatment 3 were fasted overnight from 1700h on the day before dosing (day 2). On day 3, one of the 4 groups was dosed each hour, starting at 0900h, with chlorfenvinphos at 35.5mg/kg body weight, based on body weights recorded immediately before dosing. This level of dosing had been shown in previous trials to be near-lethal. Dosing was staggered to reduce disturbance and to allow one bird in each group to be video-recorded. Chlorfenvinphos was dissolved in corn oil at two concentrations: 17.75 mg/ml and 355 mg/ml. This enabled us to administer the dose in 2.0µl corn oil /g body weight to birds in treatments 1 and 3 (high volume), and in 0.1µl corn oil /g body weight to birds in treatment 2 (low volume). The doses were administered using a positive displacement pipette (Anachem, Luton, UK), through the mouth into the proventriculus. Immediately before dosing a sheet of clean absorbant paper was placed in each cage, covering the floor and extending about 5 cm up each wall. All birds were allowed access to water *ad libitum* but denied access to food from the time of dosing.

One hour after dosing the birds were removed, anaesthetised with carbon dioxide and killed by decapitation. Carcases were dissected to check stomach contents, and the brains were removed immediately and stored at -20°C for acetylcholinesterase assay the following day. Whole brain samples were assayed by the method of Hart and Westlake (1986).

The paper from the cage floor was removed and areas of regurgitated oil (translucent patches easily distinguished from faecal material) were cut out and placed in clean glass jars at -20°C. The samples of paper were extracted in hexane and extracts were analysed using a gas chromatograph (Analytical Instruments, Cambridge, UK) equipped with DB225 column (15m x 0.53mm i.d.) and phosphorus/ sulphur selective flame photometric detector.

Video tapes of the monitored birds were examined and the times of drinking activity and attempted regurgitation were recorded.

RESULTS AND DISCUSSION

The results are summarised in Table 1. Data on the amount of food taken overnight (between 1700 on day 2 and the time of dosing on day 3) show increases by birds in treatments 1 and 2, as would be expected, from 1st group to the 4th group, although the treatment 2 bird in the 4th group treated took only 0.68g.

Table 1. Summary of results, ± standard errors. Each treatment group comprised four birds. All birds received chlorfenvinphos at 35.5 mg/kg bodyweight (weight recorded immediately before dosing). Treatment volume refers to volume of dose solution administered: low = 0.1 µl / g body weight, high = 2.0 µl / g body weight. Amounts of chlorfenvinphos regurgitated have been corrected to allow for differences in recovery rate from cage floor papers (see text).

Treatment	Food taken overnight (g)	Chlorfenvinphos regurgitated (mg)	Brain AChE (% of controls)
1. Unfasted, high volume	11.1 ± 2.0	1.64 ± 0.15	37.3 ± 6.5
2. Unfasted, low volume	6.8 ± 2.2	1.50 ± 0.17	35.0 ± 4.7
3. Fasted, high volume	0.0 ± 0.0	1.55 ± 0.11	55.0 ± 4.0

Analysis of samples of paper treated directly with the two solutions of chlorfenvinphos showed good recovery (102 %) for the solution used in treatments 1 and 3, but poorer recovery (77 %) for the more concentrated solution which was used in treatment 2. This difference is thought to have been due to greater losses from the filter paper, through volatilization, of the pesticide in the more concentrated solution. The measured recovery rates were therefore used to correct our measurements of chlorfenvinphos in the papers from the cage floors, to provide estimates of the amounts actually regurgitated (Table 1).

The estimated amount of chlorfenvinphos regurgitated ranged from 1.1 to 1.8 mg. This represented 37 - 72% of the administered dose (mean 59%). Analysis of variance showed no significant difference between treatments in the amount regurgitated (Table 1, $F_{2,9} = 0.21$, $p > 0.05$). Thus, contrary to expectation, neither dose volume nor prior fasting appeared to affect the extent of regurgitation. There was a significant difference between groups ($F_{3,6} = 12.3$, $p < 0.01$), due to reduced regurgitation in group 2 (dosed at 1000h) compared to the other groups. The reason for this is unknown. It is unlikely to have arisen from the differences between groups in food intake since the two were uncorrelated.

Brain AChE activity differed significantly between treatments, showing greater inhibition for treatments 1 and 2 than treatment 3 (Table 1, $F_{2,9} = 4.52$, $p < 0.05$). Thus overnight fasting resulted in decreased inhibition of brain AChE when measured 1 hour after dosing. Possible explanations for this result are that gut blood flow is reduced in fasted birds, resulting in slower uptake of the pesticide and delaying the time of maximal inhibition, or reduced uptake due to increased rate of passage through the gut, as no food absorption of the oil can take place. A third possible explanation is that the doses administered were significantly smaller for treatment 3 (mean 30.6 mg/kg) than treatments 1 and 2 (mean 32.6 mg/kg), when considered relative to normal free-feeding bodyweight (as recorded at 1700h on day 1). This arose because the fasted birds lost significantly more weight

between day 1 and day 3 (mean loss 14%) than was lost by unfasted birds (mean 8%). However, although the correlation between AChE activity and dose relative to free-feeding weight was negative, as expected, it was not significant ($r = -0.48$, 10 d.f., $p > 0.10$), suggesting that this was not the main cause of the difference in AChE between the treatments. There was no significant difference in brain AChE between the 4 groups dosed in successive hours ($F_{3,6} = 0.2$, $p > 0.05$), and it appeared that the effect of fasting on brain AChE was not increased by the more prolonged fasting of the treatment 3 birds in the later groups.

There was no correlation between the estimated amount of chlorfenvinphos regurgitated and brain AChE ($r = 0.04$, $p > 0.05$). This may have been due to the timing of the measurement of brain AChE activity. It might be expected that the maximal levels of inhibition would be inversely related to amount regurgitated, but occur later in birds which were fasted. This study did not investigate the time-course of inhibition because it would have required the use of many more birds, killed at different intervals after dosing.

The four birds for which behaviour was recorded on videotape engaged in 14, 3, 7 and 4 bouts of retching movements during the periods 5-19, 9-15, 6-12 and 14-20 minutes after dosing, respectively. For each bird, the period of apparent attempts to regurgitate was followed by a short period of unusually frequent drinking activity. The duration of individual bouts of retching movements ranged from under 1 second to 7 seconds. The timing observed in this study is consistent with an earlier experiment, in which starlings began regurgitating 7 - 15 minutes after dosing with 6 - 15 mg/kg chlorfenvinphos administered in gelatin capsules (Hart, 1993). Zach and Falls (1976) reported latency of regurgitation of 2-3 minutes after administration of tartar emetic to captive ovenbirds (*Seiurus aurocapillus*). Two of the twelve birds in the present study showed obvious signs of intoxication (ataxia, prostration) when removed for AChE assay 1 hour after dosing. It is likely that, had the experiment been allowed to continue, some of the birds would have died. In a separate experiment, conducted in an outdoor aviary, 3 of 9 starlings died when dosed with 35.5 mg/kg chlorfenvinphos in a dose volume of 0.1ul/g and without prior fasting (Fryday *et al.*, in prep.).

It is appropriate to review existing procedures for acute oral toxicity testing in the light of these findings. The EPA testing protocol for avian oral LD50 (EPA 1985) requires that any signs of regurgitation are recorded, although neither the method of observation nor frequency are stated. If regurgitation is observed then it is usual to repeat the test with a different species. UK regulations (MAFF 1986) require observations to be made hourly on the day of dosing, but do not specify regurgitation as a critical parameter. It is apparent from the limited data which exist on the timing and duration of regurgitation, in this and other studies, that observations should be made continuously for the first 30-60 minutes after dosing in order to ensure that any regurgitation is recorded. It is possible that birds may be inhibited from regurgitating if the observer is not concealed.

EPA regulations (EPA 1985) require all birds to be fasted for 15 hours prior to acute oral toxicity testing and UK regulations (MAFF 1986) recommend overnight fasting. The data from this study suggests that fasting has little effect on regurgitation of the administered dose. However, the results are consistent with the possibility that fasting reduces gut blood flow and hence delays pesticide uptake and the onset of symptoms. In this respect the test procedure is unrealistic because, except in the early morning, wild birds exposed to pesticides will not normally be in a fasted state. However, this disadvantage of fasting might be outweighed by the variability which would occur in tests without fasting, due to variation in the amount and type of food in the gut at the time of dosing.

EPA regulations (EPA 1985) appear to favour the use of gelatin capsules in acute oral tests for all pesticides, to eliminate the requirement for a vehicle. However, gelatin capsules are limited in their applicability, and they are susceptible to regurgitation (Hart 1993). When vehicles are required the recommended maximum volume is 0.1-1.0% body weight. UK regulations (MAFF 1986) do not express a preference for gelatin capsule or vehicle administration of pesticides nor do they specify vehicle volumes. This study showed that there was no significant difference in the amount of pesticide regurgitated when vehicle volumes were 0.1 µl/g or 2 µl/g, i.e. 0.01-0.2% body weight. Use of higher volumes of vehicle has been observed to increase the level of regurgitation when compounds with emetic properties are administered (Fairbrother pers. comm.). Therefore the vehicle volume should be reduced in order to limit the risk of regurgitation to the emetic properties of the pesticide itself, but not so far as to cause significant volatilisation, as apparently occurred with the lower vehicle volume in this study. If larger volumes are required and the compound has no emetic properties, then capsules may be preferred. In the absence of pesticide, higher volumes of carrier could be administered without regurgitation in a capsule than by gavage (Fairbrother, pers. comm.).

For different reasons a variety of birds regurgitate It is well known that predatory birds such as hawks and owls regurgitate indigestible portions of their food in the form of pellets, and scavenging birds such as the raven (*Corvus corax*) and carrion crow (*Corvus corone*) produce pellets similar to those of birds of prey (Tucker 1944). Many insectivorous species regurgitate the chitinous hard parts of their prey, e.g. blackbird (*Turdus merula*), robin (*Erithacus rubecula*) and swift (*Apus apus*) (Tucker 1944), and some species, e.g. seabirds and blue herons (*Ardea herodius*) regurgitate to feed their young (Ryan 1988, Pierce 1982). Regurgitation also occurs in response to exposure to naturally occurring chemicals, e.g. blue jays (*Cyancitta cristata*) vomit in response to ingestion of monarch butterflies (*Danus plexippers*) (Brower 1969).

Prys-Jones *et al.* (1973) reported the use of emetics in obtaining food samples from passerine species. The results suggested that more highly insectivorous species are more likely to regurgitate, and the more muscular the gizzard is the more it acts as a barrier to regurgitation. Granivorous birds appear to be less able

to regurgitate than insectivorous species as demonstrated by the differing responses of ovenbirds (*Seiurus aurocapillus*) and house sparrows (*Passer domesticus*) to emetics (Zach and Falls 1976, Prys-Jones *et al.* 1973). There have also been reports of regurgitation induced by emetics in Cassins finch (*Carpodacus cassinii*), white crowned sparrow (*Zonotrichia leucophrys*), fox sparrow (*Passerella iliaca*) and dark eyed junco (*Junco hyemalis*), all of which were less sensitive to the effects of emetics than Stellar's jays (*Cyanocitta stelleri*) (Tomack 1975). Other species reported to regurgitate include rooks (*Corvus frugilegus*) and starlings (Kadochnikov 1967); pigeons (*Columba livia*) (Dorrestein *et al.* 1983); bobwhite quail (*Colinus virginianus*) (Fairbrother pers. comm.); and a range of seabirds (Montague and Cullen 1988, Nisbet 1983). Data reported by Prys-Jones *et al.* (1973) suggest that quelea (*Quelea quelea*) do not respond to emetics. Vomiting has been reported in birds dosed with a wide range of pesticides including organophosphorus and carbamate insecticides, pyrethroids and herbicides (Hudson *et al.*. 1984). Gulls (*Larus* spp.) have been observed regurgitating in the wild, after eating leatherjackets (*Tipula* spp. larvae) on a Scottish airfield treated with chlorpyrifos (N Horton, personal communication). In the present study 37-72% of the dose was regurgitated. In summary, the available information shows that a wide range of avian species are capable of regurgitation, to varying extents.

It is the variability of regurgitation which causes the most serious problem for avian risk assessment. If the extent of regurgitation were the same for all species and chemicals, the result of a standard acute oral toxicity test could be used to provide reliable estimates of the risks to wild birds. A correction factor would have to be applied to allow for the extent of regurgitation, but at least this would be uniform. However, the extent of regurgitation varies between individuals, between species, and between chemicals (some being specifically recognised as emetics). Furthermore, it is likely that the occurrence of regurgitation in oral dosing studies is different from that in the wild, especially as the manner of exposure to pesticides in the wild will usually be very different. Thus even if the extent of regurgitation were measured as a routine part of acute toxicity tests, there would be no reliable way of using this to extrapolate to wild species.

For most types of pesticide use, avian risks are usually assessed primarily for dietary exposures, through feeding on contaminated vegetation, on vertebrate or invertebrate prey, or on the pesticide formulation itself where this has an edible base. Only for a few pesticide uses is a significant dose contained within a single bolus of food, such as would be comparable to dosing by gavage. For most pesticides, interest centers on whether a bird feeding mainly on the contaminated diet will receive a lethal dose within one day. In these more typical circumstances, the onset of toxic effects may be more gradual and the factors affecting the likelihood of regurgitation more complex. In addition, there is the possibility that birds may be able to detect and avoid food contaminated with some pesticides (eg. Avery *et al.* 1993). We therefore suggest that consideration be given to employing a suitably modified short-term dietary test method in place of the acute oral LD50,

for assessing risk from dietary exposures to pesticides. If such a method were to include monitoring of food intake, then it might also provide useful information on food avoidance as suggested by Luttik (1993), and also might be designed so as to avoid the additional suffering which occurs in some dietary tests as a result of self-starvation or anorexia (eg. Grue 1982). This method would require validation before it was adopted for routine use, especially to determine whether avoidance of contaminated food in the test was a reliable predictor of avoidance in the wild. Where a non-dietary route of exposure requires assessment, an LD50 based on injection might be more appropriate than an acute oral LD50. Further research is required to assess the potential benefits of these proposals in increasing the reliability and decreasing the cost of ecological risk assessment for pesticides.

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