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Noninvasive Characterization of the Effects of Diazinon on Pigeons

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Many questions in ecotoxicology can only be answered by studying wild animals in captivity. However, keeping stress-sensitive species in captivity may confound both experimental treatments and interpretation of results. Non-invasive research methodologies provide a means of combating these problems by reducing exposure of captive animals to humans. Automated, non-invasive methodologies may also reduce experimenter bias and research costs, while improving animal welfare and providing more detailed observations over time. Further, sensitive behavioral measures have the potential ability to detect lower-level effects of organophosphates and other compounds than would normally be characterized through traditional physiologic measures of brain and plasma cholinesterase (ChE) levels.

Non-invasive techniques are commonly used in several research disciplines. Operant psychology methodology provides sophisticated examples of the use of automated, computer-controlled environments for manipulating contingencies in the study of behavior of laboratory animals. Other non-invasive methods of data collection are routinely used in animal agriculture, e.g., automated monitoring of body mass in flocks of broiler chickens and turkeys, identification of individual animals in both companion animals and in dairy herds. However, despite the utility of these techniques, few examples exist in which multiple methods of non-invasive measurement have been used simultaneously to study the influence of xenobiotics on wild animals in captivity.

To this end, we demonstrate the use of three non-invasive techniques for characterizing the effects of an organophosphate pesticide, diazinon, on pigeons *(Columba livia).* Pigeons were used as a model for wild birds because previous research in our laboratories established plasma levels of cholinesterases in response to acute, oral doses of diazinon (Henderson et al., 1994).

MATERIALS AND METHODS

Adult male and female King pigeons were obtained from D&H Squab Farm, Modesto, California. Following a 45-day quarantine period, during which time they were treated with medicated feed containing chlortetracycline, they were fed Purina Pigeon Chow Checkers pelleted diet for the duration of the experiment. One pair of male and female birds was used in Experiment 1 and three male pigeons were used in Experiment 2. All experiments were done under approved Animal Use and Care Protocols.

A 0.9m(w) x 1.8m(l) x 0.9m(h) experimental cage constructed of 1.3cm x 7.6cm welded wire cloth was supported by a steel frame. Attached to the cage were a nest box, operant

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feeding device and key, drinking font, electronic scale, and a loop antenna mounted on the wall of the cage that encircled the drinking font. The cage was housed in a light- and climate-controlled chamber. Photoperiod was 10 hr light:14 hr dark, with light onset at 09:00 hr. Temperature was maintained at approximately 21°C, with relative humidity at approximately 50%.

A nest box constructed of black acrylic plastic (305cm[w] x 61cm[l] x 46cm[h])was attached to one end of the cage. The nest box, similar to ones used in commercial pigeon husbandry, was divided into two equal-sized compartments by an acrylic plastic partition. The rear panel of the nest box was hinged at the top to allow inspection and cleaning. The entrance to the nest box was partially reduced in size by a 10 cm high panel extending across the bottom edge of the nest box. This panel was set back 15 cm from the front edge of the nest box, forming a ledge on which pigeons could perch.

An electronically controlled pigeon feeder, pigeon response key and white stimulus light were mounted on the end of the cage opposite the nest box. The response key and opening for the food magazine were located 25 cm and 10 cm above the cage floor, respectively. Control of the operation of the feeder was provided by a microcomputer with a data acquisition board (Keithley Model DAS Series #800, Cleveland, OH). The computer controlled communications with the operant response key, remote feeder activation switch (used in training the pigeon to key peck), pellet feeder, scale, and loop antenna.

Following local analgesia with 2% lidocaine, a small transponder (AVID model #2002, Norco, CA; a.k.a. “radio-frequency ID tag”) was implanted subcutaneously in each bird through a 3 mm incision on top of the head.

Using plastic wire ties, a 38 cm diameter loop antenna (AVID, Norco, CA) was mounted onto 1.5 cm thick plywood measuring approximately 60 cm x 60 cm that replaced the wire cloth portion of the cage. Wood and plastic were used as support for the antenna because wire cloth absorbs the electromagnetic field, thereby reducing the ability of the antenna to detect the transponder. A plastic drinking font (Plasson nipple-type plastic drinking font with cup; Diversified Imports, D.I.V. Co, Inc., Lakewood, NJ) was positioned within the perimeter of the antenna.

The antenna was connected via shielded cable to the reader, which contained circuitry for detecting a transponder within the field of the antenna. A serial communications protocol in the control program determined the frequency of interrogation of the reader (1 Hz) and monitored reception of an 8-digit identification number associated with each transponder.

Birds were observed remotely from outside the chamber by video camera to facilitate operant training and at various times throughout the experiments. Birds were first habituated to repeated remote activations of the feeder until they ate readily when the feeder was operated. They were then operantly conditioned to peck the key by reinforcing successively closer approximations of pecking on the key via a remote switch that, when actuated, caused from 10 to 30 s of food presentation. After key pecking for food was learned, food was presented for 20 s after a single key peck (fixed ratio 1 schedule of reinforcement). Additional key pecks during the 20 s period extended feed presentation 20 s from the time of the last peck. This contingency for reinforcement was in place 24 hr per day, although birds normally consumed food only during the light phase. In Exp. 2, the feeder was refilled and weighed daily just after the onset of the light phase as an additional measure of feed use. The white stimulus light positioned about 5 cm above the response key was lit continuously, providing very minimal light even during the dark phase.

To standardize idiosyncratic key-pecking patterns among birds, data on key pecking for food reinforcement was transformed in the following manner. Seconds of food access earned per 15 min interval were first converted to cumulative min of feed presentation earned per 15 min interval. Then the total daily min of feed access earned on seven consecutive days were averaged to calculate average daily feed use, and each day's cumulative feed use was plotted as a fraction of average daily feed use.

To measure body mass, an electronic scale (Ohaus model #6000, Florham Park, NJ) was positioned on the floor of the cage directly below and in front of the drinking font. Birds stood on the scale while drinking, thus placing the transponder within the field of the antenna. The scale was controlled by the computer through serial communications. The control program interrogated the scale at Is intervals and stored the readings in an array, which was analyzed at 15 min intervals. Body mass readings were saved for further analysis only if a bird first generated a transponder reading by having its head within the field of the antenna and if the scale output produced a reading that was judged "stable" by the control program. The readings for each 15 min period were then averaged, stored on floppy disk, and printed as backup hard copy.

Within the antenna loop, a 6.5 cm x 11.5 cm square hole in the plywood panel permitted access of a single bird at a time to a drinking font and cup. The cup was positioned 13 cm above the surface of the scale. Slight pressure on the nipple by the beak of the bird released water, which would accumulate in the cup. Thus, the act of drinking, while standing on the scale, would position the bird's transponder within the field of the antenna.

As with responding for food, drinking "behavior" was normalized to the weekly mean with one important exception. Because diazinon treatment dramatically altered normal locomotor behavior at higher doses (the birds would often perch on the scale with their heads not in the drinking font window but still within the field of the antenna), drinking behavior on the day of treatment for all doses was excluded in calculating weekly means.

In Exp.1, a male and female pair of pigeons were given diazinon within a few min of light onset when their crops were empty. Diazinon (analytical standard, lot #184-38A, purity 99%, Chem Service, West Chester, PA) was dissolved in polyethylene glycol (PEG) at concentrations of 0.5, 1.0, and 2.0 mg/ml and pigeons were orally gavaged with doses of 0 (vehicle only control), 0.5, 1.0, and 2.0 mg/kg. A minimum of one week was allowed to pass between successive administrations of diazinon, thus ensuring that plasma cholinesterase activity reached control levels (Henderson et al. 1994). In Exp. 2, diazinon was administered as above to three male pigeons, each housed individually.

RESULTS AND DISCUSSION

Combined (male and female) responding for food in Exp. 1 (not shown) resembled the temporal pattern observed for individual pigeons in Exp. 2 (Fig. 1). Birds began eating shortly after light onset and ceased eating at dark onset. Doses of 0.5 and 1 mg/kg diazinon produced delays of about 1 and 1 ½ hr, respectively, in initial responding for food, compared either to control (O-dose) Day 0, or to the days immediately before (Day – 1) and after (Day 1) diazinon administration. This delaying effect of diazinon was dramatic at 2.0 mg/kg, when daily onset of responding for food was delayed for approximately 4 hr. But then birds began key-pecking for food reward in a manner similar to that seen at the beginning of control or non-dosed days.

Time spent by the female in the field of the antenna in Exp. 1 (operationally defined as time spent drinking) reflected the temporal pattern of responding for food, although drinking bouts typically followed the first eating bout by one or more hr. Daily onset of

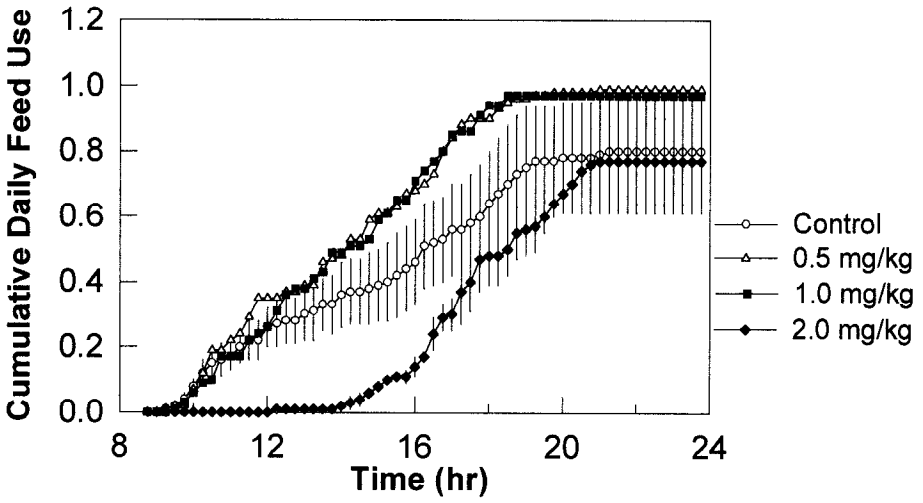

Figure 1. Cumulative daily feeding of male pigeons in Exp. 2. For clarity of presentation, standard error bars are shown for control and 2.0 mg/g doses (n=3). The daily onset of responding for food was significantly delayed by 2.0 mg/kg diazinon.

female drinking behavior, like feeding, was delayed more than 4 hr following administration of 2.0 mg/kg diazinon.

Time spent by the male in the field of the antenna resembled that of the female, with one important exception. Video observation showed the male often perching on the scale with his head in the field of the antenna, but not drinking. This was particularly evident on the day of administration of 2.0 mg/kg diazinon, when time spent in the field of the antenna suggested that the male "drank" over five times the normal daily amount. In fact, the video showed that the male was sitting at apparent ease on the scale, not drinking, and occasionally auto-preening and looking about. He was neither hunched over, nor did he display the fluffed feathers indicative of a sick bird. Thus, particularly with diazinon administration, the effect on locomotor behavior produced "artifactual" drinking.

Automatic monitoring of body weight revealed daily changes of 6 to 7% (range of weekly min/max differences in body masses of males and females) and, occasionally, as much as 9%. Bird weights were at their minima in the morning and increased with eating throughout the day. While differences of 6-7% reflect the automatically collected body masses, the actual minimum masses were probably lower on all dates, as video observation showed that birds often engaged in several bouts of feeding before their first bout of drinking, when typically they would first be weighed.

Video observation in Exp. 1 showed how social interactions between the pigeons could introduce error into the validity of automated characterization of feeding. The male pigeon was clearly dominant to the female. Often she would peck the operant response key, earning a 20 s food reinforcement, but then be prevented from eating by the threat or act of male aggression. Thus, at least for the female pigeon, the total time earned for feed access over-estimated the actual time spent eating. Such social interactions were the principal reason individual pigeons were used in Exp. 2.

Responding for food by male pigeons in Exp. 2 is shown in Fig. 1. Standard errors are included for control and 2.0 mg/kg doses to show variability, but are omitted from 0.5

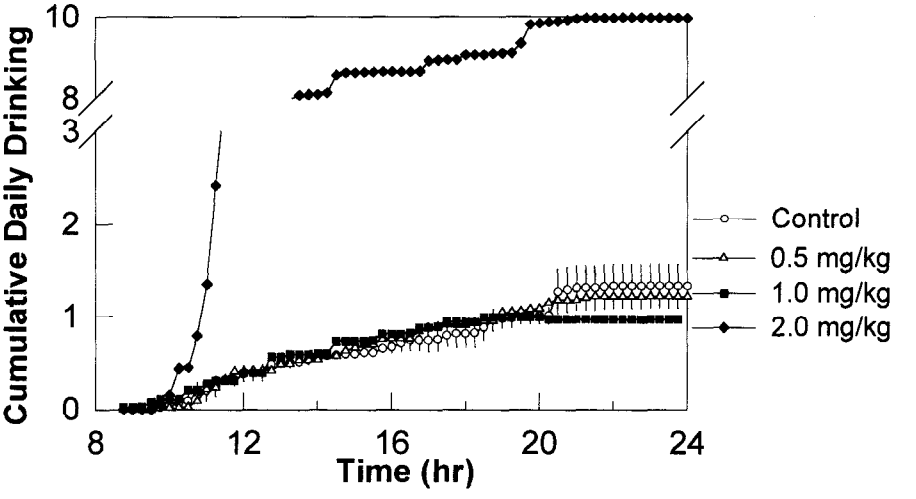

Figure 2. Cumulative daily drinking of male pigeons in Exp. 2. Data are normalized to show the cumulative daily amount of drinking. Standard error bars are shown only for the control dose. The apparent dramatic increase in "drinking" observed at 2.0 mg/kg is artifactual, reflecting hypoactivity, not thirst.

and 1.0 mg/kg doses for clarity of presentation. Compared to days on which birds were gavaged only with PEG control, on days of 0.5 and 1.0 mg/kg diazinon administration there were slight but not significant delays in onset of daily responding for food. Days after administration of diazinon at these doses were undistinguishable from controls. However, as in Exp. 1, the 2 mg/kg dose produced delays of several hours before the onset of responding for food (Fig. 1). The time from light onset until 10% of the day's total responding was reached was approximately 1 ½ to 2 hr for 0, 0.5 and 1.0 mg/kg, while it was approximately 6 ½ hr for the 2.0 mg/kg group ($P<0.05$, Tukey Test for pair-wise comparison among all treatments [$P< 0.001$ for main effect, ANOVA]). However, after food responding commenced, following the 2.0 mg/kg dose, the rate of responding resembled that observed at lower doses, and on control days and, in fact, continued for about 2 hr into the dark phase, a time when pigeons typically do not eat.

An effect of diazinon on feed use (grams of feed removed from the feeder each day) was also evident. A repeated-measures 2-way ANOVA revealed no significant main effects of either dose or day on feed use, but did detect a significant interaction between dose and day ($P<0.043$). A separate repeated-measures ANOVA of the 2.0 mg/kg dose revealed feed use on Day 0 to be significantly less than on Days -2, 3 and 4 ($P<0.05$). No other differences were significant.

As in Exp. 1, time spent in the field of the antenna, an estimate of drinking behavior, reflected eating behavior at 0, 0.5 and 1.0 mg/kg. But at 2.0 mg/kg, the artifact of perching on the scale, which was observed for male drinking in Exp. 1, was clearly evident, with apparent cumulative drinking reaching about 10 times the normal daily intake. Video observation confirmed that, during the period of 11:00 to 13:00, birds were sitting on the scale, auto-preening occasionally, but otherwise with normal appearance. Daily body weight changes of about 6% for males caged singly were similar in magnitude to those of the pair of pigeons in Exp. 1 (Fig. 3). Also, body weights throughout the day for birds treated with 0, 0.5 and 1 mg/kg were similar to each other, but values on the day of 2.0 mg/kg diazinon administration were depressed compared to the day before by an

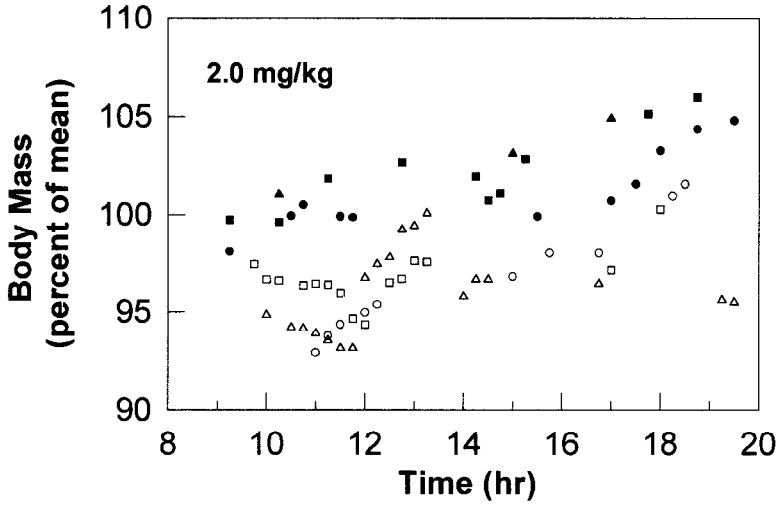

Figure. 3 Body mass changes of three individual male pigeons (square, circle, triangle) the day before (closed symbols) and day of (open symbols) administration of 2.0 mg/kg diazinon. Diazinon treatment significantly depressed mean body mass on the day of administration compared to the day before ($P<0.05$).

overall average of 6%. ANOVA of mean daily averages detected a main effect ($P<0.015$), and pair-wise comparisons detected significance only for a dose of 2.0 mg/kg (day before [102.11% ±0.53 of weekly mean] vs. day of diazinon [96.46±0.22%]; $P<0.05$, Tukey Test).

The automated, non-invasive methods we used to characterize effects of acute oral diazinon exposure in pigeons produced results consistent with previous reports of mild organophosphate intoxication in birds, namely anorexia, weight loss and hypoactivity (Grue et al. 1991; 1997). At the 2.0 mg/kg dose we observed a delayed onset in daily operant responding for food, an effect consistent with reports of reduced feed consumption following acute exposure to organophosphates in such species as common grackles *(Quiscalus quiscula;* Grue 1982) and starlings *(Sturnus vulgaris;* Hart 1993). Likewise, dietary exposure to some organophosphates reduces food intake in mallard ducks *(Anas platyrhynchos;* Keith and Mulla 1966) bobwhite quail *(Colinus virginianus;* Stromborg 1981) and house sparrows *(Passer domesticus;* Mehrotra et al. 1967) although whether this is due to food aversion or pesticide-induced anorexia is not clear. Our data show that the suppression of eating following a single oral gavage with diazinon is virtually complete, but also short-lived. After approximately 4-6 hr of suppression, responding for food commenced at a rate comparable to that observed in control (zero-dose) birds and actually continued into the dark phase.

This pattern of responding, i.e., delayed onset with responding continuing past its normal time of conclusion, could also be interpreted as a phase-delay in the circadian pattern of responding for food. Carbachol, another acetylcholinesterase inhibitor, can produce either phase-delays or phase-advances when administered intraventricularly in Syrian hamsters (Bina and Rusak, 1996) and some have reported phase-adjusting effects when it is administered peripherally to mice (Gaweda, 1998). The hypothermic effects reported in studies of organophosphate exposure in birds would also be consistent with a phase-delaying effect (e.g., Maguire and Williams 1987).

Organophosphate exposure in birds typically results in a temporary reduction in body mass, although responses to sub-lethal doses are not well documented (Grue et al., 1991). The reduction in pigeon body mass produced by 2.0 mg/kg diazinon is less than many reports of acute exposures of organophosphates, although more lethal doses are often employed, e.g., Maguire and Williams (1987). In part, our use of a remotely monitored scale to monitor sub-lethal effects of diazinon permitted such slight differences to be detected with a small number of experimental animals. Indeed, the observed 6% mean daily difference in body mass between the day before and day of 2.0 mg/kg diazinon administration was less than the observed normal dawn/dusk variation in body mass. As diurnal body mass changes of 6-7% are typical of many avian species, with smaller species often varying by much more (e.g., Baldwin and Kendeigh, 1938), treatment effects that are smaller than the magnitude of normal diurnal changes could easily be missed. This could be particularly important in studying wild animals in captivity for which numbers of animals might be severely limited because of conservation or animal welfare concerns.

Patterns of drinking reflected the general pattern of eating, although the method we used to estimate drinking produced artifacts that render it unreliable in evaluating the effects of organophosphates. Organophosphates typically induce lethargy, ataraxia (tranquility), or perching (Grue et al. 1991) all of which can be viewed as hypoactivity. In the present study, video observation of the male pigeons dosed with 2.0 mg/kg diazinon showed that apparent periods of continuous drinking were actually due to birds simply sitting, not drinking, on the electronic scale. A more accurate measure of drinking could have been achieved by other devices, e.g., an optical-interrupt sensor placed in front of the drinking font. The transponder detection system measured time spent by the birds within the field of the antenna, which involved standing on the scale while typically, but not always, drinking. As birds were only occasionally observed by video, we cannot say whether every instance of apparent high rates of drinking behavior was artifactual.

Nonetheless, the automated methods employed here show potential for monitoring the behavior of stress-sensitive species in a manner that minimizes human contact. As psychogenic stress reduces feed consumption, more invasive methods might well have reduced the sensitivity required to detect subtle effects of organophosphate toxicity on a wild species in captivity.

The non-invasive methods we used showed that treatment with vehicle alone, 0.5 or 1.0 mg/kg diazinon did not significantly alter the parameters we measured, but that 2.0 mg/kg diazinon produced delays in the onset of operant responding for food, with corresponding declines in body mass. Thus, the behavioral threshold for acute, oral administration of diazinon in pigeons appears to be between 1.0 and 2.0 mg/kg. Our earlier report found that 1.0 mg/kg diazinon depressed plasma cholinesterase levels to 20% of normal (Henderson et al. 1994). The current data demonstrate that an operant feeding apparatus, automated body mass sensing, and implanted transponders for animal identification can provide the means to obtain a finely detailed characterization of the eating and drinking patterns of birds in a typical captive environment. They also suggest that such measures could be used to monitor the effects of other xenobiotics in a minimally invasive manner, permitting captive studies of wild species that might not otherwise tolerate the human intrusion required to gather these types of data.

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