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13. ABSTRACT ***(Maximum 200 Words)***

A series of behavioral, neurochemical, and immunocytochemical studies were undertaken to characterize the possible role of insecticide exposure in the etiology of Parkinson's disease as it may relate to Gulf War Syndrome. The insecticides under study are the organophosphorus compound chlorpyrifos and the pyrethroid, permethrin given 3 times over a two week period by injection (chlorpyrifos subcutaneous and permethrin intraperitoneal). Permethrin showed an up-regulation of dopamine transport at a dose of 1.5 mg/kg, which is at least two orders of magnitude below its LD50. At higher doses of permethrin ($\geq$25 mg/kg), the increase in transport declined to a level below that of control. Toxic effects may have been involved, since immunocytochemical labeling of the caudate-putamen found that transporter staining was near control levels. Both permethrin and chlorpyrifos caused small, but statistically significant decreases in mitochondrial dehydrogenase activity. However, cytotoxicity was not reflected in levels of striatal dopamine, which were not changed by 100 mg/kg chlorpyrifos or 200 mg/kg permethrin. There was, however, an increase in dopamine turnover at 100 mg/kg chlorpyrifos, as indicated by a significant increase in titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid. Chlorpyrifos at 25-100 mg/kg caused 15-84% inhibition of acetylcholinesterase, which correlated reasonably well with effects on open field, rearing, and pole climbing behaviors. Permethrin caused a significant decline in open field behavior, which may have been related to a doubling of muscarinic receptor density in the striatum of mice treated with $\geq$50 mg/kg. These studies demonstrate significant effects on dopamine neurochemistry by these insecticides.

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INTRODUCTION

This project is focused on Parkinson's disease (PD) as a long term consequence of Gulf War Syndrome, with insecticides evaluated as causative agents. The experiments for the first year are related to Objective #1, which is to characterize any effects on biomarkers of PD over a range of doses of permethrin (PM) and chlorpyrifos (CPF). Treatments were given in three injections over a two week period, exactly as described in the proposal (Fig. 1). The plan of work emphasizes studies on permethrin, with chlorpyrifos experiments following later in the year. Results of dose-response studies, along with the observed effects on PD biomarkers, are listed below in the Body of the Report, in the order outlined in the amended proposal.

BODY

Overall, we have performed the majority, but not all, of the experiments planned for the first year. Two major factors limited our ability to complete all of the scheduled experiments. First, we lost 5 months of postdoctoral effort due to the timing of employment. Although the project started on 1/1/99, Dr. Wen Li was unable to join the group until 3/1/99. In addition, Dr. Paul Harp resigned from this study 9/30/99 in order to take a position in the State Toxicologist's Office in New Hampshire. We have just hired an excellent young scientist, Dr. Daniel Karen, into the vacant postdoctoral position, and he started work on 1/25/00. A copy of his resume is included in the Appendices. The other problem concerned a two month construction/maintenance procedure on our Laboratory Animal facility that resulted in loss of temperature control in the animal holding rooms. This problem led to heat stress in the animals and unreliable data, which was excluded from the report. This maintenance problem, which has been rectified, is described in a detailed letter from Dr. David Moore, University Veterinarian (see the last item in the Appendices).

a. Assess toxicant effects on dopamine titers and turnover by measuring the dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC) content of the striata from treated mice.

Methods: HPLC analysis was performed exactly as described in the proposal. High doses of PM and CPF were tested in these initial studies, since we felt that loss of dopamine would be an indicator of relatively more intense effects on the striatum, compared to other biomarkers.

Results and Discussion: Our first experiments with CPF at 150 mg/kg resulted in lethality in 4 of 7 mice (57%), so the top dose was reduced to 100 mg/kg, where only 4 mice died out of the 42 treated (9.5% mortality). At 50 mg/kg CPF there was 7% mortality (2 out of 28 mice), one of which was a bad ip injection. Thus, mortality below 100 mg/kg is probably unrelated to toxicant action. No mortality was observed in any of the mice treated with PM.

Once the maximal doses were established, we began the striatal dopamine analysis. Dopamine content was about 160 pmoles/mg striatum in controls (Fig. 2). High doses of CPF (100 mg/kg) and PM (200 mg/kg) had no effect on dopamine content of the striatum, compared to controls treated with appropriate solvents. There was, however, a significant elevation of DOPAC by CPF treatment, about 14% above control levels (Fig. 3). In contrast, PM treatment had no effect on DOPAC titers, which averaged about 8 pmoles/mg striatum wet weight, in controls (Fig. 3).

Loss of dopamine and DOPAC is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both neuronal electrical activity and cell death in the striatum. Elevated levels of DOPAC indicate greater turnover of dopamine in response to toxicant-induced processes (Hudson *et al.*, 1985). We assume that CPF increases turnover through its well-known ability to cause neuronal hyperexcitation through inhibition of acetylcholinesterase. We were somewhat surprised by the lack of any effect of PM, given that increased striatal DOPAC occurs following treatment with the pyrethroids deltamethrin (Kirby *et al.*, 1999) and fenvalerate (Husain *et al*, 1991).

However, fenvalerate and deltamethrin have much greater mammalian acute toxicity toxic than PM, with rat oral LD_{50}s of 31, 451, and 3801 mg/kg for deltamethrin, fenvalerate, and PM, respectively (Budavari *et al.*, 1996).

We have previously speculated that enhanced turnover of dopamine may itself be neurotoxic, because the metabolism and auto-oxidation of dopamine generates cellular oxidative stress (Dawson *et al.*, 1995) and high levels of dopamine in the brain can be neurotoxic (Filloux and Townsend, 1993). However, we would expect that cell toxicity would be reflected in a loss of striatal dopamine, but this effect was not observed, perhaps due to the short duration of exposure (two weeks). Overall, we do not think the lack of dopamine depletion by these insecticides when given alone is very surprising. If insecticides were frank parkinsonian agents, like the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), this activity would have been documented by now, given the large number of toxicity studies that have been performed over the years on pyrethroids and especially, chlorpyrifos. Subtle, predisposing effects of these compounds, or synergistic interactions, are more likely to be involved in environmental parkinsonism.

b. Assess effects on the density and kinetic properties of dopamine transporters in striatal synaptosomes from treated mice.

Methods: Exactly as described in the proposal for dopamine uptake. We have also established methods for [^{3}H]GBR12935 binding, but have not yet run this assay on treated mice.

Results and Discussion: We observed that PM treatment significantly alters maximal transport (V_{max}) of dopamine with little effect on half maximal substrate affinity (K_m). Figure 4 shows representative results for V_{max} and K_m determinations in our initial experiments having three treatment groups, two of which were treated with 100 or 200 mg/kg PM. Kinetic data given beneath Figure 4 confirms that the major effect of PM was a significant depression of V_{max}, with no effect on K_m (all K_m values have extensive overlap in their 95% confidence limits). These experiments were replicated over a broad range of doses (two experiments at each dose), and the results are summarized in the bar graph in Figure 5. The lowest doses of PM (0.2, 0.4, and 0.8 mg/kg) had little or no effect on dopamine uptake. So, these doses presumably represent the threshold for this biomarker, although we expect to see some evidence of enhanced uptake at 0.8 mg/kg in additional replicates. A peak increase in maximal transport of dopamine was observed at 1.5 mg/kg, and this effect lapsed into a depression of uptake at much higher doses (Fig. 5).

These studies were initiated at high doses (100 and 200 mg/kg PM), with the dose reduced in two-fold steps (Fig. 5). Thus, they represent a significant amount of effort, and we kept reducing the dose because we had observed an upregulation in dopamine transport in mice treated with deltamethrin (Kirby *et al.*, 1999). The dose of PM where upregulation occurs (1.5 mg/kg) is miniscule compared to its LD_{50}, and 200 mg/kg ip caused no lethality, no overt signs of toxicity, and no effect in our behavioral studies (see below). Thus, 1.5 mg/kg is at least 2 orders of magnitude less than a nonlethal dose. It is also important to note that technical permethrin is a mixture of four stereoisomers (Fig. 6), only one of which (1*R*, 3*R*, *cis*) is expected to have any toxic effects in mammals, based on studies of acute lethality (Casida *et al.*, 1983). If so, the true level of toxic isomer in these studies is 0.375 mg/kg. A few more replicates of these studies around the 1.5 mg/kg point are required, so the ultimate shape of this response curve and the maximal increase in transport at 1.5 mg/kg PM remain to be finalized.

The level of increased dopamine transport caused by PM (mean of two experiments: 50% above control) is about the same as that observed for cocaine, which raises expression 50% when given 5 times/day at 40 mg/kg (Miller *et al.*, 1993). In contrast to PD, schizophrenia results from dopamine overactivity (Bowman and Rand, 1980) and dopamine uptake in postmortem brain preparations from schizophrenic patients was increased 74% over controls (Haberland and Hetey, 1987). Thus, the level of DAT overexpression in schizophrenia appears similar to that resulting from low dose

exposures to PM. Moreover, psychiatric problems are known to result from OP exposure and disturbance of consciousness has been reported for pyrethroid intoxication in man (He *et al.*, 1988).

We speculate that the dopamine transporter was up-regulated in response to increased levels of synaptic dopamine, which is consistent with findings we recently published on the structurally-related pyrethroid, deltamethrin (Kirby *et al.*, 1999). We expect to confirm upregulation of DAT by immunocytochemical labeling (biomarker f) in animals treated with 1.5 mg/kg PM. We also expect that levels of [^{3}H]GBR12935 binding will go up by a similar amount. We have recently documented that the organochlorine insecticide heptachlor virtually doubles dopamine transport, and that antibody labeling of DAT in western blots of synaptosomal protein is increased by a similar amount (Miller *et al.*, 1999). Moreover, the dose-response curve for heptachlor has a shape similar to that reported here for PM. Cytotoxicity is the simplest explanation for the depression of uptake at higher doses. We would expect toxicity to be reflected in loss of striatal dopamine, which was not observed (Fig. 2). However, there is evidence of cell stress in mice treated with PM, as measured in the MTT assay (see next section). At higher doses, toxicity in nerve terminals could mask up-regulated transporter density in synaptosomal uptake experiments; if, for example, toxic processes result in greater leakage of dopamine from presynaptic stores, *in vitro*. Thus, at higher doses, there may not be a clear correlation between uptake, GBR binding, and immunocytochemical labeling, which we do expect to see at doses near 1.5 mg/kg PM. Our studies with heptachlor (Miller et al., 1999) were not run at a range of doses wide enough to shed much light on this possibility.

Pilot studies of GBR binding in albino mice are shown in Figure 7, demonstrating our ability to perform this assay. For these studies, striatal synaptosomes are harvested, and then subjected to repeated resuspension in distilled water, followed by centrifugation, following the procedure Marks *et al.* (1986) developed for measuring [^{3}H]nicotine binding. The tissue is then stored frozen until ready for use. Equilibrium binding parameters for [^{3}H]GBR12935 are given with the binding curve in Figure 7, which also shows the dose-dependence and saturation of [^{3}H]GBR12935 binding. Autoradiographic methods of measuring [^{3}H]GBR12935 binding in brain slices found a similar K_d of 1.4 nM, but not surprisingly, a lower binding capacity (B_{max}) of 6 pmol/mg protein (Richfield, 1991), compared to our studies in well-washed synaptosomal membranes. We place a high priority on performing the GBR binding assay in mice treated with the doses of PM shown in Figure 5, for comparison with the uptake data.

Overall, these findings suggest that transporter activity/expression is a sensitive biomarker for toxicity in neurotransmitter systems where the transporter is the primary mechanism for terminating transmitter action. It is interesting to note that for cholinergic synapses, where acetylcholinesterase terminates transmitter action, chlorpyrifos slightly decreases (16-20%) choline uptake (Liu *et al.*, 1995), indicating a different regulatory mechanism. Increased transporter expression or activity would augment neuronal accumulation of endogenous or exogenous pyridinium toxins (*e.g.*, MPP^+, the oxidation product of MPTP), thereby providing a likely mechanism of synergism in our planned studies with MPTP.

c. Compare the extent of toxin-dependent actions on mitochondrial function in striatal synaptosomes by measuring thiazolyl blue dehydrogenase activity.

Methods: The thiazolyl blue (MTT) assay kit from Sigma Chemical Co. (St. Louis, Missouri), which reports on the amount of dehydrogenase activity at mitochondrial complex 1 (Slater *et al.*, 1963) was used essentially as described in the proposal. We use synaptosomes instead of brain mitochondria in order to assess respiration of mitochondria in a cellular environment approximating what they experience *in vivo*, which we hypothesize will be altered by poisoning. Accordingly respiration of isolated mitochondria in artificial media may not be an accurate reflection of their *in vivo* activity.

Results and Discussion: Preliminary experiments using the MTT assay in synaptosomes from treated mice found that it does not follow typical kinetics (Fig. 8). The reduction of MTT is linear with respect to concentration, up to about 0.5 mM, where the activity saturates and then declines at higher substrate concentrations. Comparison of curves from controls and mice treated with CPF at 13 mg/kg and controls showed significant overlap, up to 0.55 mM MTT. The 25 mg/kg CPF group actually had slightly higher levels of activity compared to controls up to saturation. Above 0.55 mM, the responses changed, with the 13 mg/kg group less than controls and the 25 mg/kg group declining to control levels. The concentration-response relationship at higher MTT concentrations is not predictive or consistent with relative activity at lower substrate levels. Given this somewhat unusual dependence on substrate concentration, in subsequent experiments we emphasized effects at concentrations up to 0.55 mM, which is at or near maximal effect. Statistically significant reductions of dehydrogenase activity, the anticipated effect of insecticides, occurring at two MTT concentrations should provide additional confidence that any observed effect is biologically significant. At the highest tested dose of CPF (100 mg/kg), there was a consistent reduction (about 20%) in MTT dehydrogenase activity (Fig. 9). There was no consistent effect of CPF at doses $\leq$50 mg/kg, but a slight increase, probably simply related to assay variability, was observed at 0.55 mM MTT. Additional replicates will clarify whether the reduction in mitochondrial function observed at 100 mg/kg is manifested across different groups of mice.

Consistent effects on MTT dehydrogenase activity were also observed after PM treatment. The results of these studies are shown in the two bar graphs of Figure 10. In the lower dose group (13, 25, and 50 mg/kg) activity was depressed at both 0.38 and 0.55 mM MTT, with a dose-response relationship especially evident at the latter substrate concentration. Reduction of mitochondrial activity at these doses is consistent with the reduced transport documented in the previous section. Surprisingly, at higher doses only 200 mg/kg PM showed a statistically significant inhibition of dehydrogenase activity (10% at 0.55 mM MTT). The reason for the lack of effect at 100 mg/kg remains unclear. Additional replicates of these studies are planned.

Toxicity to nerve terminals *in vivo* should be reflected in reduced mitochondrial function *in vitro*. We have observed small, but statistically significant reductions in mitochondrial activity, which is also observed in PD (Schapira *et al.,* 1990) and other neurodegenerative diseases (Beal *et al.*, 1993).

d. Search for anatomical evidence of general neurotoxicity within light microscopic preparations of the nigro-striatal system by examining glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for gliosis.

e. Search for anatomical evidence of neurotoxicity within specific dopaminergic neurochemical components of the nigro-striatal system using immunohistochemical staining for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH) and dopamine beta hydroxylase (DBH).

f. Confirm whether functional changes in dopamine transport are due to fluctuating levels of the dopamine transporter (DAT) protein using immunohistochemical identification.

Methods: These biomarkers are dealt with together because fixation, sectioning, staining, and analysis is similar for all these studies. Much time during the first reporting period was devoted to the hiring and training of a graduate student (Graduate Research Assistant) to cut brain tissue and perform the immunohistochemical procedures. This required some fine-tuning of our proposed protocols, primarily in terms of reagent and antibody concentrations, for use in the nigro-striatal system. In addition, the SigmaScan Pro image analysis software, which we originally proposed to use for quantitative analysis, proved difficult to work with for our intended purpose. Therefore, a

considerable period of time was devoted to development of our own novel image analysis protocol using Adobe Photoshop 5.0 and the training of personnel to use it. A description of this protocol is provided below.

First, the control and treated slices are mounted on the same slide and stained under identical conditions. Then, positions of the fields to be measured are specified for each coronal section through the striatum. This is done with the aid of a camera lucida attachment to a microscope. The external border of the caudate-putamen (CPu) is identified and traced using the external capsule as an anatomical marker. Along the arc of this external border, a mark is then made at 1/3 and 2/3 of the distance between its ventral-most point and the dorsal ventricular border of the nucleus. The central point of the CPu is then quantified and a radius is drawn from the 1/3 and 2/3 markers to the center of the CPu. Along each of these two radii, a mark is made 1/4 and 1/2 the distance from the lateral border of the nucleus to the center point of the nucleus. This yields four locations within the CPu from which measurements of immunolabel will be made. Since the CPu comprises the dorsal portion of the striatum, our procedure ensures that our samples will be taken from the dorsolateral portion of the striatum, which is the primary locus of nigral dopaminergic afference to the striatum. An example of the position of the four fields to be measured is indicated on appended Figure 11.

After specifying the four loci to be measured in each section, a digital image was captured from the nearest homogenous terminal field to each locus, using the 100X objective. The digital images from each section were then analyzed using the histogram function of Adobe Photoshop. After converting the image to grayscale, the histogram function can provide mean or median grayscale value (0-256, with 0 being black and 256 white), pixel count for a single specified gray value or total pixel count for a continuous range of grayscale values.

In general, a mean minimum acceptable grayscale value was determined for immunolabeled neuropil for each control brain. This value was also applied to a matched treated brain. A given measurement looked at the total number of pixels that were darker (smaller in grayscale value) than the mean minimum acceptable grayscale value (e.g. if mean minimum value was 102, looked at total number of pixels from grayscale values of 102 to 0).

To determine mean minimum acceptable grayscale value, first the mean value of two immunolabeled varicosities per field, judged as the minimum acceptable for measurement, was determined. Then the grand section mean across the four fields per section was determined. Finally, a grand mean for the brain was determined from each of the grand section means. Again, this minimum acceptable value of the grayscale, measured from a given control brain, was used to make pixel counts on that brain and its corresponding pesticide-matched brain. This determines the change relative to the control brain.

Pixel counts were then made as follows: For each of the four fields for a given section, the grayscale histogram was examined for the entire image. Then, the number of pixels with a value less than (darker than) the minimum acceptable grayscale value was determined. For example, if mean minimum value was 102, looked at total number of pixels from grayscale values of 102 to 0. The count for each field was used to produce a section mean of pixel counts. The mean for each section was then used to calculate a brain mean pixel count. The procedure was then repeated for the matched treated brain, and the percent change in mean pixel counts was calculated between the two brains. This percent change is one data point to be used in a matched pairs comparison of means.

Results and Discussion: We have begun processing striatal tissue for anatomical biomarkers of PD to determine the effects of each of the pesticides alone, and compared them to MPTP as a positive control. We have processed two vehicle controls and two brains each from mice treated with MPTP (30 mg/kg), a low dose of PM (1.5 mg/kg), a high dose of PM (200 mg/kg), and 100 mg/kg CPF. Only the MPTP-treated brains exhibit obvious differences between vehicle control

upon visual inspection. Examples of this change for DAT immunoreactive neuropil are illustrated in the appended Figure 12. The control image (Fig. 12A) shows intense and evenly distributed staining for DAT. After treatment with MPTP, overall staining is reduced and is less evenly distributed, as more staining is evident along the left margin of the Cpu (Fig. 12B). At higher magnification (Fig. 12 C,D), the staining appears uniform over small areas. Quantitative analysis of DAT staining following MPTP treatment detected a mean 77.1% decrease in DAT immunolabel relative to vehicle controls (Fig. 13). This is an important finding for our project since it shows that our digital analysis protocol is capable of detecting a well defined change in dopaminergic afference to the striatum that has been demonstrated in other labs (Heikkila and Sonsalla, 1992). The high dose permethrin treatment produced a minimal 7% increase in DAT immunolabel (Fig. 13). Our current sample size precludes meaningful statistical analysis of these results, but this data does suggest that there are near normal levels of DAT in the striatum of mice treated with 200 mg/kg PM. Thus, other cytotoxic factors probably underlie the reduced dopamine uptake consistently observed at this dose.

We are intrigued by the apparent non-uniform distribution of DAT in the immunocytochemical studies of MPTP, and wonder if a related phenomenon might be occurring in PM- or CPF-treated mice with respect to dopamine levels. Recall that we observed no change in dopamine content of the striatum following PM or CPF treatment (Fig. 2). However, what if some terminals are depleted of dopamine, and other increase production to compensate. A hypothetical example of this idea is shown in Figure 14. Would a uniform immunostaining for dopamine become non-uniform, reduced in some areas, but more intense in others? Immunostaining coronal sections for dopamine would address this issue.

An example of TH immunoreactive profiles from a chlorpyrifos-treated mouse is also appended (Fig. 15), simply to illustrate the staining. The quantitative analysis of this data is not finished, and more mice will be processed shortly. If a pesticide treatment causes a reduction in specific dopaminergic inputs to the striatum (TH staining is reduced), we will process brains for immunocytochemical analysis of dopaminergic cells in the substantia nigra, as well. For pesticide treatments that fail to produce any change in specific dopaminergic afference to the striatum, we will examine GFAP immunoreactivity for evidence of a more generalized neurotoxic response. Although we originally stated that we would also process brains for dopamine beta hydroxylase immunoreactivity to distinguish noradrenergic from dopaminergic striatal neuropil, it has since come to our attention that there is an insignificant noradrenergic innervation of the striatum (Aston-Jones *et al.*, 1995). We conclude that these studies are moot.

In terms of methodological improvements, we continue to fine tune our procedure in an attempt to reduce the concentration of antibody necessary for visualizable staining. This would reduce the cost of tissue processing. We are also attempting to maximize the number of brains that can be processed at one time. We have just hired an individual to fill the wage position, which will increase our tissue processing output. We expect to rapidly accelerate our processing of tissue in the second year of the project.

g. Explore toxicant effects on open field/rearing frequencies and pole climbing behaviors and search for correlations between behavioral impairment and neurochemical effects.

Methods: Behavioral experiments focused on measurements of impairment of motor function and were performed essentially as described in the proposal..

Results and Discussion: CPF had dose-dependent effects on movement, rearing, and pole climbing behavior. Open field movement (Fig. 16) was unaffected by 25 mg/kg CPF, but inhibited about by 36% at 50 mg/kg and 71% by 100 mg/kg. Similarly, rearing (Fig. 16) was not changed by 25 mg/kg CPF, but 50 mg/kg reduced rearing frequency 29% and at 100 mg/kg rearing was

reduced by 76%. Only the responses at 100 mg/kg CPF were statistically significant. In the pole traction test (Fig. 17), CPF caused a dose-dependent increase in the percentage of mice that fell from the pole that confounded our planned measurements of descent time. We will replicate this analysis in order to do conventional statistical analysis. We prefer this approach as opposed to nonparametric statistical analysis of this type of data.

Permethrin treatment had only limited effects on behavioral performance. There was a slight trend for a decrease in movement as the dose was increased from 0.2 to 200 mg/kg (Fig. 18). There was a statistically significant inhibitory effect at a dose of 50 mg/kg. The small magnitude of the effect and inherent variability of behavioral data probably contributed to the lack of significance at other doses. No effect was observed in the rearing measurements (Fig. 18) or in the descent time measurement of the pole test (Fig. 19). Descent time proved to be the most variable of all the behavioral measures. In three cases, we excluded data from individual mice that hung from the pole for more than 180 sec and never attempted to climb down (control, 13, 25, and 50 mg/kg groups, all from the same cohort of mice). A score of 180 sec for down time is more than three standard deviations from the mean. Unlike CPF, no falls were recorded after treatment of PM at any dose.

PD results in tremors, bradykinesia, and incoordination (Bowman and Rand, 1980), which are reflected in the behavioral assays we have performed.. These behavioral studies have allowed us to observe some general correlations between behavior and neurochemical effects. Thus, we expect to observe tremors and bradykinesia that should correlate with depletion of dopamine or other neurochemical effects in future studies.

h. Determine the extent of acetylcholinesterase inhibition following treatment with toxicants for comparison with other behavioral and neurochemical effects.

Methods: We used the classical method of Ellman *et al.* (1961) to determine acetylcholinesterase activity in striatal synaptosomes from treated mice. The assay measures enzyme generation of yellow color by reaction of 5,5'-dithiobis-2-nitrobenzoic acid and thiocholine when acetylthiocholine is used as the enzyme substrate. Although V_{max} and K_m values for acetylcholinesterase activity were planned, standard measurement of cholinesterase involves a single substrate concentration at an incubation time in the linear range of activity. We used a substrate concentration of 400 mM and an incubation time of 3 min.

Results and Discussion: CPF at 25, 50, and 100 mg/kg gave 15%, 58%, and 85% inhibition of acetylcholinesterase (Fig. 20). Thus, the dynamic range of inhibition is essentially covered by these doses. The maximal level of cholinesterase inhibition observed at 100 mg/kg CPF is similar to that reported for rat striatum (82-96% inhibition) treated with CPF or parathion (Liu and Pope, 1998). The extent of acetylcholinesterase inhibition by CPF typically does not correlate with effects on behavior (Nostrandt *et al.*, 1997), possibly due to compensatory changes in muscarinic receptors (Nostrandt *et al.*, 1997) and high affinity choline uptake (Liu *et al.*, 1995). In the present study, however, there was a reasonable match between enzyme inhibition and behavioral effects (movement, rearing, and falling; Figs. 16 and 17). Moreover, there was about 15% mortality at 50 mg/kg and about 20% mortality at 200 mg/kg, although the mice showed no signs of SLUD.

We also observed an effect of PM treatment on acetylcholinesterase activity. In this case, there was an increase in enzyme activity in treated mice (Fig. 21). The effect was small, typically an increase of 10-20%, and was not clearly dose-dependent, so the biological relevance is somewhat questionable. It is interesting to note that exposure to deltamethrin also caused a small but significant increase in acetylcholinesterase activity in rat brain (Husain *et al.* 1994). Perhaps this effect is an adaptive response to high levels of synaptic acetylcholine caused by the pyrethroids.

i. Define any toxicant-induced changes in cholinergic receptor density or function with respect to agonist-induced dopamine release from striatal synaptosomes.

Methods: This goal of the research actually contains several separate neurochemical measurements. We have established methods for radioligand binding studies involving [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and [^{3}H]nicotine, with nicotine binding adapted from the procedures of Marks *et al.* (1986). In addition, two methods for measuring functional cholinergic modulation of dopamine release are still under development. The first will measure loss of label by repeated application of buffer with a pipettor. Alternatively, the labeled synaptosomes will have agonists superfused over them with a peristaltic pump and loss of label will be quantified in this manner. We have not yet performed any studies on the ability of cholinergic agonists to alter release of dopamine in striatal synaptosomes from insecticide-treated mice.

Results and Discussion:

In mouse brain striatal synaptosomes from controls, we observed [^{3}H]QNB binding characteristics of K_d values in the picomolar range (13 pM) and11 pmol/mg protein for B_{max} (Fig. 22). This compares reasonably well with the values reported for heart membranes by Goodwin *et al.* (1995) of K_d = 60 pM and B_{max} = 401 fmol/mg protein. In addition, there is a good match between our Bmax value and that reported by Nostrandt *et al.* (1997), which was about 2.8 pmol/mg protein in rat striatum.

Exposing mice to PM causes an apparent upregulation of muscarinic receptors, as evidenced by an increase in the B_{max} for [^{3}H]QNB binding (Fig. 22). Compared to controls, QNB binding was increased 86% at 50 mg/kg, 131% at 100 mg/kg, and 111% at 200 mg/kg PM (Table with Fig. 22). There were also changes in K_d, but there was considerable overlap in the 95% confidence limits for these values (Table with Fig. 22). We have not performed any studies of striatal QNB binding in mice treated with CPF. However, the known effect of chlorpyrifos to down-regulate muscarinic receptors in the striatum (Chaudhuri *et al.*, 1993) makes it likely that we will confirm this effect of CPF, which is opposite that of PM. This difference is interesting, since both pesticides are expected to increase synaptic levels of acetylcholine, but differ in how this action affects receptor regulation. Systemic injection of pyrethroids (*ca.* 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic receptors (5-10%), but apparently not in the striatum (Eriksson and Fredriksson, 1991). These results however, may be suspect, since Eriksson and Fredriksson (1991) report a K_d value in the low micromolar range of what they call high affinity QNB binding. This data is several orders of magnitude different from the K_d we report (13 pM), as well as that found by others: Goodwin *et al.* (1995) for heart (K_d = 63 pM) and Niemeyer *et al.* (1995) for cat retina (K_d = 270 pM). We feel it is imperative to replicate our PM results to ensure our complete confidence in the observation of up-regulated muscarinic receptors.

It is interesting to note what is known of the role of muscarinic receptors in the control of motor behavior as it relates to our results with PM and its up-regulation of muscarinic receptors. Muscarinic agonists mimic the bradykinesia and tremor seen in PD (Gomeza *et al.*, 1999), and we observed a slight decrease in open field movement following PM treatment (Fig. 18). Perhaps the doubling in muscarinic receptor density caused by PM is in part responsible.

Although we have not yet measured effects in treated mice, we have a reasonable assay in place to perform experiments on [^{3}H]nicotine binding (Fig. 23). In these studies, nicotine shows saturable binding with specific binding about %80 of total binding at 5 nM ligand. Our K_d value of 2.1 nM is similar to the 8 nM reported by Marks *et al.* (1986). We have a number of processed tissues from treated mice that are stored frozen at –70 °C and ready to be used in binding studies (Table 1). Thus, we will be able to make rapid progress on all the binding studies in the near future.

KEY RESEARCH ACCOMPLISHMENTS

Demonstration of dopamine transport up-regulation at extremely low doses of technical permethrin (1.5 mg/kg).

Found small, but statistically significant reductions in mitochondrial activity occurred in PM- and CPF-treated mice.

Observed a large up-regulation of striatal muscarinic receptors by PM treatment.

Inhibition of acetylcholinesterase by CPF was correlated with open field, rearing, and pole traction behavioral performance.

REPORTABLE OUTCOMES

None for the past year. We anticipate two posters to be given at the Society for Neuroscience meeting in the fall of 2000, along with two research papers to be submitted for publication.

CONCLUSIONS

A number of major conclusions are derived from the first year of this project. First, the up-regulation of dopamine transport occurring at low doses of PM (1.5 mg/kg) provides a ready mechanism for synergism with pyridinium toxins, such as MPP^+. Investigating this synergism is a major goal of the second year of the project, after we perform additional studies to nail down the dose-response relationship for this effect.

Second, the loss of dopamine transport at higher doses of PM is probably related to other toxic effects, such as a reduction in mitochondrial activity. Even though the magnitude of the effect is small, any reduction in mitochondrial activity caused by PM and CPF may be significant over the long term. Although we have not yet performed GBR binding studies to estimate transporter density in striata from treated mice, our initial immunocytochemical studies suggests no deficit in DAT levels in mice treated with 200 mg/kg PM. We did not observe a loss of striatal dopamine after 100 mg/kg CPF or 200 mg/kg PM, which would have been expected if significant cytotoxicity had occurred. However, we hypothesized a "clumping" effect of dopamine could be occurring that serves to mask effects on dopamine when measured as total amount of transmitter by HPLC. We would like to try dopamine immunolabeling to see if this is indeed the case.

Third, the strong up-regulation of muscarinic receptors by PM was unexpected, and may play a role in reducing motor activity, since muscarinic agonists such as oxotremorine cause bradykinesia and tremor. This finding needs to be replicated, and lower doses run as well, to define the NOEL for this effect.

The good correlation between behavior and acetylcholinesterase inhibition for CPF is not too surprising, and these results confirm a number of previous studies done in rats and set the stage for more thorough studies of its effects in year two of this project.

These studies are significant as a body of research because they illuminate a number of significant actions in the neurotoxicology of insecticides, some of which are applicable beyond the scope of this research. The most significant finding is the upregulation of transport at low doses of PM. We have previously observed that the organochlorine heptachlor (Bloomquist *et al.*, 1998) and the pyrethroid deltamethrin increase dopamine transport (Kirby *et al.*, 1999). However, the latter studies did not include a dose-response analysis for this effect. Now, we have extended this observation to permethrin and shown that this action occurs at doses at least two orders of

magnitude below the LD_{50}. Thus, up-regulated DAT is a sensitive index of CNS exposure to insecticides and may be generalized to include other classes of neurotoxins as well. Studies on mitochondrial impairment have also provided additional significant findings, since mitochondrial dysfunction is implicated in a number of neurodegenerative diseases besides PD (Beal *et al.*, 1993). Thus, our observation of compromised mitochondrial function following insecticide exposure may broaden the possible roles of insecticide exposure in other neurological conditions.

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APPENDICES

Resume:
Daniel J. Karen
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Blacksburg, VA 24060

EDUCATION

Clemson University, PhD, Environmental Toxicology — December, 1999
University of Charleston, MS, Marine Biology — August, 1994
Emory University, BS, Biology — May, 1992

PROFESSIONAL EXPERIENCE

Research Assistantship, Department of Environmental Toxicology, Clemson University, SC.
Organized and ran GLP research project for the South Carolina Department of Natural Resources (SCDNR). Assessed the effects of hardness on copper toxicity to fathead minnow fry.

Laboratory Manager, Department of Environmental Toxicology, Clemson University, SC
Managed aquatic culture laboratory housing *H. azteca, F. heteroclitus U. imbecillis, D. magna, C. tentans, C. dubia, L. macrochirus, R. pipiens, A. vulgare,* and *P. promelas*. Managed use of aquatic exposure lab for dosing studies.

Research Assistantship, Department of Environmental Toxicology, Clemson University, SC
Organized and ran GLP project for an industrial sponsor (Silver Council). Presented data at national and international meetings in Fall, 1997. Wrote and published report.

Membership Chair, Alpha Epsilon Lambda, Clemson University, SC
Organized and managed Spring, 1998 new member selection and induction into Clemson University's graduate and professional student honor society.

President, ENTOX Graduate Group, Clemson University, SC
Initiated and implemented members' ideas. Improved library and computing facilities, participated in community service, coordinated with faculty, and organized and ran group meetings with other officers.

Vice President, ENTOX Graduate Group, Clemson University, SC
Assisted the President. Initiated and implemented members' ideas. Improved library and computing facilities, participated in community service, coordinated with faculty, and organized and ran group meetings with other officers.

Teaching Assistantship, Department of Biological Sciences, Clemson University, SC
Taught Biochemistry lab during the Fall, 1997 semester and Human Anatomy lab during the Spring, 1998 semester.

Teaching Assistantship, Department of Biological Sciences, Clemson University, SC
Assisted and taught Human Anatomy lab during the Fall, 1995 and Spring, 1996 semesters.

SPECIAL TRAINING/CERTIFICATION

WET (Whole Effluent Toxicity) Testing, SETAC Short Course, 1999
Multivariate Statistical Analysis of Ecotoxicological Data, SETAC Short Course, 1998.
Practical GIS for the non-GIS professional, SETAC Short Course, 1997
Environmental contaminant stress: Immune function in aquatic and terrestrial organisms, Carolinas SETAC Short Course, 1997
Advanced Open Water SCUBA Diver, Professional Association of Diving Instructors (PADI), 1991

DISTINCTIONS/HONORS

1999 PRIMO 10 Student Travel Award
1999 Carolinas SETAC Best Student Platform Presentation
1998 Inducted to Sigma Xi
1998 National SETAC Best Student Poster Presentation Competition, 2nd Place
1997 Inducted to Alpha Epsilon Lambda
1997 National SETAC Student Travel Award
1995 Joanna Fellowship

PROFESSIONAL MEMBERSHIP

Society of Environmental Toxicology and Chemistry
Society of Toxicology
Alpha Epsilon Lambda
Sigma Xi

PUBLICATIONS

Karen, DJ, and PE Ross. (2000). Xenobiotic impacts on the skeletal system: A review *Rev. Env. Contam. Toxicol.* (*In Prep*).

Karen, DJ, PE Ross and SJ Klaine. (2000). Sublethal impacts of pesticide exposure on *Fundulus heteroclitus*: Comparisons between lab-reared and wild-caught fish. *Env. Toxicol. Chem.* (*In Prep*).

Karen, DJ, PE Ross and SJ Klaine. (2000). A comparison of episodic chlorpyrifos and TCP exposure on *Fundulus heteroclitus. Env. Toxicol. Chem.* (*In Prep*).

Karen, DJ, SJ Klaine, and PE Ross. (1999). Further considerations of the skeletal system as a biomarker of episodic chlorpyrifos exposure. *Aquatic Tox.* (*Submitted*).

Karen, DJ, DR Ownby, GP Cobb, SJ Klaine, and TW LaPoint. (1999). Influence of Water Quality Parameters on Silver Toxicity to Rainbow Trout (*O. mykiss*), Fathead Minnows (*P. promelas*), and the Waterflea (*D. Magna*). *Env. Toxicol. Chem.* 18(1):63-70. (*Invited*)

Karen, DJ, B Draughn, M Fulton, and PE Ross. (1998). Bone strength and acetylcholinesterase inhibition as endpoints in chlorpyrifos toxicity to *Fundulus heteroclitus*. *Pest. Biochem. Physiol.* 60:167-175.

Karen, DJ, BM Joab, JM Wallin, and KA Johnson. (1998). Partitioning of Chlorpyrifos between water and an aquatic macrophyte (*Elodea densa*). *Chemosphere*. 37(8):1579-1586.

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Ownby, DR, **DJ Karen,** DP Shupack, BS Day, TW LaPoint, SJ Klaine, and GP Cobb. (1997). Using spectroscopy and voltammetry to evaluate silver activity in aquatic toxicity evaluations. In: The Fifth International Argentum Conference Proceedings: Transport, Fate, and Effects of Silver in the Environment. University of Wisconsin, Madison. USA.

Figures and Tables

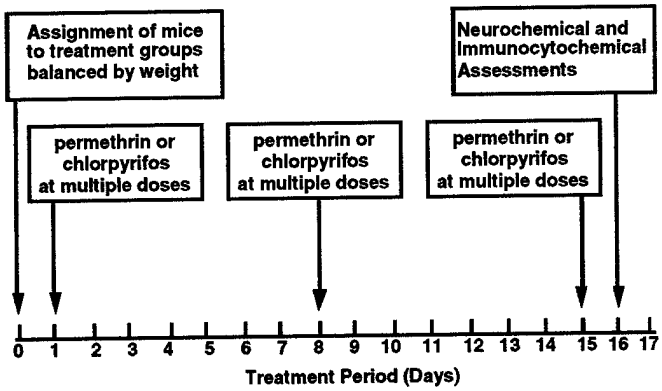


Figure 1. Subchronic treatment procedure for determining parkinsonian effects of PM and CPF. Mice not receiving toxins are injected with vehicle (methoxytriglycol [MTG] for PM and corn oil for CPF).

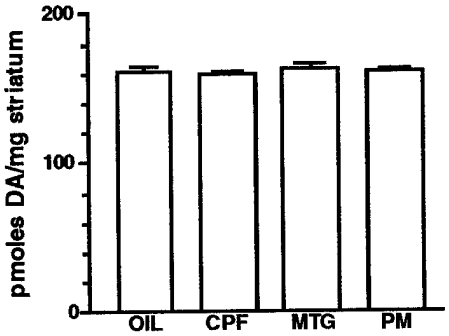


Figure 2. Effect of CPF (100 mg/kg, sc) and PM (200 mg/kg, ip) on striatal dopamine levels. In this and all subsequent figures bars represent means ± SEM. Results are compared to vehicle (corn oil for CPF and MTG for PM). No statistically significant effects were observed.

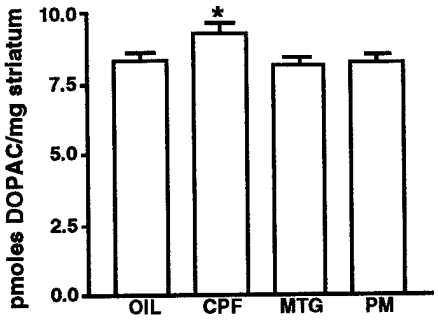
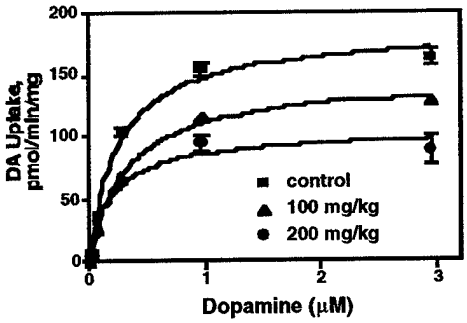


Figure 3. Effects of CPF (100 mg/kg, sc) and PM (200 mg/kg, ip) on striatal DOPAC levels. Results are compared to vehicle (corn oil for CPF and MTG for PM). Asterisk indicates statistical significance of CPF effect compared to vehicle ($p < 0.03$, unpaired T-test).



	control	100 mg/kg	200 mg/kg
Equation 1			
Variables			
BMAX	185.8	147.8	104.8
KD	0.2570	0.3244	0.2033
Std. Error			
BMAX	7.144	3.625	6.894
KD	0.03596	0.02757	0.05092
95% Confidence Intervals			
BMAX	170.7 to 200.9	140.1 to 155.5	90.22 to 119.4
KD	0.1807 to 0.3332	0.2660 to 0.3829	0.09536 to 0.3113
Goodness of Fit			
Degrees of Freedom	16	16	16
R squared	0.9775	0.9918	0.9284
Absolute Sum of Squares	1825	382.2	2041
Sy.x	10.68	4.888	11.29
Data			
Number of X values	6	6	6
Number of Y replicates	3	3	3
Total number of values	18	18	18
Number of missing values	0	0	0

Figure 4. Representative dopamine uptake studies for mice treated with PM. The table beneath the figure summarizes the data and shows the calculated kinetic values, along with their standard statistical parameters. The mathematical equation used is standard for binding studies, so the program reports B_{max} and K_d values, which are identical to V_{max} and K_m values for these transport measurements.

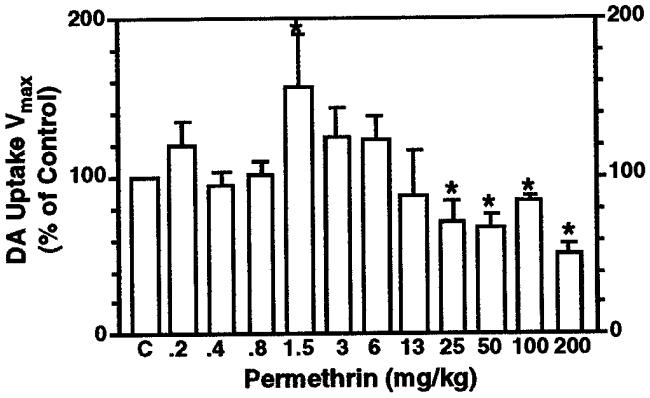


Figure 5. Dose-response studies of dopamine uptake with PM, normalized to matched controls. Asterisks indicate statistical significance in a T-test (1.5 mg/kg compared to control, $p < 0.08$) or ANOVA (all other indicated doses compared to control, Dunnett's multiple comparison test, $p < 0.01$).

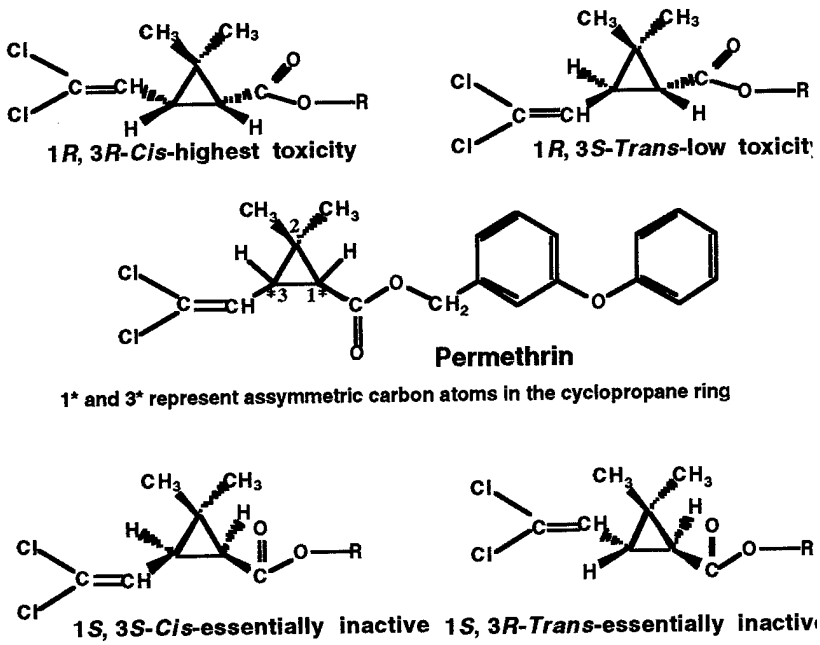


Figure 6. Molecular configuration of stereoisomers of permethrin. Structures are drawn after Elliott *et al.* (1974), along with their relative mammalian toxicities, as reported by Casida *et al.*, (1983).

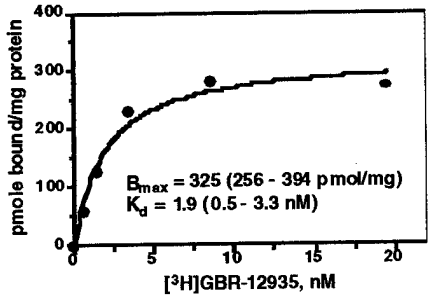


Figure 7. Typical binding isotherm of [^{3}H]GBR-12935. Symbols represent means with SEM bars. Binding parameters are also given (95% confidence limits).

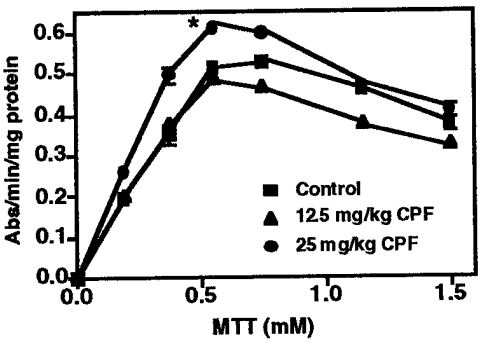


Figure 8. Assay of mitochondrial integrity with thiazolyl blue (MTT) at various concentrations. Asterisk indicates a significant difference ($p < 0.05$) between control and 25 mg/kg CPF at 0.55 mM MTT.

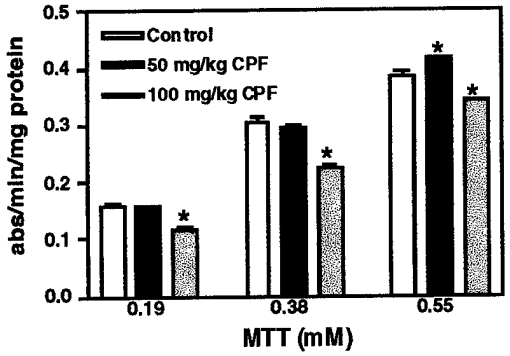


Figure 9. Assay of mitochondrial integrity with thiazolyl blue (MTT) at three concentrations in mice treated with 50 or 100 mg/kg CPF. Asterisk indicates a significant difference ($p < 0.05$) between treatment and control ((ANOVA with Student-Newmann-Keuls post test, $p < 0.05$).

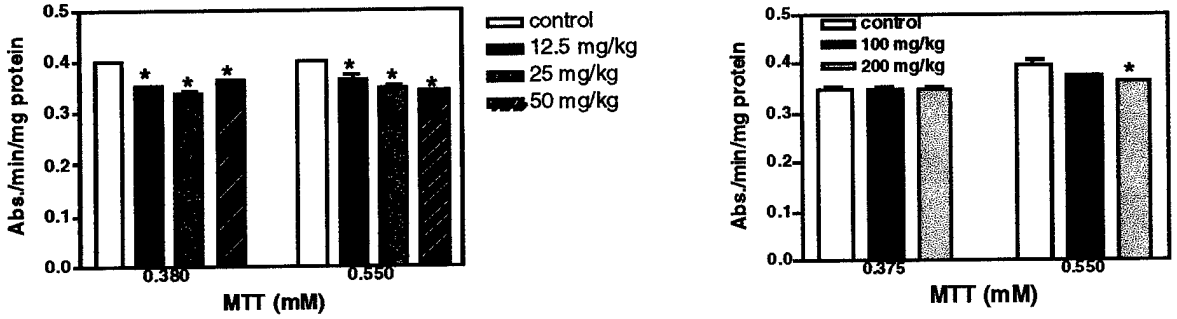


Figure 10. Reduction in MTT dehydrogenase activity in PM-treated mice. Asterisks indicate a mean level of activity significantly different from control (ANOVA with Student-Newmann-Keuls post test, $p < 0.05$).

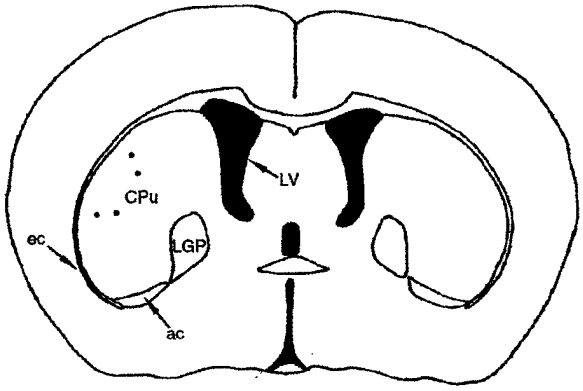


Figure 11. Representative coronal brain atlas section, at 0.1 mm caudal to bregma, showing the caudate-putamen (Cpu) and the relative position of the four regions (filled circles) typically sampled from each section. Taken from Franklin and Paxinos (1997). LV = lateral ventricle; LGP = lateral globus pallidus.

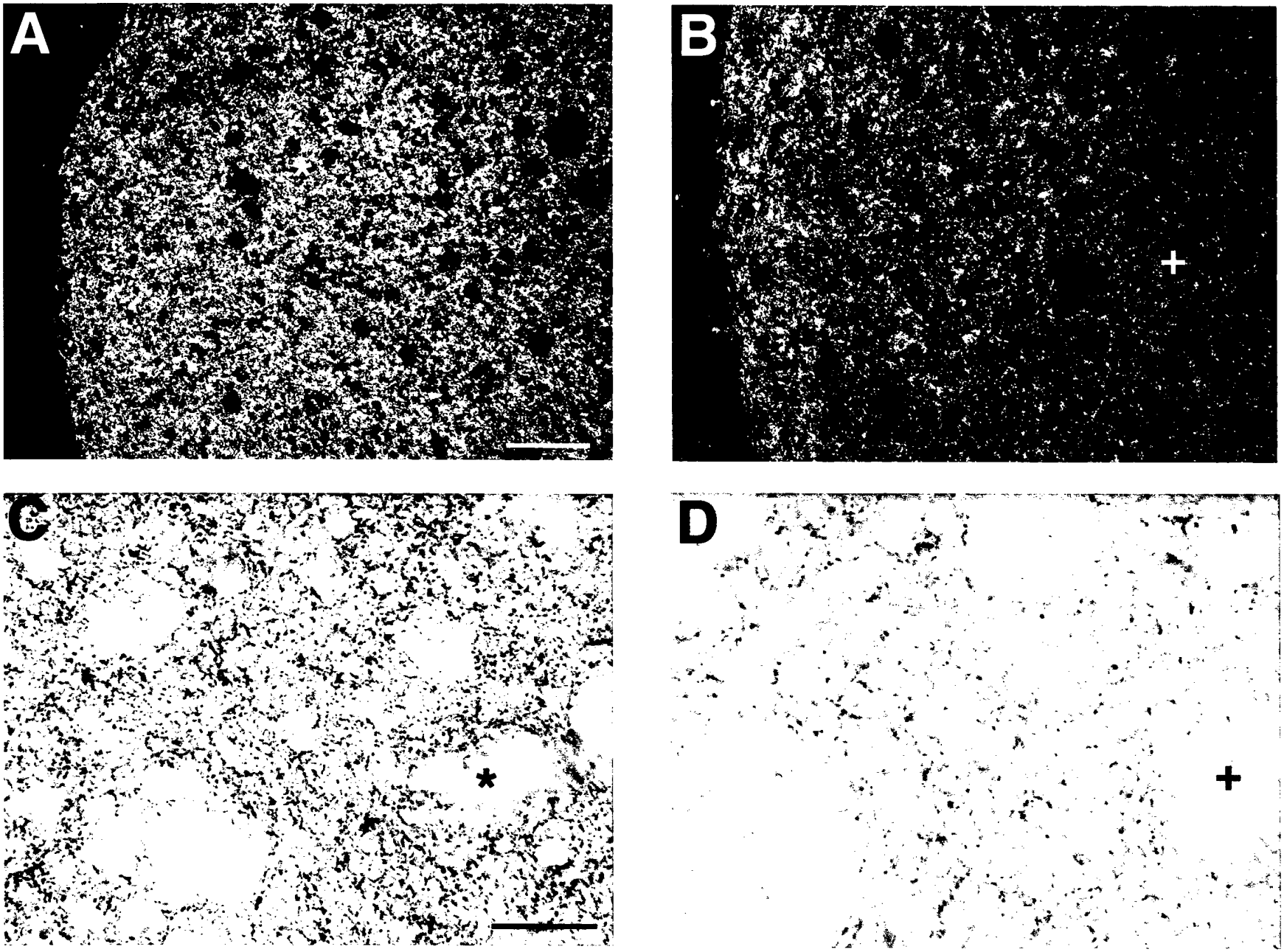


FIGURE 12

DAT immunoreactivity in striatal sections (15 um) from control (A,C) and MPTP-treated (B,D) mice. A and B are darkfield images, C and D are higher magnification brightfield images. Asterisks in A and C represent identical locations in the two images, as do the crosses in B and D. The white bar in A = 100um and the black bar in B = 25um. B is same magnification as A and D is same magnification as C. Images of MPTP sections were photographed at same exposure as controls.

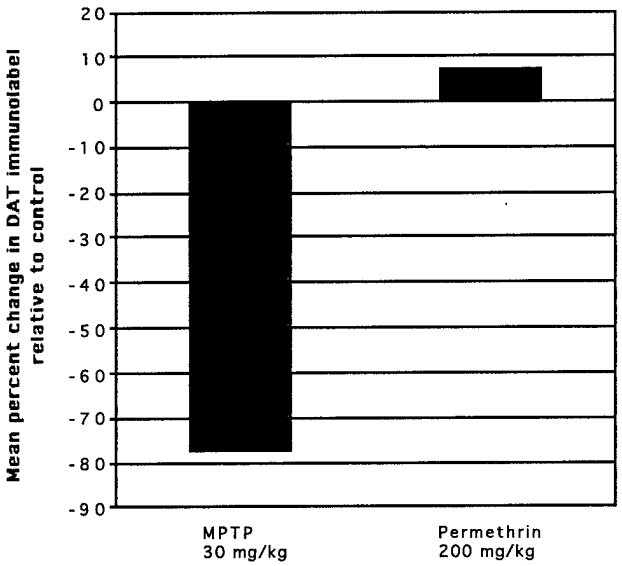
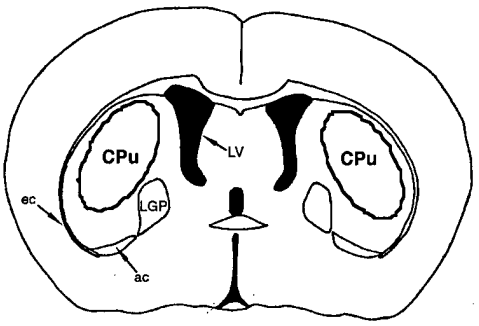
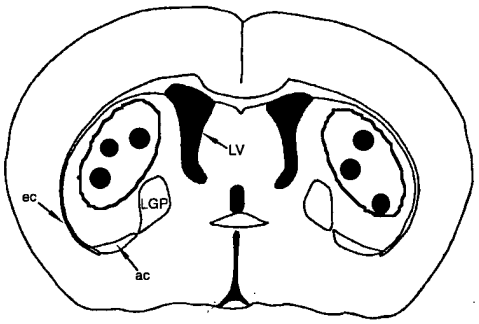


Figure 13. Graphical analysis of effects of MPTP and PM treatment on DAT labeling.



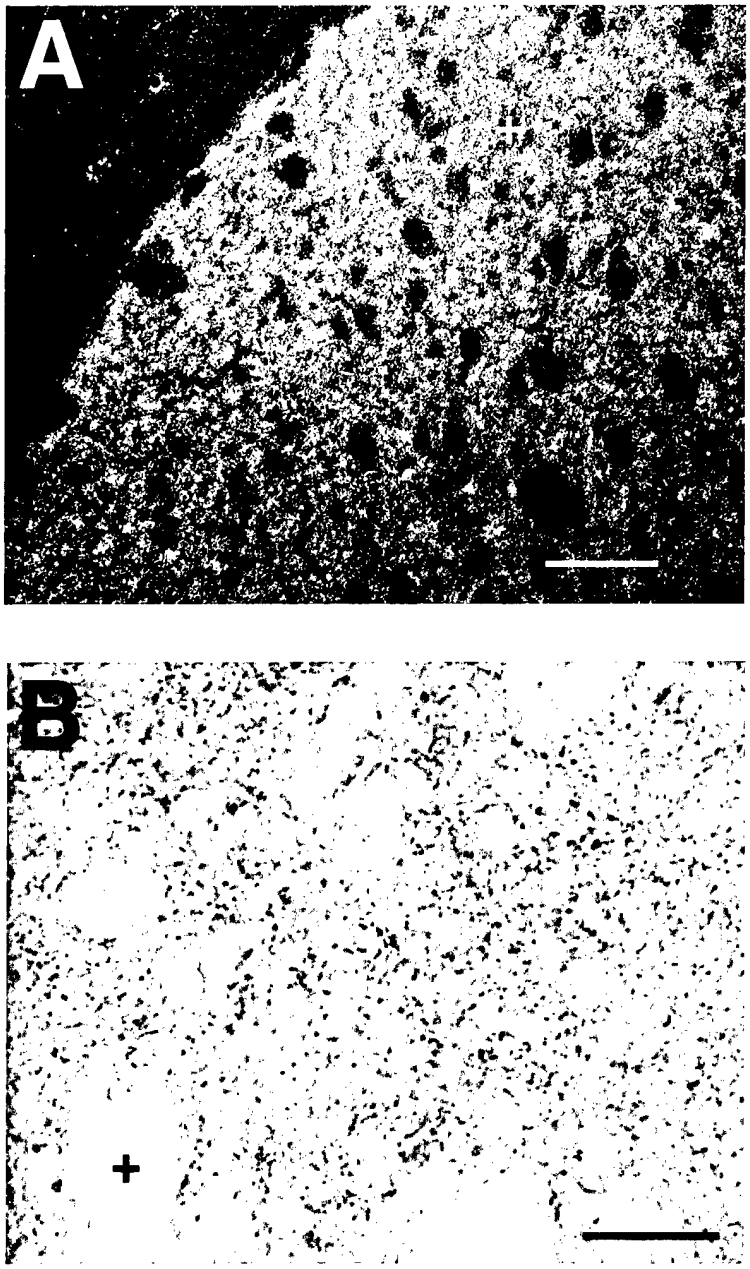
DA Distribution Before Treatment



DA Distribution After Treatment

Figure 14. Hypothetical distribution of dopamine (DA) in the caudate putamen (CPu) of control mice (top) and neurotoxin-treated mice (bottom). Light stippling in the top figure represents normal immunostaining of dopamine in the Cpu. Following toxicant treatment, large areas of the CPU are clear, indicating loss of dopamine, but others stain more intensely. Such an effect might occur as a homeostatic mechanism to restore overall dopamine levels in the CPu.

FIGURE 15



TH immunoreactivity in a striatal section (15 um) from a mouse treated with 100 mg/kg chlorpyrifos. A is a darkfield image and B is a higher magnification brightfield image of a portion of image A. Crosses represent identical locations on the two images. The white calibration bar in A is 100 um and the black bar in B is 25 um.

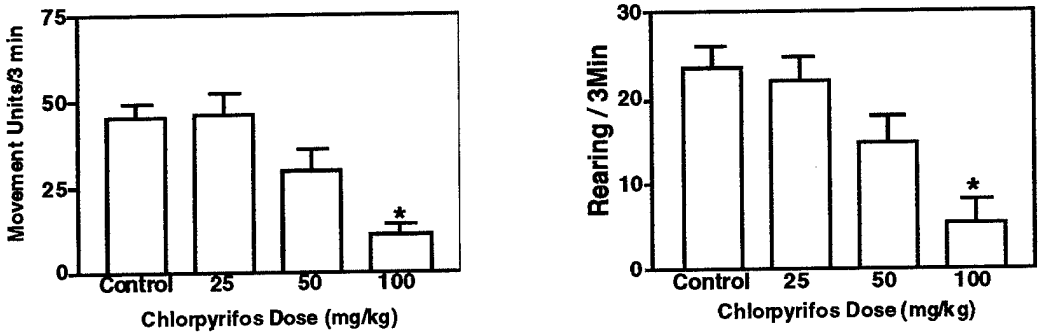


Figure 16. Movement (left) and rearing (right) behaviors in mice treated with CPF. Asterisks indicate a mean level of activity significantly different from control (ANOVA with Student-Newmann-Keuls post test, $p < 0.05$).

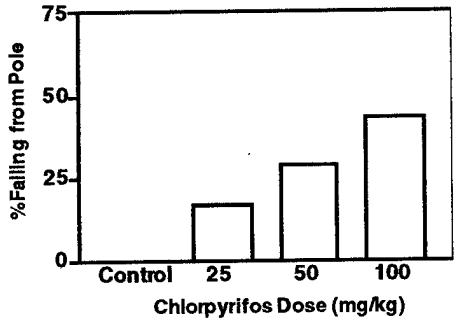


Figure 17. Mice that fell from the pole following CPF treatment. Since this effect was calculated as a percentage from each group of treated mice, it will be need to be replicated. We prefer this approach as opposed to nonparametric statistical analysis.

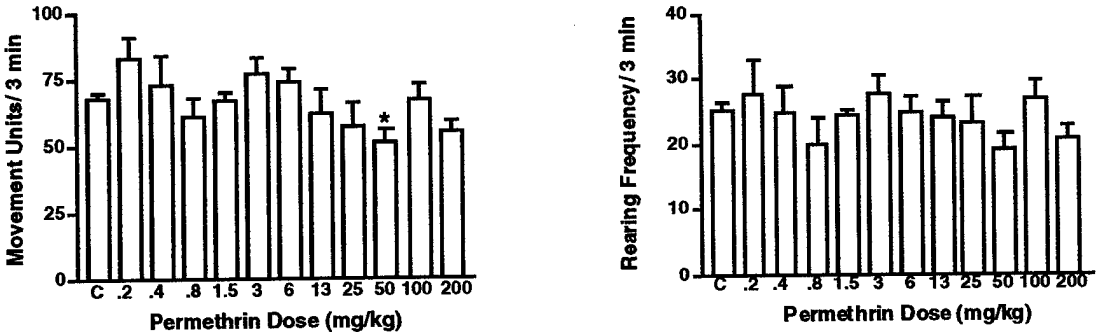


Figure 18. Movement (left) and rearing (right) behaviors in mice treated with PM. Asterisk indicates a mean level of activity significantly different from control (ANOVA with Student-Newmann-Keuls post test, $p < 0.05$).

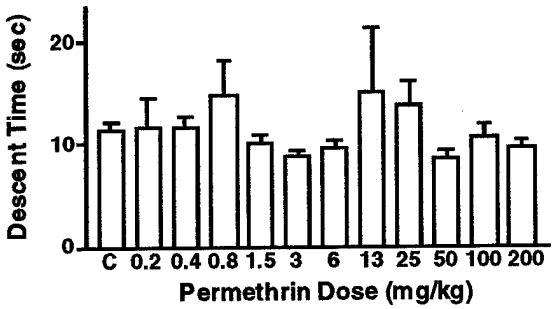


Figure 19. Mean descent time for mice treated with PM in the pole traction test. No statistically significant effects were observed in descent time, and none of the mice fell from the pole.

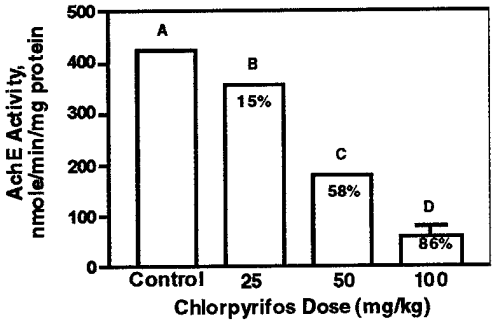


Figure 20. Acetylcholinesterase activity in the striatum following treatment with the indicated doses of CPF. The % inhibition at each dose is given inside the bars. Bars labeled by different letters are significantly different at the $p < 0.05$ level (ANOVA, Student-Newmann-Keuls post test).

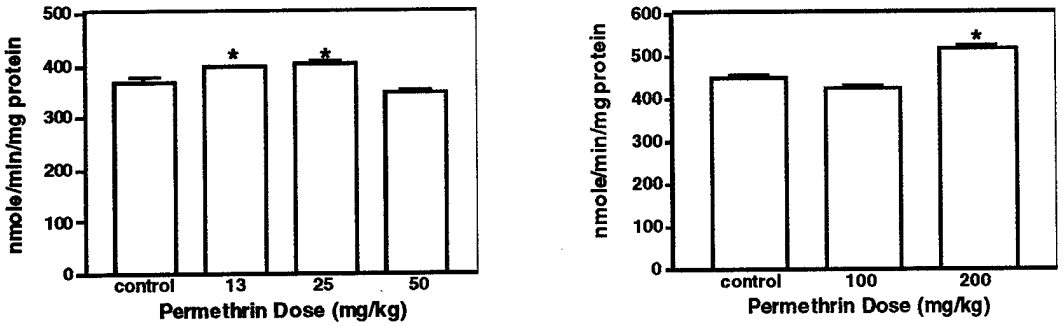
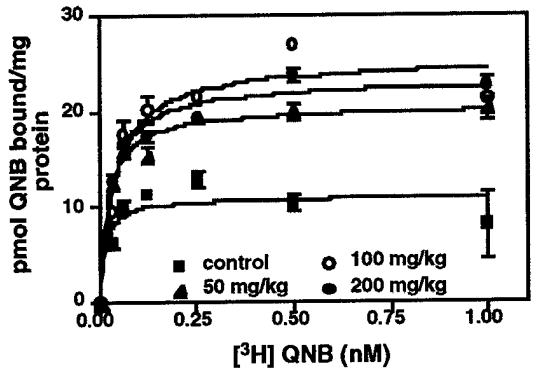


Figure 21. Acetylcholinesterase activity in the striatum following treatment with the indicated doses of PM. Asterisks indicate a mean level of enzyme activity significantly different from control (ANOVA with Student-Newmann-Keuls post test, $p < 0.05$).



	control	50 mg/kg	100 mg/kg	200 mg/kg
Equation 1				
Variables				
BMAX	11.08	20.48	25.35	23.17
KD	0.01275	0.02143	0.03635	0.02767
Std. Error				
BMAX	1.068	0.5424	1.567	0.6744
KD	0.01007	0.003461	0.01079	0.004344
95% Confidence Intervals				
BMAX	8.757 to 13.41	19.30 to 21.66	21.94 to 28.77	21.70 to 24.64
KD	-0.009200 to 0.03470	0.01389 to 0.02897	0.01283 to 0.05986	0.01821 to 0.03714
Goodness of Fit				
Degrees of Freedom	12	12	12	12
R squared	0.7370	0.9760	0.9032	0.9732
Absolute Sum of Squares	66.00	14.74	99.20	20.74
Sy.x	2.345	1.108	2.875	1.315
Data				
Number of X values	7	7	7	7
Number of Y replicates	2	2	2	2
Total number of values	14	14	14	14
Number of missing values	0	0	0	0

Figure 22. Upregulation of striatal muscarinic receptors by treatment with PM. Significant effects were only observed on B_{max} , as judged by the nonoverlap in the 95% confidence limits.

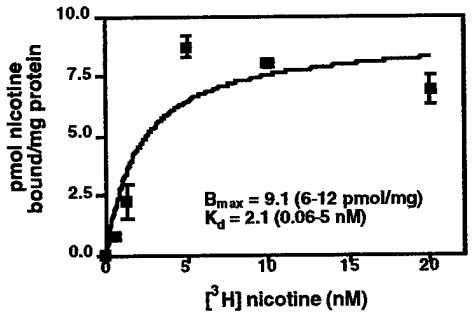


Figure 23. Binding assay for [^{3}H]nicotine in striatal membranes from mice, based on the procedures of Marks *et al.* (1986)..

Table 1. Frozen, processed tissue samples from treated mice currently available for binding studies. P1 = first pellet composed of cell bodies and large cellular fragments. P2 = crude synaptosome fraction enriched in membranes from presynaptic nerve terminals.

Compound	Dose	Pellet
Corn oil	Control	P1
CPF	25	P1
CPF	50	P1
CPF	100	P1
Corn oil	Control	P2
CPF	25	P2
CPF	50	P2
CPF	100	P2
MTG	Control	P1
PM	25	P1
PM	50	P1
PM	100	P1
PM	200	P1
MTG	Control	P2
PM	25	P2
PM	50	P2
PM	100	P2
PM	200	P2

Virginia Tech
VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Office of the University Veterinarian & Animal Resources

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14 January 2000

MEMORANDUM

TO: Dr. Jeffrey R. Bloomquist
Entomology

FROM: David M. Moore DM

SUBJECT: Environmental Extremes Experienced by Study Animals

This is intended to document the environmental extremes to which your study animals (in the study funded by the US Army) were exposed to over approximately 60 days at the end of 1999, and to explain the events which led to those conditions.

During March of 1999, both Dave Gemmell (Central Vivarium Manager/Supervisor) and I were contacted by the Virginia Tech Physical Plant Department and advised of the necessity to shut down the HVAC system in Litton-Reaves Hall which supplied a significant area of the building, including the Central Vivarium where your mice were housed. The shutdown was necessary to replace the primary exhaust fan unit which served the area. This would require the use of a massive crane to pluck the existing unit from the top of the 4 story component of the building, a crew of various trades to effect modifications to the mechanical systems to accept installation of the new unit, and use of the crane to hoist the new unit to the roof for installation. We were advised that this task would take approximately 3 weeks to complete. We were also advised that during that timeframe, the indoor air quality unit would be cleaning the interior of the ductwork and treating/coating the interior surfaces with an antimicrobial finish. Both Dave and I have experienced 3 previous major HVAC shutdowns, two which were planned, and one which was an emergency. We recognized, through our past experience, that the most ideal time of year to effect repairs was in late fall, as the cooler outside temperatures would facilitate maintenance of interior temperatures within the Vivarium. Thus we won a concession to delay the project until late October of 1999.

Dave and I discussed contingencies for the three-week outage. One option was to transfer all animals to other facilities on campus, but full occupancy at all facilities precluded that option. A second option was to completely depopulate the Vivarium, but a number of longterm ongoing studies could not afford the loss of valuable animals. A third option was to secure alternative facilities off-campus, but costs for rent, renovation, moving, and utilities would have exceeded three times the annual budget of the Central Vivarium for that 3 week period. The fourth option, which was selected, involved pre-planning with Physical Plant to acquire and install mobile HEPA-filtered air conditioning units and temporary ductwork to temper the air and maintain cooling within the Vivarium during the 3 week period that the main HVAC was down.

Approximately two days before the system was set to be shut down, Dave Gemmel was informed that the process of cleaning the ductwork would take 6 weeks, not the 3 weeks as previously stipulated. Based on indoor air quality requirements stipulated by the Commonwealth of Virginia Department of Environmental Quality, we could not prevent the workers from performing the duct cleaning at that time. Visits to the other campus

facilities confirmed that no space was available at that time for transfer of the animals. Two main HEPA-filtered air conditioning units were delivered and installed, along with ductwork, on October 17 and 18, 1999. Dave Gemmell coordinated schedules with the air quality crew to ensure that animal rooms were depopulated, by transferring animals to an open room, before entry of personnel for cleaning. All areas where cleaning took place were isolated with floor to ceiling plastic drapes.

On October 18, 1999 the HVAC system supplying the Central Vivarium was shut down. Overnight, there was a 7.2°F increase in the temperature in the room housing your mice. Unbeknownst to us, the mechanical room located below Rooms 7 & 8 was generating significant heat which directly impacted room temperatures within the Vivarium. In the 3 previous HVAC shutdowns mentioned earlier, none had involved shutdown of all primary and backup exhaust fans. The duct cleaning necessitated shutdown of all exhaust fans. Neither we nor Physical Plant had anticipated the significant impact of heat generation and transfer from the mechanical room on the Vivarium temperatures, as this was the first time in 18 years, since the building has been on line, that full shutdown of the HVAC system had occurred. Upon recognizing the increased temperatures, Dave Gemmell contacted Physical Plant to request the immediate installation of additional cooling units and fans. Two more units were delivered that same day, but required a water source for their operation. Special connectors had to be ordered, and were not received for approximately 5 days. A third unit was also delivered later that day, but it was determined that the breaker/circuit was not configured to accommodate the additional load. This required coring of the 1' concrete ceiling to run a line from another circuit. This took approximately 4 days to complete before the hookup was made.

Further compounding the situation was an unexpected heat wave at the end of October and into November, with outside temperatures approximately 30-40°F higher than would be typically expected. Our decision to initiate the project in late October was predicated on my 14 years in Blacksburg, and the relatively stable late Fall weather. Initially, Physical Plant configured the main HEPA-filtered air conditioning units to draw supply air from the hallway, but after Dave Gemmell and I pointed out that the system should be drawing outside air to ensure lower temperatures, they complied and installed additional ducting to the outside. However, higher than normal daytime and nighttime temperatures overrode any benefit of outside supply air, and temperatures again climbed.

By the 11th of November, the additional air conditioning units had been brought on line, and temperatures were moderated, but still not back to nominal levels. Around this time, a small number of the mice belonging to you and Dr. Klein were transferred to the CVM Phase IV facility. Space constraints precluded transfer of the remainder of your animals.

We were then told that duct cleaning was behind schedule, and might continue through the first week in December. Another warm front during the last week in November caused room/facility temperatures to climb again. After additional delays, the HVAC system was restored to full operation on December 27,1999.

I want to state, for the record, and for the benefit of your study sponsor, that you, as PI for your project, had not been adequately consulted prior to this shutdown related to the scope and magnitude of the potential environmental extremes, and this precluded you from exercising your scientific judgement to plan for this contingency. We recognize that had you, and we, known that the temperature extremes would have been that extensive, you could have delayed portions of your study to avoid the influence of environmental variables that were ultimately experienced. Our decisions and actions in this matter were predicated on our combined 38 years of lab animal facility operation and management, and our combined 24 years of operation of the Central Vivarium at Virginia

Tech. Our decisions were undermined, as previously stated, by unforeseen complications with generation of excessive heat in the basement mechanical room, with atypical/aberrant warm fronts during the late Fall months of 1999, and with abrogated agreements regarding adherence to schedules/timelines by the indoor air quality management and staff. It was fortunate that the temperature extremes (up to a high of 92°F) did not result in the death or illness of animals housed within the Central Vivarium during that 2 month period. And to date, no evidence of clinical illness has been found in any of the animal species housed within the Vivarium. But undoubtedly, the high temperatures had either a subtle or a profound effect on the data which you collected.

Attached are the room logs for Room 7 for the months of October, November, and December 1999, which provide the daily high and low temperatures for the room. For comparative purposes, room logs for November and December 1998 for Room 8 (where your animals were housed at the time) have been provided. A comparison of those temperatures is provided in two graphs attached to this memo.

If you or your sponsor require any additional information/data regarding this matter, please let know, and I will be happy to assemble and provide the requested information.

DAILY ACTIVITY ROOM LOG

Room # 7 Month Oct Year 1999 Study ID _____

Date	Spec. Code*	Temp		Check feed/ water per SOP 117 (√)	Check autowater per SOP 106 (√)	Check time clock per SOP 105 (√)	Initial/date	Floors cleaned per SCP 119 S = Sweep (√) M = Mop (√)			Health Checks per SCP 105 (initial/date)	
		Hi	Lo					S	M	Initial/date	AM	PM
1	M	21	21	✓	✓	✓	PKS 10/1/99	✓	✓	PKS 10/1/99	PKS 10/1/99	PKS 10/1/99
2	M	21	21	✓	✓	✓	PS 10/2/99	✓		PS 10/2/99	PS 10/2/99	PS 10/2/99
3	M	21	20	✓	✓	✓	PS 10/3/99	✓		PS 10/3/99	PS 10/3/99	PS 10/3/99
4	M	21	20	✓	✓	✓	DJU 10-4-99	✓	✓	DJU 10-4-99	DJU 10-4-99	DJU 10-4-99
5	M	21	20	✓	✓	✓	DJU 10-5-99	✓	✓	DJU 10-5-99	DJU 10-5-99	DJU 10-5-99
6	M	21	20	✓	✓	✓	DJU 10-6-99	✓	✓	DJU 10-6-99	DJU 10-6-99	DJU 10-6-99
7	M	21	20	✓	✓	✓	PS 10/7/99	✓	✓	PS 10/7/99	PS 10/7/99	PS 10/7/99
8	M	21	20	✓	✓	✓	LDC 10-8-99	✓	✓	LDC 10-8-99	LDC 10-8-99	DJU 10-8-99
9	M	21	21	✓	✓	✓	PS 10/9/99	✓		PS 10/9/99	PS 10/9/99	PS 10/9/99
10	M	21	21	✓	✓	✓	PS 10/10/99	✓		PS 10/10/99	PS 10/10/99	PS 10/10/99
11	M	21	21	✓	✓	✓	PS 10/11/99	✓	✓	PS 10/11/99	PS 10/11/99	PS 10/11/99
12	M	22	20	✓	✓	✓	DJU 10-12-99	✓	✓	DJU 10-12-99	DJU 10-12-99	DJU 10-12-99
13	M	22	20	✓	✓	✓	DJU 10-13-99	✓	✓	DJU 10-13-99	DJU 10-13-99	DJU 10-13-99
14	M	21	20	✓	✓	✓	DJU 10-14-99	✓	✓	DJU 10-14-99	DJU 10-14-99	DJU 10-14-99
15	M	22	20	✓	✓	✓	DJU 10-15-99	✓	✓	DJU 10-15-99	DJU 10-15-99	DJU 10-15-99
16	M	21	20	✓	✓	✓	PS 10/16/99	✓		PS 10/16/99	PS 10/16/99	PS 10/16/99
17	M	21	21	✓	✓	✓	PS 10/17/99	✓		PS 10/17/99	PS 10/17/99	PS 10/17/99
18 (Shut down)	M	23	22	✓	✓	✓	DJU 10-18-99	✓	✓	DJU 10-18-99	DJU 10-18-99	DJU 10-18-99
19	M	27	26	✓	✓	✓	DJU 10-19-99	✓	✓	DJU 10-19-99	DJU 10-19-99	DJU 10-19-99
20	M	28	27	✓	✓	✓	DJU 10-20-99	✓	✓	DJU 10-20-99	DJU 10-20-99	DJU 10-20-99
21	M	29	29	✓	✓	✓	DJU 10-21-99	✓	✓	DJU 10-21-99	DJU 10-21-99	DJU 10-21-99
22	M	30	30	✓	✓	✓	DJU 10-22-99	✓	✓	DJU 10-22-99	DJU 10-22-99	DJU 10-22-99
23	M	30	30	✓	✓	✓	PS 10/23/99	✓		PS 10/23/99	PS 10/23/99	PS 10/23/99
24	M	30	30	✓	✓	✓	PS 10/24/99	✓		PS 10/24/99	PS 10/24/99	PS 10/24/99
25	M	31	29	✓	✓	✓	DJU 10-25-99	✓	✓	DJU 10-25-99	DJU 10-25-99	DJU 10-25-99
26	M	31	30	✓	✓	✓	DJU 10-26-99	✓	✓	DJU 10-26-99	DJU 10-26-99	DJU 10-26-99
27	M	31	28	✓	✓	✓	DJU 10-27-99	✓	✓	DJU 10-27-99	DJU 10-27-99	DJU 10-27-99
28	M	29	28	✓	✓	✓	DJU 10-28-99	✓	✓	DJU 10-28-99	DJU 10-28-99	DJU 10-28-99
29	M	31	31	✓	✓	✓	DJU 10-29-99	✓	✓	DJU 10-29-99	DJU 10-29-99	DJU 10-29-99
30	M	31	31	✓	✓	✓	PS 10/30/99	✓		PS 10/30/99	PS 10/30/99	PS 10/30/99
31	M	32	31	✓	✓	✓	PS 10/31/99	✓		PS 10/31/99	PS 10/31/99	PS 10/31/99

*Species Code:

M=mouse	GP=guinea pig	G=gerbil	GR=grouse
R=rat	C=cat	H=hamster	S=sheep
RB=rabbit	CK=chicken	Q=quail	Other:

Room # 7 Month NOV Year 1999 Study ID _____

Date	Spec. Code*	Temp		Check feed/ water per SOP 117 (√)	Check autowater per SOP 105 (√)	Check time clock per SOP 105 (√)	Initial/date	Floors cleaned per SCP 119 S = Sweep (√) M = Mop (√)		Health Checks per SCP 105 (initial/date)		
		Hi	Lo					S	M	Initial/date	AM	PM
1	M	33	32	✓	✓	✓	DJW 11-1-99	✓	✓	DJW 11-1-99	DJW 11-1-99	DJW 11-1-99
2	M	33	32	✓	✓	✓	DJW 11-2-99	✓	✓	DJW 11-2-99	DJW 11-2-99	DJW 11-2-99
3	M	28	28	✓	✓	✓	DJW 11-3-99	✓	✓	DJW 11-3-99	DJW 11-3-99	DJW 11-3-99
4	M	28	26	✓	✓	✓	DJW 11-4-99	✓	✓	DJW 11-4-99	DJW 11-4-99	DJW 11-4-99
5	M	28	28	✓	✓	✓	DJW 11-5-99	✓	✓	DJW 11-5-99	DJW 11-5-99	DJW 11-5-99
6	M	29	28	✓	✓	✓	PS 11/6/99	✓		PS 11/6/99	PS 11/6/99	PS 11/6/99
7	M	30	28	✓	✓	✓	PS 11/7/99	✓		PS 11/7/99	PS 11/7/99	PS 11/7/99
8	M	30	27	✓	✓	✓	DJW 11-8-99	✓	✓	DJW 11-8-99	DJW 11-8-99	DJW 11-8-99
9	M	28	27	✓	✓	✓	DJW 11-9-99	✓	✓	DJW 11-9-99	DJW 11-9-99	DJW 11-9-99
10	M	27	26	✓	✓	✓	DJW 11-10-99	✓	✓	DJW 11-10-99	DJW 11-10-99	DJW 11-10-99
11	M	26	25	✓	✓	✓	DJW 11-11-99	✓	✓	DJW 11-11-99	DJW 11-11-99	DJW 11-11-99
12	M	25	25	✓	✓	✓	DJW 11-12-99	✓	✓	DJW 11-12-99	DJW 11-12-99	DJW 11-12-99
13	M	25	25	✓	✓	✓	PS 11/13/99	✓		PS 11/13/99	PS 11/13/99	PS 11/13/99
14	M	25	24	✓	✓	✓	PS 11/14/99	✓		PS 11/14/99	PS 11/14/99	PS 11/14/99
15	M	25	23	✓	✓	✓	DJW 11-15-99	✓	✓	DJW 11-15-99	DJW 11-15-99	DJW 11-15-99
16	M	25	23	✓	✓	✓	DJW 11-16-99	✓	✓	DJW 11-16-99	DJW 11-16-99	DJW 11-16-99
17	M	24	22	✓	✓	✓	DJW 11-17-99	✓	✓	DJW 11-17-99	DJW 11-17-99	DJW 11-17-99
18	M	24	23	✓	✓	✓	DJW 11-18-99	✓	✓	DJW 11-18-99	DJW 11-18-99	DJW 11-18-99
19	M	24	23	✓	✓	✓	DJW 11-19-99	✓	✓	DJW 11-19-99	DJW 11-19-99	DJW 11-19-99
20	M	25	23	✓	✓	✓	SC 11/20/99	✓		SC 11/20/99	SC 11/20/99	SC 11/20/99
21	M	26	26	✓	✓	✓	PS 11/21/99	✓		PS 11/21/99	PS 11/21/99	PS 11/21/99
22	M	28	26	✓	✓	✓	DJW 11-22-99	✓	✓	DJW 11-22-99	DJW 11-22-99	DJW 11-22-99
23	M	29	28	✓	✓	✓	DJW 11-23-99	✓	✓	DJW 11-23-99	DJW 11-23-99	DJW 11-23-99
24	M	30	28	✓	✓	✓	DJW 11-24-99	✓	✓	DJW 11-24-99	DJW 11-24-99	DJW 11-24-99
25	M	28	28	✓	✓	✓	DJW 11-25-99	✓	✓	DJW 11-25-99	DJW 11-25-99	DJW 11-25-99
26	M	28	28	✓	✓	✓	SC 11-26-99	✓	✓	SC 11/26/99	SC 11/26/99	SC 11/26/99
27	M	28	26	✓	✓	✓	SC 11/27/99	✓		SC 11/27/99	SC 11/27/99	SC 11/27/99
28	M	28	26	✓	✓	✓	SC 11/28/99	✓		SC 11/28/99	SC 11/28/99	SC 11/28/99
29	M	28	26	✓	✓	✓	SC 11/29/99	✓	✓	SC 11/29/99	SC 11/29/99	SC 11/29/99
30	M	25	24	✓	✓	✓	DJW 11-30-99	✓	✓	DJW 11-30-99	DJW 11-30-99	DJW 11-30-99
31												

*Species Code:

M=mouse	GP=guinea pig	G=gerbil	GR=grouse
R=rat	C=cat	H=hamster	S=sheep
RB=rabbit	CK=chicken	Q=quail	Other:

LABORATORY ANIMAL RESOURCES

DAILY ACTIVITY ROOM LOG

Room # 7 Month Dec Year 1998 Study ID _____

Date	Spec. Code*	Temp		Check feed/ water per SOP 117 (√)	Check autowater per SOP 106 (√)	Check time clock per SOP 106 (√)	Initial/date	Floors cleaned per SOP 119 S = Sweep (√) M = Mop (√)			Health Checks per SOP 106 (initial/date)	
		Hi	Lo					S	M	Initial/date	AM	PM
1	M	28	25	✓	✓	✓	DJN 12-1-98	✓	✓	DJN 12-1-98	DJN 12-1-98	DJN 12-1-98
2	M	27	26	✓	✓	✓	DJN 12-2-98	✓	✓	DJN 12-2-98	DJN 12-2-98	DJN 12-2-98
3	M	27	26	✓	✓	✓	DJN 12-2-98	✓	✓	DJN 12-3-98	DJN 12-3-98	DJN 12-3-98
4	M	27	26	✓	✓	✓	SC 12/4/98	✓		SC 12/4/98	SC 12/4/98	SC 12/4/98
5	M	27	25	✓	✓	✓	SC 12/5/98	✓		SC 12/5/98	SC 12/5/98	SC 12/5/98
6	M	29	27	✓	✓	✓	DJN 12-6-98	✓	✓	DJN 12-6-98	DJN 12-6-98	DJN 12-6-98
7	M	29	27	✓	✓	✓	MQS 12-7-98	✓	✓	MQS 12-7-98	MQS 12-7-98	MQS 12-7-98
8	M	24	24	✓	✓	✓	SC 12/8/98	✓	✓	SC 12/8/98	SC 12/8/98	SC 12/8/98
9	M	25	23	✓	✓	✓	MQS 12-9-98	✓	✓	MQS 12-9-98	MQS 12-9-98	MQS 12-9-98
10	M	25	23	✓	✓	✓	DJN 12-10-98	✓	✓	DJN 12-10-98	DJN 12-10-98	DJN 12-10-98
11	M	25	24	✓	✓	✓	SC 12-11-98	✓		SC 12/11/98	SC 12/11/98	SC 12/11/98
12	M	27	26	✓	✓	✓	SC 12/12/98	✓		SC 12/12/98	SC 12/12/98	SC 12/12/98
13	M	27	27	✓	✓	✓	SC 12/13/98	✓	✓	SC 12/13/98	SC 12/13/98	SC 12/13/98
14	M	28	28	✓	✓	✓	DJN 12-14-98	✓	✓	DJN 12/14/98	DJN 12/14/98	DJN 12-14-98
15	M	25	23	✓	✓	✓	DJN 12-15-98	✓	✓	DJN 12-15-98	DJN 12-15-98	DJN 12-15-98
16	M	26	23	✓	✓	✓	DJN 12-16-98	✓	✓	DJN 12-16-98	DJN 12-16-98	DJN 12-16-98
17	M	26	23	✓	✓	✓	MQS 12-17-98	✓	✓	MQS 12-17-98	MQS 12-17-98	MQS 12-17-98
18	M	25	24	✓	✓	✓	SC 12/18/98	✓		SC 12/18/98	SC 12/18/98	SC 12/18/98
19	M	25	25	✓	✓	✓	SC 12/19/98	✓		SC 12/19/98	SC 12/19/98	SC 12/19/98
20	M	27	24	✓	✓	✓	DJN 12-20-98	✓	✓	DJN 12-20-98	DJN 12-20-98	DJN 12-20-98
21	M	25	24	✓	✓	✓	DJN 12-21-98	✓	✓	DJN 12-21-98	DJN 12-21-98	DJN 12-21-98
22	M	26	23	✓	✓	✓	DJN 12-22-98	✓	✓	DJN 12-22-98	DJN 12-22-98	DJN 12-22-98
23	M	26	23	✓	✓	✓	DJN 12-23-98	✓	✓	DJN 12-23-98	DJN 12-23-98	DJN 12-23-98
24	M	25	23	✓	✓	✓	DJN 12-24-98	✓	✓	DJN 12-24-98	DJN 12-24-98	DJN 12-24-98
25	M	24	23	✓	✓	✓	SC 12/25/98	✓		SC 12/25/98	SC 12/25/98	SC 12/25/98
26	M	25	24	✓	✓	✓	SC 12/26/98	✓		SC 12/26/98	SC 12/26/98	SC 12/26/98
27	M	25	23	✓	✓	✓	DJN 12-27-98	✓	✓	DJN 12-27-98	DJN 12-27-98	DJN 12-27-98
28	M	24	20	✓	✓	✓	DJN 12-28-98	✓	✓	DJN 12-28-98	DJN 12-28-98	DJN 12-28-98
29	M	22	20	✓	✓	✓	DJN 12-29-98	✓	✓	DJN 12-29-98	DJN 12-29-98	DJN 12-29-98
30	M	22	20	✓	✓	✓	DJN 12-30-98	✓	✓	DJN 12-30-98	DJN 12-30-98	DJN 12-30-98
31	M	22	21	✓	✓	✓	MQS 12-31-98	✓	✓	MQS 12-31-98	MQS 12-31-98	MQS 12-31-98

*Species Code:

M=mouse	GP=guinea pig	G=gerbil	GR=grouse
R=rat	C=cat	H=hamster	S=sheep
RB=rabbit	CK=chicken	Q=quail	Other:

LABORATORY ANIMAL RESOURCES

DAILY ACTIVITY ROOM LOG

Room # 9 Month Nov. Year 1998 Study ID _____

Date	Spec. Code*	Temp		Check feed/ water per SOP 117 (√)	Check autowater per SOP 106 (√)	Check time clock per SOP 106 (√)	Initial/date	Floors cleaned per SOP 119 S = Sweep (√) M = Mop (√)			Health Checks per SOP 106 (initial/date)	
		Hi	Lo					S	M	Initial/date	AM	PM
1	M	23	22	✓	✓	✓	EB 11-1-98	✓		EB 11-1-98	EB 11-1-98	EB 11-1-98
2	M	22	22	✓	✓	✓	EB 11-2-98	✓	✓	EB 11-2-98	EB 11-2-98	EB 11-2-98
3	M	22	22	✓	✓	✓	EB 11-3-98	✓	✓	EB 11-3-98	EB 11-3-98	EB 11-3-98
4	M	22	22	✓	✓	✓	MCB 11-4-98	✓	✓	MCB 11-4-98	MCB 11-4-98	MCB 11-4-98
5	M	24	23	✓	✓	✓	MCB 11-5-98	✓	✓	MCB 11-5-98	MCB 11-5-98	MCB 11-5-98
6	M	25	22	✓	✓	✓	EB 11-6-98	✓	✓	EB 11-6-98	EB 11-6-98	EB 11-6-98
7	M	23	22	✓	✓	✓	EB 11-7-98	✓		EB 11-7-98	EB 11-7-98	EB 11-7-98
8	M	23	22	✓	✓	✓	EB 11-8-98	✓		EB 11-8-98	EB 11-8-98	EB 11-8-98
9	M	22	22	✓	✓	✓	DSW 11-9-98	✓	J	DSW 11-9-98	DSW 11-9-98	DSW 11-9-98
10	M	23	22	✓	✓	✓	EB 11-10-98	✓	✓	EB 11-10-98	EB 11-10-98	EB 11-10-98
11*	M	24	22	✓	✓	✓	EB 11-11-98	✓	J	EB 11-11-98	EB 11-11-98	EB 11-11-98
12	M	24	22	✓	✓	✓	KES 11-12-98	✓	✓	KES 11-12-98	KES 11-12-98	KES 11-12-98
13	M	22	22	✓	✓	✓	EB 11-13-98	✓	✓	EB 11-13-98	EB 11-13-98	EB 11-13-98
14	M	22	22	✓	✓	✓	EB 11-14-98	✓		EB 11-14-98	EB 11-14-98	EB 11-14-98
15	M	22	22	✓	✓	✓	EB 11-15-98	✓		EB 11-15-98	EB 11-15-98	EB 11-15-98
16	M	23	22	✓	✓	✓	DSW 11-16-98	✓	✓	DSW 11-16-98	DSW 11-16-98	DSW 11-16-98
17	M	23	22	✓	✓	✓	EB 11-17-98	✓	✓	EB 11-17-98	EB 11-17-98	EB 11-17-98
18	M	22	22	✓	✓	✓	EB 11-18-98	✓	✓	EB 11-18-98	EB 11-18-98	EB 11-18-98
19	M	22	22	✓	✓	✓	EB 11-19-98	✓	✓	EB 11-19-98	EB 11-19-98	EB 11-19-98
20	M	23	22	✓	✓	✓	EB 11-20-98	✓	✓	EB 11-20-98	EB 11-20-98	EB 11-20-98
21	M	23	23	✓	✓	✓	EB 11-21-98	✓		EB 11-21-98	EB 11-21-98	EB 11-21-98
22	M	23	22	✓	✓	✓	EB 11-22-98	✓		EB 11-22-98	EB 11-22-98	EB 11-22-98
23	M	23	21	✓	✓	✓	EB 11-23-98	✓	✓	EB 11-23-98	EB 11-23-98	EB 11-23-98
24	M	23	21	✓	✓	✓	EB 11-24-98	✓	✓	EB 11-24-98	EB 11-24-98	EB 11-24-98
25	M	24	21	✓	✓	✓	EB 11-25-98	✓	✓	EB 11-25-98	EB 11-25-98	EB 11-25-98
26	M	24	21	✓	✓	✓	EB 11-26-98	✓		EB 11-26-98	EB 11-26-98	EB 11-26-98
27	M	24	21	✓	✓	✓	EB 11-27-98	✓	✓	EB 11-27-98	EB 11-27-98	EB 11-27-98
28	M	24	21	✓	✓	✓	EB 11-28-98	✓		EB 11-28-98	EB 11-28-98	EB 11-28-98
29	M	24	21	✓	✓	✓	EB 11-29-98	✓		EB 11-29-98	EB 11-29-98	EB 11-29-98
30	M	24	24	✓	✓	✓	EB 11-30-98	✓	✓	EB 11-30-98	EB 11-30-98	EB 11-30-98
31												

*Species Code:

M=mouse	GP=guinea pig	G=gerbil	GR=grouse
R=rat	C=cat	H=hamster	S=sheep
RB=rabbit	CK=chicken	Q=quail	Other:

LABORATORY ANIMAL RESOURCES

DAILY ACTIVITY ROOM LOG

Room # 8 Month Dec. Year 1998 Study ID _____

Date	Spec. Code*	Temp		Check feed/ water per SOP 117 (√)	Check autowater per SOP 106 (√)	Check time clock per SOP 106 (√)	Initial/date	Floors cleaned per SOP 119 S = Sweep (√) M = Mop (√)			Health Checks per SOP 106 (initial/date)	
		Hi	Lo					S	M	Initial/date	AM	PM
1	M	24	21	✓	✓	✓	12-1-98	✓	✓	12-1-98	12-1-98	12-1-98
2	M	24	21	✓	✓	✓	12-2-98	✓	✓	12-2-98	12-2-98	12-2-98
3	M	24	22	✓	✓	✓	KES 12-3-98	✓	✓	KES 12-3-98	KES 12-3-98	KES 12-3-98
4	M	24	23	✓	✓	✓	12-4-98	✓	✓	12-4-98	12-4-98	12-4-98
5	M	24	23	✓	✓	✓	12-5-98	✓		12-5-98	12-5-98	12-5-98
6	M	24	23	✓	✓	✓	12-6-98	✓		12-6-98	12-6-98	12-6-98
7	M	24	23	✓	✓	✓	12-7-98	✓	✓	12-7-98	12-7-98	12-7-98
8	M	24	23	✓	✓	✓	12-8-98	✓	✓	12-8-98	12-8-98	12-8-98
9	M	24	23	✓	✓	✓	12-9-98	✓	✓	12-9-98	12-9-98	12-9-98
10	M	24	22	✓	✓	✓	12-10-98	✓	✓	12-10-98	12-10-98	12-10-98
11	M	24	23	✓	✓	✓	12-11-98	✓	✓	12-11-98	12-11-98	12-11-98
12	M	23	22	✓	✓	✓	KES 12-12-98	✓		KES 12-12-98	KES 12-12-98	KES 12-12-98
13	M	24	23	✓	✓	✓	KES 12/13/98	✓		KES 12/13/98	KES 12/13/98	KES 12/13/98
14	M	23	22	✓	✓	✓	12-14-98	✓	✓	12-14-98	12-14-98	12-14-98
15	M	23	22	✓	✓	✓	12-15-98	✓	✓	12-15-98	12-15-98	12-15-98
16	M	23	22	✓	✓	✓	12-16-98	✓	✓	12-16-98	12-16-98	12-16-98
17	M	23	22	✓	✓	✓	12-17-98	✓	✓	12-17-98	12-17-98	12-17-98
18	M	23	22	✓	✓	✓	12-18-98	✓	✓	12-18-98	12-18-98	12-18-98
19	M	23	22	✓	✓	✓	KES 12-19-98	✓		KES 12-19-98	KES 12-19-98	KES 12-19-98
20	M	23	22	✓	✓	✓	KES 12-20-98	✓		KES 12-20-98	KES 12-20-98	KES 12-20-98
21	M	23	23	✓	✓	✓	KES 12-21-98	✓	✓	KES 12-21-98	KES 12-21-98	KES 12-21-98
22	M	23	22	✓	✓	✓	KES 12-22-98	✓	✓	KES 12-22-98	KES 12-22-98	DJW 12-22-98
23	M	23	22	✓	✓	✓	12-23-98	✓	✓	12-23-98	12-23-98	12-23-98
24	M	23	22	✓	✓	✓	KES 12-24-98	✓		KES 12-24-98	KES 12-24-98	KES 12-24-98
25	M	23	22	✓	✓	✓	12-25-98	✓		DJW 12-25-98	12-25-98	12-25-98
26	M	23	22	✓	✓	✓	KES 12-26-98	✓		KES 12-26-98	KES 12-26-98	KES 12-26-98
27	M	23	22	✓	✓	✓	KES 12-27-98	✓		KES 12-27-98	KES 12-27-98	KES 12-27-98
28	M	23	22	✓	✓	✓	KES 12-28-98	✓	✓	KES 12-28-98	KES 12-28-98	KES 12-28-98
29	M	23	23	✓	✓	✓	KES 12-29-98	✓	✓	KES 12-29-98	KES 12-29-98	KES 12-29-98
30	M	23	23	✓	✓	✓	12-30-98	✓	✓	12-30-98	12-30-98	12-30-98
31	M	23	23	✓	✓	✓	12-31-98	✓		12-31-98	12-31-98	12-31-98

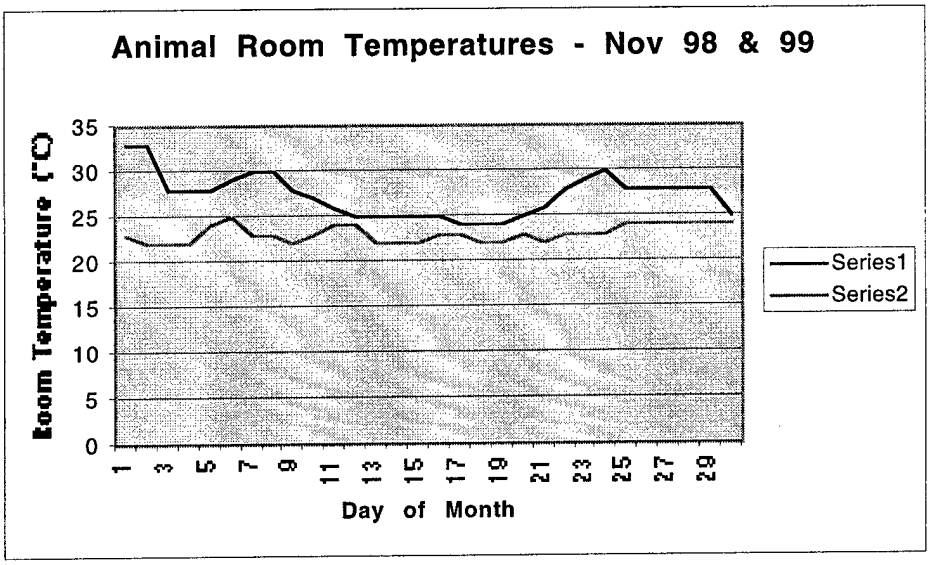
*Species Code:

M=mouse	GP=guinea pig	G=gerbil	GR=grouse
R=rat	C=cat	H=hamster	S=sheep
RB=rabbit	CK=chicken	Q=quail	Other:

ANIMAL ROOM TEMPERATURES (°C)
FOR ROOMS 7 & 8
IN THE CENTRAL VIVARIUM
Litton-Reaves Hall
Virginia Tech
Blacksburg, VA

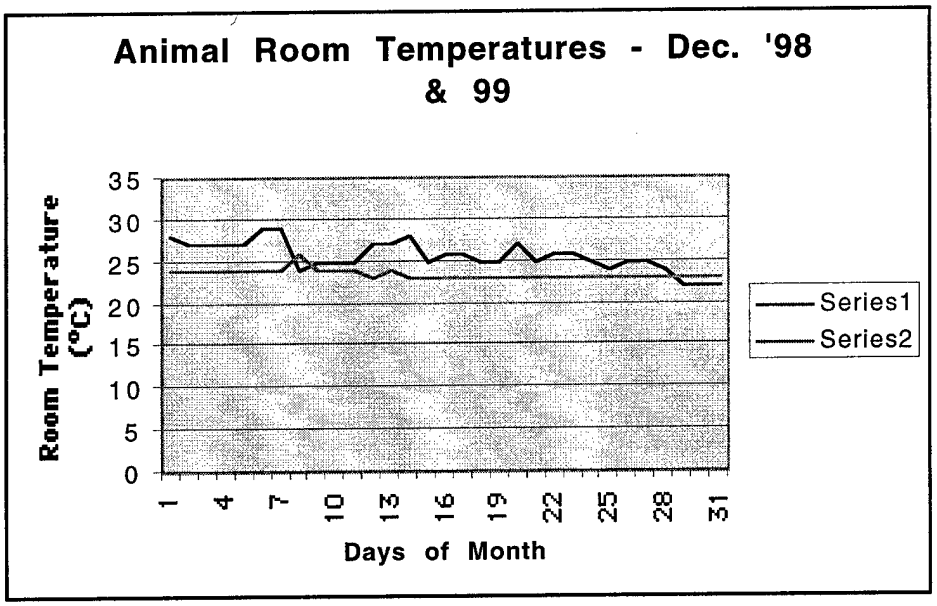
Rm 7 Oct 99 Hi Temps prior to shutdown	Rm 7 Oct 99 Hi Temps after shutdown	Rm 7 Oct 99 Hi Temps full month	Rm 7 Oct 99 Lo Temps prior to shutdown	Rm 7 Oct 99 Lo Temps after shutdown	Rm 7 Oct 99 Lo Temps full month	Rm 7 Nov 99 Hi Temps	Rm 7 Nov 99 Lo Temps	Rm 7 Dec 99 Hi Temps	Rm 7 Dec 99 Lo Temps	Rm 8 Nov 98 Hi Temps	Rm 8 Nov 98 Lo Temps	Rm 8 Dec 98 Hi Temps	Rm 8 Dec 98 Lo Temps
21	27	21	21	26	21	33	32	28	25	23	22	24	21
21	28	21	21	27	21	33	32	27	26	22	22	24	21
21	29	21	20	29	20	28	28	27	26	22	22	24	22
21	30	21	20	30	20	28	26	27	26	22	22	24	23
21	30	21	20	30	20	28	28	27	27	24	23	24	23
21	30	21	20	30	20	29	28	29	27	25	22	24	23
21	31	21	20	29	20	30	28	29	27	23	22	24	23
21	31	21	20	30	20	30	27	24	24	23	22	26	23
21	31	21	21	28	21	28	27	25	23	22	22	24	23
21	29	21	21	28	21	27	26	25	23	23	22	24	22
21	31	21	21	31	21	26	25	25	24	24	22	24	23
22	31	22	20	31	20	25	25	27	26	24	22	23	22
22	32	22	20	31	20	25	25	27	27	22	22	24	23
21	Σ 390	21	20	Σ 380	20	25	24	28	28	22	22	23	22
22	u=30	22	20	u=29.23	20	25	23	25	23	22	22	23	22
21		21	20		20	25	23	26	23	23	22	23	22
21		21	21		21	24	22	26	23	23	22	23	22
23		23	22		22	24	23	25	24	22	22	23	22
Σ 383		27	Σ 368		26	24	23	25	25	22	22	23	22
u=21.28		28	u=20.44		27	25	23	27	24	23	22	23	22
		29			29	26	26	25	24	22	22	23	23
		30			30	28	26	26	23	23	22	23	22
		30			30	29	28	26	23	23	22	23	22
		30			30	30	28	25	23	23	21	23	22
		31			29	28	28	24	23	24	21	23	22
		31			30	28	28	25	24	24	21	23	22
		31			28	28	26	25	23	24	21	23	22
		29			28	28	26	24	20	24	21	23	22
		31			31	28	26	22	20	24	21	23	23
		31			31	25	24	22	20	24	24	23	23
		32			31	Σ 820	Σ 784	22	21	Σ 691	Σ 656	23	23
		Σ 773			Σ 748	u=27.33	u=26.13	Σ 795	Σ 745	u=23.03	u=21.87	Σ 727	Σ 692
		u=24.9			u=24.12			u=25.65	u=24.03			u=23.45	u=22.32

"High" Temperature Readings



Series 2, bottom line = Nov. 1998
u=23.03 (73.45°F)
Series 1, top line = Nov. 1999
u=27.33 (81.19°F)

"High" Temperature Readings



Series 2, bottom line = Dec. 1998
u=23.45 (74.21°F)
Series 1, top line = Dec. 1999
u=25.65 (78.11°F)