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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Chlorpyrifos (P.C. Code 059101) - Toxicology Data Review

FROM: Susan L. Makris, M.S.
Toxicology Branch I
Health Effects Division (7509C)

TO: Deborah Smegal, Ph.D.
Reregistration Branch 3
Health Effects Division (7509C)

THRU: Alberto Protzel, Ph.D., Branch Senior Scientist
Toxicology Branch I
Health Effects Division (7509C)

TASK ID: DP Barcode: D254907 Submission: S559875
PC Code: 059101 Chemical: Chlorpyrifos

REGISTRANT: Dow AgroSciences LLC

Action requested: Review the following supplementary data:

Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; SAS Statistical Output. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, Undated. MRID not assigned. Unpublished.

Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; Report Supplement 2 - Reanalysis of Morphometric Data. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, March 19, 1999. MRID 44787301. Unpublished.

These data were submitted in supplement to the following study:

Hoberman, A. M. (1998) Developmental neurotoxicity study of chlorpyrifos

administered orally via gavage to CrI:CD®BR VAF/Plus® presumed pregnant rats. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, May 1, 1998. MRID 44556901. Unpublished.

Hoberman, A.M. (1998) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to CrI:CD®BR VAF/Plus® presumed pregnant rats; Report Supplement 1 (pathology data). Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K- 044793-109, September 23, 1998. MRID 44661001. Unpublished.

Background Information

A developmental neurotoxicity study with chlorpyrifos (MRID 44556901), including a separate supplementary report addressing terminal pathology data (MRID 44661001), was submitted to the Agency by the Registrant (Dow AgroSciences). In the review of these data (HED Doc. No. 013081), it was noted that there were outstanding issues which had not been adequately addressed by the original study report documents. The study was graded **unacceptable**, due to an inadequate presentation of the statistical data analysis which resulted in the inability to determine the definitive developmental neurotoxicity NOAEL and LOAEL for the offspring.

One of the outstanding critical issues concerned review of the morphometric data for Subset 1 (PND 12) and Subset 4 (PND 66) males and females in the developmental neurotoxicity study with chlorpyrifos (HED Doc. No. 013081). These issues originated with the manner in which the statistical analyses of the brain measurements were presented and interpreted. The original report stated that the statistical tests were conducted at $\alpha = 0.02$; it was also unclear exactly how PND 12 and PND 66 data were analyzed, and whether the analysis had included data from mid- and low-dose measurements.

Revised EXECUTIVE SUMMARY

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (GD 6) through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on GD 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F₁ generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation

were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.

The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F₁ generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); bodyweight gains were reduced in these animals during the same period (↓5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17-19%) and the Subset 4 (PND 66) high-dose males (↓10%). For the F₁ generation adults, body weights of the high-dose males were decreased at PND 22 through 66 (↓11-17% vs controls). High-dose F₁ adult females also weighed less than controls at PND 22 (↓17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (↓13% vs controls) and PND 40-66 interval (↓7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (↓13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/ litter (↓27%) and live litter size at culling (↓16%), pup viability index (↓29%), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing

retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high dose male and female pups on PND 14 (↓56% in males and ↓37% in females), and increased in high dose females on PNDs 18 and 22 (↑51% on both days). On PND 61, motor activity was increased for both sexes (↑16-17%). There was a statistically significant increase (↑16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 (not statistically significant) compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑13% vs controls), reduced anterior to posterior measurement of the cerebellum (↓24% vs controls), reduced height of the cerebellum (↓14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), and decreased thickness of the hippocampal gyrus (↓9% vs controls). High-dose female pups had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), decreased width of the caudate-putamen (↓10% vs controls), and decreased thickness of the hippocampal gyrus (↓12% vs controls). In Subset 4 F1 animals, killed on PND 66, morphometric analysis revealed significantly decreased parietal cortex measurements in high-dose (↓5%) and mid-dose (↓4%) females, as compared to controls. Decreases in the thickness of the hippocampal gyrus in high-dose females (↓7%) resulted in contradictory statistical results when compared to controls; decreases in mid-dose (↓4%) females as compared to control were not found to be statistically significant. There was no evaluation of the morphometric data for low dose females at PND 66. Brain weight in high dose females was similar to control brain weight at day 66 (↓0.3%).

It is not possible to definitively classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, adverse findings in the adult (~PND 66) offspring, i.e., alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequellae of developmental exposure to chlorpyrifos.

Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined.**

Study Classification and Additional Information Required

While the offspring NOAEL and LOAEL have not yet been identified for this developmental neurotoxicity study, it is recognized that the study was well-conducted according to Agency guideline §83-6, and under GLP regulations. Remaining questions can be resolved with

additional information and statistical analysis, but there are no outstanding concerns regarding the quality of the animal data. It is recommended that the classification of the study be changed from **unacceptable** to **guideline-unacceptable** at this time, pending submission and review of additional morphometric data for PND 66 low-dose females (parietal cortex and hippocampus measurements).

cc: Kathleen Raffaele (7509C)
Mark Hartman (7508W)

EPA Reviewer: Susan L. Makris, M.S. _____

TOX 1 (7509C)

EPA Secondary Reviewer: Kathleen Raffaele, Ph.D. _____

RAB3 (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Developmental Neurotoxicity Study - Rat; Supplemental Analytical Data

OPPTS Number: 870.6300 OPP Guideline Number: §83-6

DP BARCODES: D251533, D254907 SUBMISSION CODES: S552530, S559875

P.C. CODE: 059101

TOX. CHEM. NO.: 219AA

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Dursban

CITATION: Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; SAS Statistical Output. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, Undated. MRID not assigned. Unpublished.

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Supplemental to:

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Hoberman, A.M. (1998) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; Report Supplement 1 (pathology data). Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, September 23, 1998. MRID 44661001. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268

I. Background Information

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II. Additional Data Submitted by Registrant

The Registrant submitted Report Supplement 2 - Reanalysis of Morphometric Data (MRID 44787301), to address the concerns of the Agency reviewers, and to present their views on the interpretation of the study data. Previously (December 4, 1998), the Registrant had also provided a volume of information entitled *Copy of Statistical Output from SAS File* to Agency reviewers (no MRID assigned), to the Health Effects Division (HED). A review of the information contained within these submissions follows.

A. Statistical Output from SAS File

The SAS output file submitted to the agency confirmed the original statistical findings, and included sufficient information to enable reviewers to determine how the original statistical analysis of the morphometric data was performed. The output file also included the statistics for some of the statistical interactions not included in the original report. Contrary to what was reported in the original review (HED document No. 013081, based on the original study report), the procedures used in the original statistical analysis appear to have been as follows:

- 1) The original Analysis of Variance (ANOVA) included data from the high-dose and control groups only (both sexes and time points); data from the mid- and low-dose groups (both sexes and time points at PND 12, mid-dose females at PND 66) were not included.
- 2) Detailed results of statistical findings were reported in the original review, and will not be repeated here, except to note that the results were not entirely in agreement with those of the hippocampal gyrus and parietal cortex measurements were: a) statistically significant decreases in hippocampal gyrus measurements, when high dose and control data for both sexes were compared across time points (main effect of Group, $p=0.001$); differences were consistent across sex and across time (interactions were not statistically significant: Group X Time and Group X Sex interactions both had p -values greater than 0.5); b) statistically

significant decreases in parietal cortex measurements, when high dose and control data were compared across time points (main effect of Group, $p=0.02$); differences were again consistent across time points and sexes (interactions were not statistically significant: Group X Time, $p=0.25$; Group X Sex, $p=0.098$), although the consistency of effect across sexes was less compelling.

B. Report Supplement 2 - Reanalysis of Morphometric Data (MRID 44787301)

Supplement 2 consists of two parts: a statistical reanalysis of the brain morphometric data; and arguments supporting the Registrant's interpretation of the morphometric findings. Copies of three cited references were also included in the submission.

1. Brain Weight and Morphometric Measurements

Statistical analysis of morphometric data

In this submission, brain morphometric data were reanalyzed using statistical procedures different from those used in the original report. In the current reanalysis, separate ANOVAs were conducted for each time point; data for males and females were also analyzed separately. In addition, all available treatment groups were included in each analysis (for day 12, all doses were evaluated for both sexes; for day 66, control and high dose males, control, mid, and high dose females). If the ANOVA was statistically significant at an alpha level of $p < 0.05$, the data from each dose level were compared to control by Dunnett's test conducted at $\alpha = 0.05$.

The results of the reanalysis provided in the supplement are summarized below in Table 1. The individual and summary morphometric data for PND 66 animals are presented in Attachment 1. The data for high-dose PND 12 male pups demonstrate significant decreases in the measurements of anterior to posterior cerebellum and cerebellum height. For high-dose PND 12 females, the caudate/putamen measurement was significantly decreased. At the mid-dose, only the cerebellum height was significantly decreased for PND 12 females. For PND 66 adult offspring, significant decreases in the measurement of the parietal cortex were noted for mid- and high-dose females. The decrease in the measurement of the hippocampal gyrus was not statistically different from control for either sex or time point. No significant differences were observed for any brain measurement for males at PND 66. The study pathologist dismissed the significant decreases in the parietal cortex of mid- and high-dose PND 66 females as being related to variability in the data; historical morphometric data were not provided to support this position.

As noted above, the current findings are not in complete agreement with those reported previously. Note that (for example), in contrast with the results of the earlier analysis, the differences between high dose and control for the hippocampal gyrus are no longer statistically significantly different for either sex or time point. Findings for parietal cortex remain significant only for high dose females at day 66; the difference between controls and mid-dose females (not included in the previous analysis) was found to be statistically significant.

Table 1: Brain weight and linear measurements expressed as percent of control	
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Variable	PND 12						PND 66		
	Males			Females			Males	Females	
	Low	Mid	High	Low	Mid	High	High	Mid	High
Brain weight	110.6	105.5	88.5*	98.9	97.6	91.3*	100.0	101.1	97.4
AP cerebrum	107.1	104.7	93.8	102.4	103.2	98.0	101.8	100.1	99.4
AP cerebellum	105.6	102.0	75.5*	95.3	103.7	94.3	99.7	99.7	97.6
Frontal cortex	100.9	100.3	94.4	100.9	98.5	99.4	98.7	100.2	98.9
Parietal cortex	108.4	108.4	94.0	99.7	99.1	94.5	102.1	95.8*	94.9*
Caudate/putamen	100.0	103.2	99.3	93.3	96.0	90.3*	98.0	99.1	105.0
Corpus callosum	103.4	99.0	100.0	93.2	99.0	89.1	92.8	105.2	95.4
Hippocampal gyrus	111.1	107.5	91.2	97.4	99.6	88.5	98.3	96.3	93.2
Cerebellum height	98.6	97.5	85.8*	90.4	88.8*	91.3	99.1	97.4	99.0
Ext germinal layer	103.1	107.6	101.3	94.0	106.5	105.6	-----	-----	-----

Statistical analyses were performed before conversion to percent of control.
 * p<0.05, Dunnett's.
 Data extracted from MRID 4478301, page 12.
 Note that findings for the ANOVAs were also significant for AP cerebellum, parietal cortex, and hippocampal gyrus for day 12 males, in the absence of significant effects for individual comparisons.

Variability in morphometric measurements

The Registrant argues that the limited sample size (n=6/sex) used for morphometric measurements (as specified by EPA testing guideline §83-6) resulted in increased variability and is a confounding factor in the interpretation of the morphometric data in this developmental neurotoxicity study with chlorpyrifos. Additionally, it was suggested that technical aspects related to histological processing of brain tissues, such as variation in brain size, difficulty in standardizing brain sections, degree of tissue dehydration during processing, and differences in section level that result from “facing in” procedures used in preparing slides from tissue blocks, may have contributed to any variability observed in the PND 12 morphometric measurements in this study. EPA reviewers note, however, that high variability in the data would be more likely to contribute to a lack of statistical significance in the face of true differences than to lead to spurious findings of statistical significance in the absence of true differences. More importantly, an examination of the individual morphometric data from this study reveals an absence of excessive overall variance in both the PND 12 and 66 measurements; the average coefficient of variation for the data at each time point are below 8% (analysis by Dr. Kevin Crofton, USEPA, National Health and Environmental Effects Laboratory).

Agency reviewers considered significant differences noted in the morphometric data to be treatment-related. The morphometric data for PND 12 pups demonstrate significant decreases in various parameters for males and females; these data are consistent with the significantly reduced brain weight data for both sexes at this age. In contrast to the Registrant's interpretation, it could be argued that the small sample size contributes to the inability to detect statistically significant differences from control for endpoints other than those currently

identified for PND 12. For example, examination of the individual data indicates that the statistically significant decreases in the hippocampal measurements identified in the original analysis, but not in the current re-analysis, may also be treatment-related at the high-dose for both sexes at PND 12 and for females at PND 66; there were also some indications of a possible effect for the mid-dose females at PND 66.

In addition to the apparent decrease in the hippocampal gyrus measurements on PND 66, the morphometric findings in the parietal cortex of female offspring indicate a treatment-related effect at the mid- and high-dose levels (1 and 5 mg/kg/day). The significant reduction in the parietal cortex measurement occurs only in females on PND 66 and was not observed in either sex at PND 12 in the current reanalysis (although the original statistical analysis did identify statistically significant differences at the high dose, which were consistent across time and sex). However, the decrease cannot be attributed to individual outliers at either the mid- or high-dose (see Attachment 1). Further analysis, conducted by Dr. Karl Jensen (USEPA, National Health and Environmental Effects Laboratory), demonstrates the following:

- a) The coefficient of variation for the PND 66 parietal cortex measurements is 3%, indicating a lack of excessive variance from any source, biological or technical.
- b) These data are within the historical range for adult female parietal cortex measurements, established by an examination of the individual morphometric data from several developmental neurotoxicity studies submitted to the Agency in support of pesticide registration.
- c) Examination of the individual parietal cortex measurements in the chlorpyrifos developmental neurotoxicity study reveals that the minimal difference between values is 24 μ m, and that all values are divisible by 24. Consequently it is reasonable to assume that for parietal cortex, this is a raw unit of measurement; the minimal raw unit of measurement may be considered a reasonable threshold for detection of differences. The differences between the mid- or high-dose group means are between 3 to 4 raw units, and thus at least 3 times the value of a minimal threshold for detection.
- d) As indicated in the study report, an ANOVA indicates that the effect of dose is significant, and a Dunnett's test indicates that the high- and mid-dose values are different from control. An analysis with Scheffe's test confirms this finding and further indicates that the high- and mid-dose values are not different from one another. Therefore, the effect is a result of treatment, but the magnitude of the effect is not dependant on the dosage.

In conclusion, the morphological alterations in the parietal cortex of female offspring at PND 66 are both statistically and biologically significant at the mid- and high-dose levels and are a clear indication that the structure of the brain has been altered by treatment. As a result, evaluation of the brain measurements for the low-dose PND 66 females becomes critical to establish the offspring NOAEL for this study. As previously noted in the Agency review of MRID 44556901, no brain measurements were presented in the data for low- and mid-dose males or for low-dose females at PND 66; it is presumed that these were not measured. At a minimum, the parietal cortex and hippocampal gyrus measurements from the low-dose females should be provided to the Agency to further examine this issue and establish a NOAEL for this endpoint.

The interrelationship of pup body weight, brain weight, and morphometric measurements

There is no disagreement between the Agency reviewers and the Registrant regarding the characterization of effects seen on pup body and brain weight in this study. High dose pups were observed to weigh less than control throughout lactation and had significantly reduced absolute

brain weights at PND 12 necropsy, although relative brain weights were increased. No effects attributable to treatment were noted in the pup body and brain weight data for the low- or mid-dose groups.

The Registrant attributes these alterations in pup body weight, brain weight, and morphometrics to postnatal undernutrition of the pups, citing a study by Peeling and Smart (1994) which concluded that body weight, brain weight, and brain morphometrics are related. In attempting to demonstrate the correlation between brain weight and morphometrics in this study, the Registrant averaged the relative values (% control) for the nine distinct morphometric measurements by sex for the high dose group, and compared these values and average high-dose brain weight values against controls. Specifically, for males the average morphometric value was 92.8% of control and the average brain weight was 88.5% of control; for females, the average morphometric value was 94.5% of control and the average brain weight was 91.3% of control (MRID 44787301, page 12). These comparisons, however, are an inappropriate and inconclusive manipulation of the data, since a numerical value derived from averaging the relative values for all external and internal morphometric measurements is not meaningful. Such a derived number would not evaluate the differences between alterations in growth patterns or disruptions in discrete areas of the brain, which could be differentially altered as an adverse consequence of treatment.

While the Agency reviewers agree with the Registrant that body weight, brain weight, and brain morphometrics can be related in preweaning rat pups, high-dose observations on pups in this study cannot be dismissed on that basis. At a dose which is unequivocally toxic to adult animals, and in the presence of information from a companion study (MRID 44648102) that fetuses and pups were exposed in the offspring at PND 12 are interpreted by Agency reviewers as being related to treatment. It is not possible to separate the origin or interrelationship of these specific effects, and classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, findings observed in the adult (~PND 66) offspring, long after exposure *via* the dam has ceased, namely alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequelae of developmental exposure.

2. Pup Undernutrition and Maternal Neglect

The Registrant has proposed that pup body and brain weight decrements during lactation were the result of undernutrition which was attributable to maternal toxicity or to maternal neglect. The inference made is that any treatment-related alterations in structure or function in the offspring were not the result of a direct effect of exposure to chlorpyrifos during development.

As support for this hypothesis, the work of Peeling and Smart (1994) and Carney et al. (1998) are cited. These two studies demonstrate 1) that undernutrition during early development causes slower body and organ growth, although brain tissue is “spared,” and 2) that pups of feed-restricted dams grew more slowly than pups of dams that were not feed-restricted. In the

developmental neurotoxicity study with chlorpyrifos, food consumption of the dams was measured during gestation and lactation. High-dose values were statistically similar to control values, although a 5-15% decrease in food consumption (g/day) was observed during early lactation, and a 5-12% decrease in relative food consumption (g/kg/day) was observed during the treatment period. These small decreases in food consumption could not be termed “undernutrition” or “feed restriction,” and it is very unlikely that they would have resulted in the alterations to offspring development as described in Peeling and Smart and in Carney et al.

An attempt was also made to link the preweaning motor activity deficits in the chlorpyrifos rats with maternal undernourishment. Gramsbergen and Westerga (1991) demonstrated a transient delay in locomotor development of pups from dams that were 40% feed-restricted throughout gestation and lactation. In the chlorpyrifos study, the decreased motor activity at the high dose was observed on PND 14, 18, and 22 for males and on PND 14 only for females. Increased motor activity, which was observed in females on PND 18 and 22, was also observed in both sexes at PND 61. The non-transient nature of the alterations to motor activity in the chlorpyrifos study, following only non-significant maternal food consumption deficits during the first few days after delivery, is an indication that the insult is probably not comparable to that induced experimentally in the Gramsbergen and Westerga study.

Furthermore, there is no support for the supposition (as proposed by the Registrant) that offspring findings at the high dose (5 mg/kg/day) should be attributed solely to maternal neglect. At that dose level, maternal brain cholinesterase was significantly inhibited (to 10% of control), cholinergic clinical signs (fasciculations, hyperpnea, and/or hyperactivity) of unknown duration and severity were observed in most dams during lactation following chlorpyrifos administration on an average of 2 separate dosing days (only during lactation days 1-6, although dams were dosed daily through lactation day 10), and maternal weight gain was decreased in late gestation (days 17-20) and early lactation (postnatal days 0-3). While the type of pup weight deficits and decreased survival observed at 5 mg/kg/day during the early postnatal period are not inconsistent with lack of maternal care, there is no evidence in the reported results that the effects on the offspring in this study were, in fact, due solely to maternal neglect. For example, the maternal clinical observation data (MRID 44556901, Table B15, p 138) did not indicate that the dams were not gathering the pups into the nest, remaining with their litters in the nest, or grooming the pups. There was no indication anywhere in the reported data that there was an alteration in high dose maternal lactation and nursing behavior. The clinical observations on live pups (report Table 24, p 205) did not describe the absence of visible milk in the stomach (often described as a “milk spot”). The gross necropsy data (report Table B28, p 220) indicated a lack of milk in the stomach of 12 pups of one litter that had died on PND 1, and in 2 pups from separate litters that had died on lactation days 2 and 5, although a total of 48 liveborn pups (from 13 litters) died and were necropsied between birth and postnatal day 5. Therefore, the statement that “neonatal deaths often were associated with a lack of milk in their stomachs” (page 8 of MRID 44787301) is not supported by the data. Nevertheless, even had there been overwhelming evidence of a lack of nourishment to the offspring, it would be impossible to discern whether nursing was interrupted or altered by treatment-related toxicity to the dams or to the pups, especially since there is evidence in the data of a companion study (MRID 44648102) that chlorpyrifos was available to the pups in maternal milk. An additional important consideration is that the

observed treatment-related effects on the offspring at the high dose level also included continued postweaning body weight deficits, increased motor activity on PNDs 18 and 22 (females) and PND 61 (both sexes), decreased peak response and increased latency to peak response in auditory startle habituation tests on PND 23 and 62, decreased PND 12 brain weight, and alterations in internal brain measurements at PND 12 in both sexes and PND 66 in females. Many of these findings continued past the time of weaning. Full recovery of body weight, motor activity, auditory startle, and morphometric alterations did not occur during the posttreatment period (of approximately 55 days), even though the rate of posttreatment weight gain appeared comparable among control and treated groups.

III. Conclusions

Several issues had remained outstanding following the review of the developmental neurotoxicity study with chlorpyrifos (MRID 44556901). These have been resolved in part by Supplement 2. Questions regarding the statistical treatment of the behavioral data, which were raised in HED Doc. No. 013081, were not addressed. These included the lack of some variance performed on replicate data such as motor activity or auditory startle, and the absence of an explanation for the use of a significance level (alpha) of 0.02. However, the issues surrounding the statistical analyses of these data were not judged by Agency reviewers to be critical to the overall interpretation of the study or the determination of the lowest adverse effect level for the offspring. The issue of greater concern and impact was the statistical analysis and interpretation of morphometric data for the offspring. Sufficient data were provided in the SAS Output file and in Supplement 2 (information on the statistical methodology, and the results of statistical analyses of the mid-dose group morphometric data) to enable the Agency reviewers to arrive at an independent conclusion.

The maternal findings and effect levels were not reexamined and remain as indicated below:

The maternal toxicity NOAEL was not observed.

The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

However, based upon the discussions above, the offspring conclusions have been revised:

Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined.**

C. Study Classification

While the offspring NOAEL and LOAEL have not yet been identified for this developmental neurotoxicity study, it is recognized that the study was well-conducted according to Agency guideline §83-6, and under GLP regulations. Remaining questions can be resolved with additional information and statistical analysis, but there are no outstanding concerns regarding the quality of the animal data. It is recommended that the classification of the study be changed

from **unacceptable** to **guideline-unacceptable** at this time, pending submission and review of additional morphometric data for PND 66 low-dose females (parietal cortex and hippocampus measurements).

D. Revised EXECUTIVE SUMMARY:

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (GD 6) through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on GD 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F₁ generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.

The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F₁ generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND

22 in Subset 4 high-dose animals (\downarrow 5-19%); bodyweight gains were reduced in these animals during the same period (\downarrow 5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (\downarrow 17-19%) and the Subset 4 (PND 66) high-dose males (\downarrow 10%). For the F₁ generation adults, body weights of the high-dose males were decreased at PND 22 through 66 (\downarrow 11-17% vs controls). High-dose F₁ adult females also weighed less than controls at PND 22 (\downarrow 17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (\downarrow 13% vs controls) and PND 40-66 interval (\downarrow 7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (\downarrow 13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/ litter (\downarrow 27%) and live litter size at culling (\downarrow 16%), pup viability index (\downarrow 29%), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high dose male and female pups on PND 14 (\downarrow 56% in males and \downarrow 37% in females), and increased in high dose females on PNDs 18 and 22 (\uparrow 51% on both days). On PND 61, motor activity was increased for both sexes (\uparrow 16-17%). There was a statistically significant increase (\uparrow 16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 (not statistically significant) compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (\downarrow 9% vs controls), increased relative brain weights (\uparrow 13% vs controls), reduced anterior to posterior measurement of the cerebellum (\downarrow 24% vs controls), reduced height of the cerebellum (\downarrow 14% vs controls), decreased thickness of the parietal cortex (\downarrow 6% vs controls), and decreased thickness of the hippocampal gyrus (\downarrow 9% vs controls). High-dose female pups had reduced absolute brain weights (\downarrow 9% vs controls), increased relative brain weights (\uparrow 14% vs controls), decreased thickness of the parietal cortex (\downarrow 6% vs controls), decreased width of the caudate-putamen (\downarrow 10% vs controls), and decreased thickness of the hippocampal gyrus (\downarrow 12% vs controls). In Subset 4 F₁ animals, killed on PND 66, morphometric analysis revealed significantly decreased parietal cortex measurements in high-dose (\downarrow 5%) and mid-dose (\downarrow 4%) females, as compared to controls. Decreases in the thickness of the hippocampal gyrus in high-dose females (\downarrow 7%) resulted in contradictory statistical results when compared to controls; decreases in mid-dose (\downarrow 4%) females as compared to control were not found to be statistically significant. There was no evaluation of the morphometric data for low dose females at PND 66. Brain weight in high dose females was similar to control brain weight at day 66 (\downarrow 0.3%).

It is not possible to definitively classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, adverse findings in the adult (~PND 66) offspring, i.e., alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequelae of developmental exposure to chlorpyrifos.

Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined.**

REFERENCES (cited by the Registrant):

Carney, E., B. Scortichini and J. Crissman (1998) Feed restriction during in utero and neonatal life: effects on reproductive and developmental end points in the CD rat. *Toxicologist* 42, Suppl. 1(abstract 506):102-103.

Gramsbergen, A. and J. Westerga. (1991) Locomotor development in undernourished rats. *Behavioral Brain Research* 48:57-64.

Peeling, A.N. and J.L. Smart. (1994) Review of literature showing that undernutrition affects the growth rate of all processes in the brain to the same extent. *Metabolic Brain Disease* 9(1):33-42.

Attachment 1

Individual Morphometric Data - Adult (PND 66) F1 Generation Male Rats										
Rat No.	Brain Wt.	Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum	
				0 mg/kg/day						
405	2.221	15.6	5.5	1920	1656	2928	317	1488	5040	
414	2.249	16.2	5.9	1872	1800	2976	269	1680	4944	
416	2.264	15.6	5.4	1656	1728	2784	269	1656	5376	
418	2.371	15.5	5.6	1680	1680	2544	230	1752	5280	
463	2.394	16.5	5.7	1824	1824	2640	250	1584	4896	
478	2.300	16.0	6.0	1800	1848	2928	259	1680	5376	
Mean	2.300	15.900	5.683	1792.000	1756.000	2800.000	265.667	1640.000	5152.000	
S.D.	0.069	0.400	0.232	104.797	79.478	176.145	29.049	91.913	218.211	
				5 mg/kg/day						
403	2.266	15.9	6.0	1728	1728	2784	259	1536	5040	
443	2.298	16.0	5.8	1704	1824	2592	250	1608	5376	
444	2.310	16.2	5.4	1872	1872	2736	250	1488	5040	
451	2.318	16.0	5.6	1824	1824	2880	211	1680	5376	
454	2.282	16.5	5.7	1800	1728	2784	259	1608	5328	
466	2.317	16.5	5.5	1680	1776	2688	250	1752	4464	
Mean	2.299	16.183	5.667	1768.000	1792.000	2744.000	246.500	1612.000	5104.000	
S.D.	0.021	0.264	0.216	75.387	58.131	97.980	17.942	95.297	350.980	
Data extracted from MRID 44787301, page 13. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.										

Attachment 1 - continued

Rat No.	Individual Morphometric Data - Adult (PND 66) F1 Generation Female Rats									
	Brain Wt.	Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum	
806	2.092	16.0	5.3	1704	1824	2784	211	1728	4896	
807	2.060	15.3	5.6	1776	1776	2496	259	1728	5184	
825	2.155	15.5	5.5	1704	1800	2400	221	1728	5136	
835	2.125	15.5	5.7	1680	1824	2640	278	1680	4896	
846	2.191	16.0	5.8	1776	1800	2592	250	1776	4992	
870	1.993	15.4	5.2	1824	1728	2544	250	1608	4992	
Mean	2.103	15.617	5.517	1744.000	1792.000	2576.000	244.833	1708.000	5016.000	
S.D.	0.071	0.306	0.232	56.114	36.133	131.161	24.766	57.633	120.479	
				1 mg/kg/day						
809	2.005	15.5	5.5	1680	1680	2400	240	1632	4992	
828	2.201	15.8	5.4	1824	1728	2400	288	1776	4704	
839	2.055	15.7	5.3	1680	1680	2592	259	1416	4704	
841	2.142	15.3	5.3	1680	1728	2736	259	1608	4992	
853	2.187	16.2	6.0	1824	1704	2784	259	1752	5040	
862	2.172	15.3	5.5	1800	1776	2400	240	1680	4896	
Mean	2.127	15.633	5.500	1748.000	1716.000	2552.000	257.500	1644.000	4888.000	
S.D.	0.079	0.344	0.261	75.004	36.398	178.097	17.604	129.467	150.008	

Data extracted from MRID 44787301, pages 14'. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.

Attachment 1 - continued

Rat No.	Brain Wt.	Individual Morphometric Data - Adult F1 (PND 66) Generation Female Rats (continued)							
		Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum
843	2.129	15.5	5.3	1752	1632	2832	221	1632	5088
848	1.993	15.6	5.3	1728	1728	2784	250	1560	4944
849	2.022	15.1	5.3	1776	1632	2640	211	1584	5232
858	2.054	15.8	5.5	1824	1752	2784	259	1440	4992
859	2.012	15.4	5.4	1608	1704	2640	221	1680	4608
874	2.079	15.7	5.5	1656	1752	2544	240	1656	4944
Mean	2.048	15.517	5.383	1724.000	1700.000	2704.000	233.667	1592.000	4968.000
S.D.	0.050	0.248	0.098	79.478	55.599	112.228	18.886	86.755	207.569

Data extracted from MRID 44787301, page 14. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.

SignOff Date: 3/3/00

DP Barcode: D254907

HED DOC Number: 014014

Toxicology Branch: TOX1

DATA EVALUATION RECORD

CHLORPYRIFOS

Study Type: §83-6; Developmental Neurotoxicity Study of Chlorpyrifos Administered Orally via Gavage to Crl:CD[®]BR VAF/Plus[®] Presumed Pregnant Rats

Work Assignment No. 3-56 (MRID 44556901)

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
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Prepared by

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Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Susan Makris, M.S. _____
Toxicology Branch 1 (7509C)

EPA Secondary Reviewer: Kathleen Raffaele, Ph.D. _____
Toxicology Branch 2 (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Developmental Neurotoxicity Study - Rat
OPPTS Number: 870.3600 OPP Guideline Number: §83-6

DP BARCODE: D247891, D250250 SUBMISSION CODE: S546162, S550086
P.C. CODE: 059101 TOX. CHEM. NO.: 219AA

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Dursban

CITATION: Hoberman, A. M. (1998) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, May 1, 1998. MRID 44556901. Unpublished.

Hoberman, A.M. (1998) (pathology) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, May 1, 1998. MRID 44661001. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268

EXECUTIVE SUMMARY:

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (GD 6) through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on GD 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F₁ generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10

pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.

The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F₁ generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); bodyweight gains were reduced in these animals during the same period (↓5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17-19%) and the Subset 4 (PND 66) high-dose males (↓10%). For the F₁ generation adults, body weights of the high-dose males were decreased at PND 22 through 66 (↓11-17% vs controls). High-dose F₁ adult females also weighed less than controls at PND 22 (↓17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (↓13% vs controls) and PND 40-66 interval (↓7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (↓13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/ litter (↓27%)

and live litter size at culling (↓16%), pup viability index (↓29%), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high dose male and female pups on PND 14 (↓56% in males and ↓37% in females), and increased in high dose females on PNDs 18 and 22 (↑51% on both days). There was a statistically significant increase (↑16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 (not statistically significant) compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑13% vs controls), reduced anterior to posterior measurement of the cerebellum (↓24% vs controls), reduced height of the cerebellum (↓14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), and decreased thickness of the hippocampal gyrus (↓9% vs controls). High-dose female pups had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑14% vs controls), thickness of the parietal cortex (↓6% vs controls), width of the caudate-putamen (↓10% vs controls), and thickness of the hippocampal gyrus (↓12% vs controls). In Subset 4 F1 animals, killed on PND 66, morphometric analysis revealed decreased parietal cortex measurements (↓5%) and decreased thickness of the hippocampal gyrus (↓7%) in high-dose females. These measurements were also decreased in mid-dose females (parietal cortex, ↓4%; hippocampal gyrus, ↓4%). The statistical significance of the differences in mid-dose females was not evaluated, and there was no evaluation of low dose females. Brain weight in high dose females was similar to control brain weight at day 66 (↓0.3%).

Due to inadequate presentation of the statistical data analysis, it was not possible to determine the definitive developmental neurotoxicity NOAEL and LOAEL for the offspring.

The tentative developmental neurotoxicity LOAEL is 5 mg/kg/day.

The tentative NOAEL is 1 mg/kg/day.

This study in the rat is classified **unacceptable (§83-6)** and does not satisfy the guideline requirements for a developmental neurotoxicity study. The study may be upgradable, following submission of more complete statistical analysis.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Chlorpyrifos

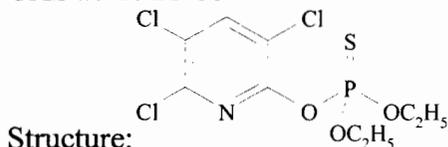
Description: Technical, white solid

Lot/Batch #: MM930503-17 (TSN 100227)

Purity: $99.8 \pm 0.1\%$ a.i.

Storage conditions: Room temperature

CAS #: 2921-88-2



2. Vehicle: Mazola[®] corn oil

Lot/Batch #: 108A7

3. Test animals: Species: Rat

Strain: CrI:CD[®]BR VAF/Plus[®] (Sprague-Dawley)

Age at mating: Approximately 3½ months

Weight at mating: 227-293 g

Source: Charles River Laboratories, Inc., Portage, Michigan

Housing: Individually in stainless-steel wire-bottomed cages; all dams moved to individual nesting boxes with corn cob bedding (bed-o'cobs[®]) no later than GD 20

Diet: Certified Rodent Diet[®] #5002, ad libitum except as noted during behavior evaluations

Water: R.O.(reverse osmosis) water, ad libitum except as noted during behavior evaluations

Environmental conditions:

Temperature (nominal): 64 to 79°F

Humidity (nominal): 30-70%

Air changes: ≥10/hr, HEPA filtered

Photoperiod: 12 hrs dark/12 hrs light

Acclimation period (P): 6 weeks

B. PROCEDURES AND STUDY DESIGN (see appended Figure 1, a study schematic extracted from study report, page 24)

1. In life dates - start: 7/29/97 end: 1/9/98

2. **Mating:** One female was placed with one breeder male of the same strain and source in the male's cage for a cohabitation period of maximum 7 days. The day of positive identification of spermatozoa in the vaginal smear and/or a copulatory plug observed *in situ* was termed gestation day (GD) 0; at this time, both animals were returned to their individual cages.
3. **Animal Assignment:** F₀ females were randomly assigned (weight-ordered) to dose groups as indicated in Table 1. F₁ pups (5/sex/litter) were randomly selected from available litters on postnatal day 5. Of these, 4 pups/sex/litter were selected (Table 1) and assigned to testing subsets (Table 2) as available.

Table 1. Animal assignment.

Test Group	Dosage (mg/kg/day)	F ₀ generation females	F ₁ generation pups c	
			Males	Females
Control ^a	0	25 ^b	80	78
Low	0.3	25 ^b	80	79
Mid	1	25 ^b	80	80
High	5	25 ^b	64	69

a Control animals received vehicle only.

b An additional 5 rats/dose F₀ generation female rats were mated, assigned to a satellite portion of the study, and sacrificed on gestation day 20 for blood and brain sample collection and cholinesterase (ChE) analysis.

c Number of available litters for control through high-dose groups: 25, 24, 24, 23; nominal number of pups per sex to be assigned per group = 80.

4. **Dose selection rationale:** The low- and mid- doses were chosen based on a chronic study indicating that dosages of 1 mg/kg/day had no effect on the level of brain ChE. The high-dose was chosen based on substantial inhibition of brain ChE and histopathological changes in the adrenal zona fasciculata at 5 mg/kg/day.
5. **Dosage preparation and analysis:** Test substance formulations were prepared once at the beginning of the study. Analyses for substance stability and concentration verification were performed. Homogeneity and concentration analyses were performed on 0 (vehicle), 0.3, 1, and 5 mg/ml dose formulations. Stability analyses were performed on 0.06 and 1 mg/ml formulations that were stored for 42 days (5/13/97-6/24/97). Storage temperature was not reported.

Results - Homogeneity and concentration analysis: 96-100% of nominal.

Stability Analysis: The 0.06 mg/ml formulation was 103 ± 0.0006- 0.0028% of nominal; the 1 mg/ml dose was 103 ± 0.01% of nominal.

The analytical data indicated that the mixing procedure was adequate and the variance between nominal and actual dosage to the study animals was acceptable.

6. Dosage administration: All doses were administered once daily to F₀ generation females by gavage on GD 6 through of lactation day 11 for dams that delivered a litter (day of delivery designated as lactation day or postnatal day 1) and through GD 24 for rats that did not deliver a litter. The dosing volume of the solutions was 1 mg/kg. Dosing was based on the daily body weights.

C. OBSERVATIONS

1. F₀ Generation Observations and Evaluations - Dams were inspected weekly during the predosing period, on GD 0, and twice daily during the dosing period for signs of toxicity and mortality. During the dosing period, the animals were observed daily at 3-4 hours postdose by someone unfamiliar with the rat's dose group for indications of autonomic dysfunction, abnormal posture, abnormal movement and behavior, and unusual appearance. Body weight data were evaluated and recorded weekly during the pretreatment period, on GD 0, and daily during the dosing period, lactation, and day of sacrifice. F₀ generation rats were also evaluated for the following:

- pregnancy status
- duration of gestation (GD 0 to the time the first pup was delivered)
- delivered litters
- gestation index
- implantation sites
- natural delivery
- number of stillborn or dying pups
- litter sizes (all pups delivered)
- pup viability at birth
- clinical observations during lactation

Food consumption data were recorded on GD 0 during the predosing interval and then daily during the dosing period through lactation day 14. Maternal behavior was evaluated daily during the 22-day postpartum period and was recorded on lactation days 1 (birth), 5, 12, 18, and 22; any variations in behavior were recorded on the day observed. In addition, 20 mated F₀ generation females (5/dose group) were assigned to a satellite portion of the study for blood and brain sample collection and subsequent ChE analyses of the brain, plasma, and erythrocyte samples.

2. F₁ Generation Observations and Evaluations - The F₁ generation pups were examined for viability at birth. Pup viability was evaluated for each litter and evaluations of viability continued at least twice daily for the 22-day postpartum period. On lactation day 5, the litters were randomly standardized to 10 pups/litter (5 males/5 females when possible) and pups were randomly assigned to one of four subsets for continued observation. On lactation day 12, the litters were randomly standardized to eight pups/litter (4 males/4 females when possible). All pups were evaluated for the following:

- pups found dead or cannibalized (Day 1 and Days 2 to 5)
- surviving pups/litter (Day 1, Day 5 [preculling] and Day 5 [postculling])
- live litter size (Day 1, Day 5 [preculling] and Day 5 [postculling])
- time to pinna detachment
- time to eye opening

The pups in each litter were counted and evaluated for any abnormal physical signs once daily during the 22-day postpartum period. Body weight, sex, and observed nursing behavior were recorded on lactation days 1, 5, 12, 18, and 22, and any variations in behavior were recorded on the day observed.

3. F₁ Generation Subset Observations and Neurobehavioral Evaluations: On PND 5, 80 pups/sex/dose (nominally one male and one female/litter/dose) were randomly assigned to Subsets 1, 2, 3, or 4 (Table 2).

All pups in Subset 1 were sacrificed on PND 12. Fixed brain weights were determined in 10 Subset 1 pups/sex/dose and neuropathological examinations were performed on 6 of those pups/sex/dose. The remaining 10 pups/sex/dose were necropsied for gross lesions.

In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92; these Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22.

The Subset 3 pups (1 pup/sex/litter/dose) were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation.

In Subset 4 pups, the following were performed:

- Clinical observations were recorded on PNDs 40 and 66. Body weights were recorded on PNDs 1, 5, 12, 18, 22, 40, and 66. Food consumption was recorded on PNDs 23-30, 40-47, and 59-66;
- Pinna unfolding and eye opening were monitored daily beginning on PND 2 and PND 12, respectively, until each pup achieved the criterion; (pinna unfolding was also monitored in all other rats because monitoring started prior to litter standardization and subset assignment on PND 5);
- Males were evaluated for the age of preputial separation beginning on PND 39 and females were evaluated for the age of vaginal patency beginning on PND 28. Body weights were recorded on the day the criterion was attained; and
- Between PNDs 66-71, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 of these 10 pups/sex/dose and all remaining animals (10/sex/dose) were necropsied for gross lesions.

Table 2. F₁ generation subset observations and neurobehavioral evaluations.

Subset Number	Observations	Days of Sacrifice
1	A total of 80 pups/sex (20/sex/dose) were assigned to Subset 1. 10 pups/sex/dose were selected for fixed brain weights on PND 12; of these, 6 pups/sex/dose were subsequently selected for neuropathological examination on PND 12 (total of 24 male and 24 female pups evaluated).	All pups were sacrificed on PND 12.
2	A total of 80 pups/sex (20/sex/dose) were assigned to Subset 2. 8 pups/sex/dose were selected for evaluation of learning and memory between PNDs 23- 25 and between PNDs 62- 92 (total of 32 male and 32 female rats evaluated).	All rats were sacrificed on PNDs 97 to 101 following the last test.
3	1 pup/sex/litter/dose (a total of 80 male and 80 female pups) were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62.	All rats were sacrificed on PNDs 63 and 64 following the last auditory startle test.
4	1 pup/sex/litter/dose (total of 80 male and 80 female pups) were assigned to Subset 4. 10 pups/sex/dose were selected for fixed brain weights between PNDs 66- 71; of these, 6 pups/sex/dose were subsequently selected for neuropathological examination between PNDs 66- 71 (total of 24 male and 24 female rats evaluated).	All rats were sacrificed between PNDs 66 and 71.

For brain weight measurements and neuropathological evaluations, the brains from 10 pups/sex/dose from Subset 1 (PND 12) and Subset 4 (PNDs 66-71) were weighed following fixation with neutral buffered 10% formalin. Of the 10 pups/sex/dose, 6 from each group were selected for neuropathology evaluation. Brains from Subset 1 animals were fixed by immersion in neutral buffered 10% formalin. Subset 4 animals (PND 66-71) were perfused in situ with neutral buffered 10% formalin after administration of heparin and an anesthetic (not specified). Subsequently, the spinal cord and peripheral nerves from the hindlimbs were exposed and immersed in neutral buffered 10% formalin prior to shipment to a consulting lab for processing.

Spatial delayed alternation was evaluated in 8 pups/sex/dose from Subset 2 on PNDs 23-25 and 62-92 in a Plexiglas[®] T-maze. The test was conducted on food and/or water deprived animals. F₁ generation pups were deprived of food and water and the adult rats were deprived of water only. The test included maze acclimation, acquisition training, and delay training. Maze acclimation included goal box training and forced runs. Acquisition training consisted of a forced run immediately followed by a choice run. Delay training was conducted similarly to acquisition training with a time delay added between the for

Motor activity was evaluated for a 1 hour period on one Subset 3 pup/sex/litter on PNDs 14, 18, 22, and 61. The number of movements was tabulated at 5-minute intervals. The apparatus monitored up to 32 cages on one rack during each session. Each animal was tested in the same location on the rack during the 4 test sessions. On PNDs 22 and 61, body temperature measurements were performed on all animals immediately following

completion of the motor activity testing.

On PND 23 and 62, an auditory startle habituation test was performed on the same animals tested for motor activity. The animals were given a 5 minute acclimation period and then the startle response was measured in 50 identical trials at a sound level of 120 dBA with a 10 second inter-trial interval. For each rat, peak response amplitude (corrected for baseline) and response latency were averaged over ten-trial blocks; the averages of these 10-trial blocks were then computed for each dose group (yielding 5 blocks per group), and these averages were compared among the dose groups.

4. Sacrifice and Pathology - F₀ Generation - All maternal rats were sacrificed by carbon dioxide asphyxiation. Rats that died or were killed for humane reasons were examined for the cause of death or moribund condition on the day the observation was made. These rats were evaluated for gross lesions, pregnancy status, and uterine contents. Any female that mated, but did not deliver a litter was euthanized on GD 25 and examined for gross lesions. To confirm the absence of implantation sites, the uteri were stained with 10% ammonium sulfide. Any dams whose entire litter was born dead or died prior to weaning of their litter were killed after the last remaining pup was found dead or missing, or presumed cannibalized; of these F₀ females, a gross necropsy was performed on the thoracic, abdominal, and pelvic viscera. Dams that littered were sacrificed on Lactation day 22, a gross necropsy with examination of the thoracic, abdominal, and pelvic viscera was performed, and the number and distribution of implantation sites was recorded. Satellite dams were sacrificed on GD 20, four to five hours post dosing. Blood was collected from the inferior vena cava and the brain was removed; ChE analyses were performed on brain, plasma, and erythrocyte samples.
5. Sacrifice and Pathology - F₁ Generation - All pups were euthanized by carbon dioxide asphyxiation, excluding the Subset 1 and 4 pups assigned to brain weight measurements and neuropathology examination.

Pups Found Dead on PND 1: To assess viability of any pups that appeared stillborn or that died before the initial viability examination of the litter, the lungs were removed and immersed in water. Pups with lungs that sank were considered stillborn, while pups with lungs that floated were considered liveborn and to have died shortly after birth. Pups with gross lesions were placed in Bouin's fluid for possible future examination.

Pups Found Dead or Moribund Sacrificed on PNDs 2 to 22: Pups found dead in the nesting boxes were necropsied and evaluated for cause of death. Pups with gross lesions were preserved in Bouin's fluid.

Pups Not Selected for Continued Observation: All pups chosen for culling were sacrificed and necropsied on PND 5 and offspring with gross lesions were placed in Bouin's solution. F₁ generation pups not assigned to one of the four subsets were sacrificed on PND 22, examined for gross lesions, and necropsied.

Scheduled Sacrifice of F₁ Generation Pups Selected for Continued Observation:

On PND 12, all pups in Subset 1 were sacrificed and examined for gross lesions; pups selected for neuropathological evaluation were prepared as described in section 3. Upon completion of all postweaning behavior and developmental examinations, all Subset 2 and 3 rats were sacrificed and examined for gross lesions. On PND 66 to 71, Subset 4 pups were sacrificed and examined for gross lesions; any Subset 4 pups chosen for brain weight and neuropathological evaluation were sacrificed and processed as previously described in section 3.

Neuropathological evaluation: For Subset 1 pups selected for neuropathological evaluation, the following parameters were evaluated: fixed brain weight, external measurements (cerebrum and cerebellum), eight brain sections (paraffin embedded, stained with hematoxylin and eosin), seven additional linear morphometric measurements. All dose groups were evaluated, for a total of 48 rats (6/sex/group). For Subset 4 pups selected for neuropathological evaluation, the following parameters were evaluated: fixed brain weight; external measurements (as above); sections from brain, spinal cord, Gasserian ganglia, nerve roots, and dorsal root ganglia (paraffin embedded, stained with hematoxylin and eosin, luxol fast blue/cresyl violet, and Bielschowsky's technique); sections of sciatic, tibial, peroneal, and sural nerves (embedded in glycol methacrylate, stained with hematoxylin and eosin, toluidine blue, and Bielschowsky's technique); six additional linear morphometric measurements. Control and high dose group animals were evaluated for both sexes (6/sex/group), and mid-dose group females were evaluated, for a total of 30 rats.

D. DATA ANALYSIS

1. Statistical Analyses: Parametric data were analyzed using Bartlett's Tests (run at $\alpha = 0.001$) or factorial repeated-measure analyses (Rep-ANOVA). In the event of statistical significance, one dose level was removed and the Rep-ANOVA was rerun to determine if a significant difference still existed between the remaining groups. Step-down analyses were conducted at $\alpha = 0.02$. Nonparametric data were evaluated using either the Kruskal- Wallis ($\alpha = 0.02$) or Chi-square test for proportions ($\alpha = 0.02$).

Note that although study authors used $p < 0.02$ as their cut-off for statistical significance, most studies consider findings to be statistically significant if $p < 0.05$. In addition, note that the above-described analyses do not include any pairwise comparisons (i.e., the statistical significance of the differences between, for instance, the high dose and low dose for specific measured values at any particular time point were not evaluated). Note also that only selected main effects and interactions from the statistical analysis were presented. For example, in the analysis for auditory startle habituation, p-values were presented for main effect of group, and for the interactions of [Group X Sex], [Group X Time], and [Group X Time X Block]; main effects for [Sex], [Time], and [Block], or interactions of (e.g.) [Group X Block], [Group X Sex X Time], etc. were not presented.

Parental Data: Group mean bodyweights and food consumption were analyzed using a

Rep-ANOVA. Clinical signs were analyzed using the Chi-square test for proportions.

Maternal Data: The number of pregnant rats, gestation index (number of rats with live offspring/number of pregnant rats), live and stillborn pups, and dams with live/stillborn pups were analyzed using the Kruskal-Wallis and Wilcoxon tests. The duration of gestation, total pups delivered, dams with all pups dying days 1 to 5 postpartum, pups found dead or presumed cannibalized, and implantation sites were analyzed using the Chi-square test for proportions.

Litter Data: Litter size at weighing and sex ratio were analyzed using the Kruskal-Wallis and Wilcoxon tests. Pup viability indices (number of live pups on day 5 [precullying]/number of pups on day 1) were analyzed using Chi-square test for proportions. Bodyweights, food consumption, and body temperature were analyzed by Rep-ANOVA. Clinical signs were analyzed using the Chi-square test for proportions.

Behavioral Data: Auditory startle, motor activity, and spatial delayed alternation were analyzed using Rep-ANOVA.

Developmental Data: Pinna unfolding, vaginal patency, and preputial separation were tested by Kruskal-Wallis followed by a Wilcoxon test. Eye opening was analyzed using the Chi-square test for proportions.

Morphometric Data: All morphometric data were analyzed by ANOVA.

2. Historical Control Data: No data were submitted.
3. Positive Control Data: Positive control data for spatial delayed alternation methodology, motor activity, developmental neurotoxicity, and morphometric measurement were provided.

A brief methodology was submitted for spatial delayed alternation to assess learning and working memory of adult and pup rats. Testing consisted of three phases: maze acclimation, acquisition training, and delay training. Results showed an increase in the learning curve during the acquisition training phase and an increase in the delay between the forced and the choice run indicating a decrease in the retention curve. Maze acclimation data were not submitted.

Positive control data were submitted for the following chemicals (doses): acrylamide (45 mg/kg/day), d-amphetamine (0.75 mg/kg/day), MK-801 (10 mg/kg/day) and trimethyltin chloride (8 mg/kg/day) in a study evaluating motor activity, startle habituation, and neurohistological effects. No startle habituation data were provided. Several statistically significant differences were found in male rats during the motor activity testing when comparing mean \pm S.D. between vehicle control and the test chemical. Statistically significant parameters for the male rats in motor activity testing follow: during session 2, differences were found in the number of movements when treated with acrylamide and d-amphetamine and time spent in movement when dosed with d-amphetamine; session 3 revealed differences in the number of movements when treated with acrylamide and d-

amphetamine and time spent in movement when dosed with d-amphetamine; session 4 showed significant differences in the number of movements and time spent in movement when rats were treated with acrylamide and d-amphetamine. Increased mortality and increased frequencies and numbers of neurological clinical observations were found when male rats were treated with acrylamide. No significant changes were found in the males with regard to mean \pm S.D. for terminal body weights, brain weights, or ratios (%) of brain weight to terminal body weight between treated groups and control.

Statistically significant differences were also found in female rats during the motor activity testing when comparing mean \pm S.D. between vehicle control and the test chemical. Statistically significant parameters for the female rats in motor activity testing follow: sessions 2, 3, and 4 indicated significant differences in the number of movements and time spent in movement when females were treated with acrylamide and d-amphetamine. Increased frequencies and numbers of neurological clinical observations were found in female rats when treated with acrylamide, MK-801, and trimethyltin. No significant changes were found in the females with regard to mean \pm S.D. for terminal body weights, brain weights, or ratios (%) of brain weight to terminal body weight between treated groups and control.

Another study submitted related to the developmental neurotoxicological effects of lead nitrate. Pregnant rats were given lead nitrate orally by gavage from GD 6 through lactation day 10 at doses of 0 (vehicle), 5, or 50 mg/kg/day. Observations made in the high-dose group included: significant increases in the number and frequency of neurotoxicological clinical observations in the dams and significantly reduced food consumption values throughout gestation and early lactation, significant decreases in maternal body weights during lactation, significant numbers of stillborn and dying pups on PND 1 to 4, average pup weights per litter were reduced on PND 1-7, and significant differences in body weights and food consumption values in the F₁ generation during the early postweaning period. Brain weights of high dose PND 11 F₁ generation females were significantly different from the vehicle control. In the low-dose, females of the F₁ generation showed significant differences from the vehicle control in whole brain weights, telencephalon, and cerebellum weights. No differences existed between the three dose groups in the functional evaluations of the F₁ generation rats which included: motor activity monitored for 1 hour periods on PND 13, 17, 21, and 60 (\pm 2), auditory startle habituation measured on PND 22 and 60 (\pm 2), performance in a passive avoidance task during the early postweaning period, and performance of adult rats in an M-maze watermaze task. Indices of sexual maturation were unaffected by treatment. Neurohistological examination of high dose group adult rats did not reveal any pathology.

The final positive control study submitted compared the gross and microscopic morphometric measurements of 10- and 12-day old pups. The ages of 10 and 12 days were selected to possibly predict either an *in utero* neurotoxic effect or delayed brain development. Results indicated the mean values for all of the neuroanatomic measurements were higher for the 12 day-old rat pups than for the 10 day-old pups, but considerable overlap existed in the data. Despite the data overlap between the two age groups and the presence of moderate intra-age group variability in the measurements for

each location, statistically significant increases (day 12 vs. day 10 rat pups) were found for the following brain regions: anterior-posterior length of the cerebrum, anterior-posterior length of the cerebellum, thickness of the frontal cortex, width of the caudate-putamen, and height of the cerebellar cortex.

II. RESULTS

A. MATERNAL TOXICITY

1. Mortality and Clinical Observations - There were no unscheduled mortalities in the F₀ maternal animals. No treatment-related clinical signs occurred.
2. Autonomic Function - High-dose (5 mg/kg/day) animals had increased signs of autonomic function toxicity, manifested at the postdose examination at the end of gestation (day 21 or 22) as fasciculations (6/25 treated vs 0/25 controls, p=0.001), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls, p=0.001), hyperpnea (8/24 treated vs 0/25 controls, p=0.001), and hyperreactivity response to handling (17/24 treated vs 2/25 controls, p=0.001). In the mid-dose group (1 mg/kg/day), hyperpnea was only observed in 1/24 dams and was not considered to be treatment-related due to the low incidence. Hyperreactivity was also observed in the low- (7/24 treated) and mid-dose (2/24 treated) groups, but was not considered treatment-related because the response was not dose-dependent. Data are shown in Table 3.

Table 3. Autonomic function toxicity observations during gestation and lactation in dams dosed with chlorpyrifos from gestation day 6 to lactation day 11 (total number of times finding was observed/no. of animals with finding observed at least once during the designated period).^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5
	Gestation			
Total # Animals	25	25	25	25
Fasciculations	0	0	0	6/6*
Ataxia	0	0	0	1/1
	Lactation			
Total # Animals	25	24	24	24
Hyperreactivity	3/2	7/7	3/2	27/17*
Fasciculations	0	0	0	30/16*
Hyperpnea	0	0	1/1	10/8*

a Data extracted from the study report Table B1, pages 120 and 121.

* Significant at p=0.001; p values represent overall significance of analysis when all groups are included, findings during lactation were no longer significant when the high dose was removed from the analysis.

3. **Bodyweight** - No significant differences occurred in maternal bodyweights and bodyweight gains during gestation or lactation. There was, however, a significant [Group X Time] interaction for body weight changes during gestation (i.e., there were differences among groups at some time points, but not at other time points, $p=0.0059$; $p=0.0419$ when the high dose group was removed from the analysis; no further analyses were done). Slightly lower gestation day 20 body weight values at the high dose were attributed by the study author to higher mean litter size and lower average pup weights in this group as compared to control. Bodyweight gains in the high-dose group were reduced during days 16-20 of gestation ($\downarrow 11\%$, Table 4a). Although the statistical significance of bodyweight gains for this specific time period is unknown, the lower body weight gain was considered to be related to treatment by the study author. During lactation, maternal bodyweight gains in the high-dose group were reduced ($\downarrow 16\%$, Table 4b) immediately after parturition. However, the bodyweight gains of all groups were small compared to absolute bodyweight, and the values were highly variable; therefore, the differences were not considered to be unequivocal evidence of treatment-related toxicity.

Table 4a. Mean bodyweight gains (g) during gestation in dams dosed with chlorpyrifos from gestation day (GD) 6 to lactation day 11.^a

Interval	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
GD 0-6	26.2	22.4	26.1	23.8
GD 6-12	18.9	23.3	23.6	22.4
GD 12-16	22.2	25.0	22.1	22.2
GD 16-20	49.2	51.4	51.6	43.9
GD 6-20	90.2	99.7	97.3	88.5
Overall - GD 0-20	116.5	122.2	123.4	112.3

a Data extracted from the study report Table B3, page 123.

(*) Rep-ANOVA: significant effect of [Group X Time], $p=0.0059$; the analysis was no longer statistically significant when the high dose group was removed; data were not further analyzed.

Table 4b. Mean bodyweight gains (g) during lactation in dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Interval	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Days 1-4	6.8	4.6	5.0	1.1
Days 12-14	3.0	1.0	1.6	7.0
Days 12-22	-0.6	1.8	9.2	5.0
Days 1-12	33.4	31.0	29.6	33.5
Overall - Days 1-22	32.9	32.7	38.8	38.6

a Data extracted from the study report Table B5, page 126.

(*) Rep-ANOVA: [Group X Time] interaction, $p=0.0406$; data not further analyzed.

4. **Food Consumption** - Food consumption values (g/kg/day and g/animal/day) in all treated groups were not significantly different from controls during gestation or lactation (Rep-ANOVA, main effect of [Group] for absolute food consumption during lactation, $p=0.0352$; not further analyzed). High-dose dams consumed slightly less food (g/day) during early lactation ($\downarrow 4-15\%$ vs control), and relative food consumption (g/kg/day) values were decreased ($\downarrow 5-12\%$) during treatment. Although the differences were minor and not significantly different (defined as $p<0.02$), they were attributed to treatment by the study author.
5. **Test Substance Intake** - This parameter was not necessary since this was a gavage study and daily doses were based on daily bodyweight measurement.
6. **Pregnancy Status and Litter Data** - There were no observed effects on maternal performance parameters of pregnancy rate, gestation index, length of gestation, number of implantation sites, and number of live or dead pups. There were no abortions. Observations are presented in Table 5.

Table 5. Pregnancy observations.^a

Observation	Dose (mg/kg/day)			
	0	0.3	1	5
Animals Assigned (Mated)	25	25	25	25
Animals Pregnant	25	24	24	24
Pregnancy Rate (%)	(100)	(96)	(96)	(96)
Number with Live Litters	25	24	24	23
Number with No Liveborn Pups	0	0	0	1
Gestation Index (%)	100	100	100	95.8
Duration of Gestation (days)	23.1 \pm 0.5	23.2 \pm 0.4	23.0 \pm 0.5	23.0 \pm 0.2
Total Implantations(FTG)	339	333	340	341
Implantations/Dam	13.6 \pm 3.2	14.5 \pm 2.0	14.2 \pm 2.4	14.2 \pm 1.6
Total Live Pups(FTG) ^b	308	319	311	292
Live Pups/Dam	12.3 \pm 3.1	13.3 \pm 1.9	13.0 \pm 2.4	12.7 \pm 2.4
Total Dead Pups(FTG) ^b	1	1	5	3
Dead Pups/Dam	0.0 \pm 0.2	0.0 \pm 0.2	0.2 \pm 0.5	0.1 \pm 0.6
Mean % Male Pups	53.0	51.9	53.0	49.0

a Data extracted from the study report Tables B10 and B11, pages 131 through 133.

b Includes only animals with live litters.

FTG - Full term gestating females

7. **Cholinesterase Activity** - Brain ChE activity was decreased in the high-dose ($\downarrow 90\%$) and the mid-dose ($\downarrow 18\%$) dams (Table 6) as compared to control. Erythrocyte ($\downarrow 41-99\%$) and plasma ($\downarrow 43-92\%$) ChE activity were decreased in a dose-dependent manner in all treated groups. The study report does not indicate whether these data were analyzed statistically.

Table 6. Mean (\pm S.E.) plasma, erythrocyte, and brain cholinesterase activity as % of control group at gestation day 20 in dams (5/group) dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Sample	Dosage (mg/kg/day)		
	0.3	1	5
Plasma	56.70 \pm 2.69	31.13 \pm 4.07	8.46 \pm 1.19
Erythrocyte	58.74 \pm 16.10	15.59 \pm 6.80	0.13 \pm 0.15
Brain	99.72 \pm 1.48	82.11 \pm 2.80	10.18 \pm 0.92

a Data extracted from the study report Appendix K, pages 606 through 613. Cholinesterase measurements conducted by Dr. Stephanie Padilla (USEPA, NHEERL, RTP, NC).

B. F₁ GENERATION TOXICITY - PUPS

- Pup Bodyweights:** Body weights for all F₁ pups, recorded prior to selection to Subsets, are presented in Table 7a. In the high-dose group, pup (male and female) body weights were significantly reduced (\downarrow 8-15%, $p=0.0001$) at PND 1 and 5 (pre- and post-culling). Analysis of male and female bodyweights separately showed that these two groups reacted similarly to treatment.

F₁ pup bodyweights (Table 7b) were reduced from birth to PND 22 in Subset 4 high-dose males (\downarrow 5-19%) and females (\downarrow 6-17%). Additionally, F₁ pup bodyweight gains (Table 7c) were reduced from birth to PND 22 in Subset 4 high-dose males (\downarrow 5-28%) and females (\downarrow 8-30%).

Table 7a. Mean pup body weights (g) (recorded prior to selection to Subset) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Interval (days postpartum)		Dosage (mg/kg/day)			
		0	0.3	1	5 (*)
Day 1	Males	6.6	6.7	6.4	6.1
	Females	6.3	6.2	6.1	5.6
Day 5 (Preculling)	Males	9.9	10.2	10.3	8.8
	Females	9.6	9.7	9.7	8.2
Day 5 (Postculling)	Males	9.8	10.2	10.1	8.8
	Females	9.4	9.6	9.5	8.2

a Data extracted from the study report Table B11, page 134.

(*) Rep-ANOVA, significant main effect of [Group] ($p=0.0001$), and significant [Group X Time] interaction ($p=0.0001$). The analysis was no longer statistically significant after removal of the high dose group; data were not further analyzed.

Table 7b. Selected, mean bodyweights of F₁ pups (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Day Postpartum	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
1	6.6	6.7	6.7	6.3
5	9.8	10.3	10.2	8.6
12	24.4	25.6	25.4	19.8
18	40.4	41.9	41.2	35.0
22	54.3	55.8	55.2	45.1
Females				
1	6.2	6.2	6.0	5.8
5	9.4	9.7	9.6	8.3
12	23.7	24.6	23.8	19.6
18	39.0	40.1	39.0	33.8
22	51.8	53.4	51.6	42.8

a Data extracted from the study report Tables F3 and F4, pages 419 and 420.

(*) Rep-ANOVA, analysis included PND 1, 5, 12, 18, 22, 40, and 66; main effect of [Group], $p=0.0001$, [Group X Time], $p=0.0001$; analysis was no longer statistically significant when the high dose group was removed; note that the analysis includes PND 40 and 66 (see Table 13a). No further statistical analysis was submitted.

Table 7c. Selected, mean bodyweight gains of F₁ pups (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

PND Interval	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
1-5	3.2	3.6	3.6	2.3
5-12	14.6	15.3	15.2	11.2
12-18	16.0	16.3	15.8	15.2
18-22	13.8	13.9	14.0	10.1
Females				
1-5	3.2	3.4	3.6	2.5
5-12	14.3	14.9	14.2	11.3
12-18	15.4	15.5	15.2	14.2
18-22	12.8	13.3	12.6	8.9

a Data extracted from the study report Tables F5 and F6, pages 421 and 422.

(*) Rep-ANOVA, analysis included days 1-5, 5-12, 12-18, 18-22, 22-40, and 40-66; significant main effect of [Group] ($p=0.0001$), significant [Group X Sex] interaction ($p=0.0307$), significant [Group X Time] interaction ($p=0.0001$). Analysis was no longer statistically significant when the high dose group was removed. Note that analysis included days 22-40 and 40-66 (see Table 13b).

2. **Pup Survival Indices:** There were overall significant group differences due to the high-dose group for the following parameters: surviving pups per litter ($\downarrow 27\%$, $p=0.002$) and live litter size at PND 5 ($\downarrow 16\%$, $p=0.018$), pup viability index ($\downarrow 29\%$, $p=0.001$), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls, $p=0.001$; days 2 to 5 - 24.7% treated vs 1.3% controls, $p=0.001$). Dams with all pups dying were

increased in the high-dose group (3/23 treated vs 0/25 controls, $p=0.024$). Data are shown in Table 8.

Table 8. Pup survival data from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Observation (days postpartum)	Dose (mg/kg/day)			
	0	0.3	1	5 (*)
Viability Index (day 5)	98.7	98.7	98.1	69.9 c
Surviving Pups/Litter (day 5)	12.2	13.1	12.7	8.9 c
Live Litter Size (day 5)	12.2	13.1	12.7	10.2 b
Pups Found Dead or Missing (day 1, %)	0.0	0.3	0.6	7.2 c
Pups Found Dead or Missing (days 2-5, %)	1.3	0.9	1.3	24.7 c
Dams with all Pups Dying (days 1-5)	0	0	0	3 a

a Data extracted from the study report Tables B10 and B11, pages 131 through 134.

(*) Statistically significant effects, a: $p=0.024$; b: $p<0.02$; c: $p<0.002$; no longer significant after removal of high dose group.

3. Developmental Landmark Data: The mean day of pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls, $p=0.0265$) in the high-dose group (Table 9). Although statistical significance (at $p<0.02$) was not achieved, this finding was judged to be treatment-related and was considered to be consistent with findings of decreased pup body weight at the high-dose. Eye opening was not significantly impacted by treatment with chlorpyrifos ($p=0.099$).

Table 9. Development Landmark Data.^a

Observation (Mean Day to Criteria for all Pups)	Dose (mg/kg/day)				
	0	0.3	1	5	
Pinna Unfolding	3.5	3.5	3.6	4.0	
Eye opening	Males	14.6	14.6	14.7	14.9
	Females	14.4	14.0	14.4	14.6

a Data extracted from the study report Table B13, page 136.

4. Clinical Observations: There were no clinical observations for the F₁ pups (Subset 4) that were considered to be treatment-related. There was no effect on body temperature in adult Subset 3 F₁ rats at PND 22 and 61.
5. Neurobehavioral Evaluations

Spatial Delayed Alternation - A total of 31-32 Subset 2 pups/sex were evaluated for the effect of exposure to chlorpyrifos on learning and memory on PNDs 22-25 and 62-92.

The data were recorded as percent correct responses during acclimation over multiple block trials and a time delay. A regression line was fitted to the individual rat data and the slope and intercept were analyzed. The slope represented the decay of information just learned, while the intercept at extrapolated time 0 represented non-memory factors such as motivation, sensory-motor processes, and attention. There were no statistically significant differences between the groups in the average acquisition and delayed response (statistical analysis compared the slope and intercept of regression lines fitted to delayed response data for each group). Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 with PNDs 62-92. Maze acclimation data were not presented. (See also attached Figures 2A-2D, from study report pages 102-105).

Motor Activity - A total of 78-79 Subset 3 pups/sex were evaluated for motor activity. There was no statistically significant main effect of [Group] for the Rep-ANOVA evaluating mean motor activity for all treatment groups on PNDs 14, 18, 22, and 61 ($p=0.630$). However, there was a statistically significant ($p=0.0141$) [Group X Time] interaction (attributed to the high dose group). On day 14, total activity was decreased for both high dose males and females (37-56%). Although total activity remained decreased for males at day 18 (15%) and day 22 (4%), there was a large increase for females (51%) at both time points. On day 61, activity was increased for both males and females (16- 17%). Insufficient statistical analyses were performed to determine the statistical significance of the differences at these various time points (for example, [Group X Sex X Time] and [Group X Sex X Time X Block] interactions were not presented). The study authors stated this effect was spurious because no consistent change in motor activity was noted across time points. Agency reviewers consider the effects on motor activity at the high dose to be treatment related. (See also attached Figures 3A-3H, from study report pages 106-113).

Body Temperature - Body temperature measurements taken on PNDs 23 and 61 immediately following completion of the motor activity testing were similar in all groups. (See also attached Figure 4, from study report page 114).

Auditory Startle Habituation - A total of 76-79 Subset 3 pups/sex were evaluated for auditory startle habituation on PNDs 23 and 62. There was an increase ($\uparrow 16-25\%$) in the latency to peak response on PND 23 in the high-dose animals compared to concurrent controls (Table 10). At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls (main effect of [Group], days 23 and 62, $p=0.0049$; [Group X Time] interaction, $p=0.1076$; when Group 4 is removed, the analysis is no longer significant). Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 compared to the controls (main effect of [Group], $p=0.0737$; [Group X Time] interaction, $p=0.0442$). Note that not all potential interactions were presented in the analysis, most notably [Group X Sex X Time] and [Group X Sex X Time X Block] (see also attached Figures 5A-5D, from study report pages 115-118).

Table 10. Auditory startle habituation [mean peak response amplitude (g) and mean latency (sec) to peak response] in F1 offspring (Subset 3)].^a

Observation/Study Days	Dose Group (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
Mean Peak Response/PND 23	56.6 ± 22.9	63.7 ± 30.1	56.9 ± 21.2	40.5 ± 10.0
Mean Peak Response/PND 62	219.7 ± 100.2	156.3 ± 69.5	171.3 ± 92.4	168.3 ± 80.5
Latency to Peak Response/PND 23	39.3 ± 7.1	38.5 ± 8.4	39.2 ± 9.4	49.1 ± 16.0
Latency to Peak Response/PND 62	36.5 ± 6.5	39.0 ± 9.2	37.5 ± 5.6	40.8 ± 11.6
Females				
Mean Peak Response/PND 23	57.9 ± 18.1	57.6 ± 16.0	55.7 ± 17.4	48.7 ± 20.5
Mean Peak Response/PND 62	146.6 ± 81.2	145.5 ± 89.2	97.0 ± 47.6	133.7 ± 82.3
Latency to Peak Response/PND 23	37.1 ± 8.8	36.8 ± 7.0	38.2 ± 7.0	43.0 ± 7.5
Latency to Peak Response/PND 62	39.3 ± 9.2	41.4 ± 9.4	45.6 ± 11.3	43.1 ± 8.8

a Data extracted from the study report Tables E3 and E4, pages 304-307; results for peak response amplitude were adjusted for baseline response (in the absence of stimulus).

(*) Rep-ANOVA: significant main effect of latency to peak response (main effect of [Group], days 23 and 62, $p=0.0049$; [Group X Time] interaction, $p=0.1076$; when high-dose was removed, the analysis was no longer significant). Additionally, the peak response amplitudes were decreased on PNDs 23 and 62 (main effect of [Group], $p=0.0737$; [Group X Time] interaction, $p=0.0442$).

6. Sacrifice and Pathology at PND 12

Macroscopic Pathology F₁ Generation Pups - There were no treatment-related gross pathology lesions.

Terminal Body Weights and Brain Weights of F₁ Generation Pups - Compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17- 19%) and the Subset 4 (PND 66) high-dose males (↓10%). Absolute brain weights of the Subset 1 high-dose animals were reduced (↓9%) and their brain/bodyweight ratios were increased (113-114%) compared to the controls (Table 11). The study pathologist concluded that the differences in brain size in the Subset 1 animals may be due to undernutrition. Brain size in the Subset 4 animals was similar in all groups (PND 66).

Table 11. Mean terminal bodyweights, brain weights, and brain/bodyweight ratios from F₁ pups (Subset 1) at PND 12.^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males (n=10)				
Body weight (g)	23.5	27.4	25.9	19.4

Brain weight (g)	1.284	1.409	1.356	1.173
Brain/body weight (%)	5.480	5.161	5.260	6.205
Females (n=10)				
Body weight (g)	23.1	23.2	23.1	18.8
Brain weight (g)	1.284	1.279	1.273	1.171
Brain/body weight (%)	5.585	5.535	5.540	6.355

a Data extracted from the study report Tables C2 and C4, pages 228 and 230.

(*) Statistical findings: Note that both day 12 and day 66 findings were included in the same statistical analysis; 1) Terminal body weight: main effect of [Group], $p=0.004$; [Group X Sex] interaction, $p=0.0149$; when high dose was excluded from analysis, [Group X Sex] interaction $p=0.0273$; 2) Brain weight: main effect of [Group], $p=0.0001$, [Group X Time] interaction, $p=0.0276$; when high dose was excluded from analysis, main effect of [Group], $p=0.0275$; 3) Brain/body weight (%): main effect of [Group], $p=0.0001$, [Group X Time] interaction, $p=0.001$; when high dose was excluded from analysis, there were no significant findings.

Microscopic Pathology F₁ Generation Pups - There were no treatment-related microscopic neuropathology lesions.

Brain Morphometry - Morphometry measurements performed on PND 12 pups (Subset 1) detected a number of alterations, including: reduced anterior to posterior measurement of the cerebellum ($\downarrow 24\%$), reduced height of the cerebellum ($\downarrow 14\%$), a decrease in parietal cortex thickness ($\downarrow 6\%$), and a decrease in the thickness of the hippocampal gyrus ($\downarrow 9\%$) in the high-dose males (Table 12). The same treatment-related findings were noted in high-dose female pups: Subset 1 high-dose female pups had decreased cerebellar height ($\downarrow 9\%$) and anterior to posterior length ($\downarrow 4\%$), reduced thickness of the parietal cortex ($\downarrow 6\%$), reduced width of the caudate-putamen ($\downarrow 10\%$), and reduced thickness of the hippocampal gyrus (neuropathologic alterations were detected in any of the animals and that the differences that were detected should be attributed to the observed reduction in brain size only. However, we consider these effects to be treatment-related at both time points (see below for day 60 findings). Note that findings from both day 12 and day 60 were included in the same statistical analysis; day 12 findings included all treatment groups, day 60 findings included control males and females, high-dose males and females, and mid-dose females (see table footnote). No analysis excluding the high dose group were performed for morphometric findings, therefore the statistical significance cannot be attributed to high dose animals only. Since both time points were included in the analysis, treatment cannot be attributed to a single treatment day. Several possible interactions were not evaluated, including [Group X Time X Sex].

Table 12. Selected, mean morphometric data at PND 12 from F1 pups (Subset 1) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males (n=6)				
Cerebellum (Ant. to Post.; mm)	3.267 \pm 0.308	3.450 \pm 0.345	3.333 \pm 0.186	2.467 \pm 0.550
Cerebellum (Height; μ m)	3504 \pm 128.8	3456 \pm 17.17	3416 \pm 200.0	3008 \pm 504.0

Parietal Cortex Thickness (μ m)	1336 \pm 56.1	1448 \pm 58.1	1448 \pm 32.8	1256 \pm 138.0
Caudate-Putamen Width (μ m)	2240 \pm 84.1	2240 \pm 108.0	2312 \pm 93.16	2224 \pm 147.7
Hippocampal Gyrus Thickness (μ m)	904 \pm 93.2	1004 \pm 114.0	972 \pm 54.2	824 \pm 65.6
Females (n=6)				
Cerebellum (Ant. To Post.; mm)	3.183 \pm 0.223	3.033 \pm 0.320	3.300 \pm 0.167	3.000 \pm 0.310
Cerebellum (Height; μ m)	3512 \pm 200.0	3176 \pm 130.3	3120 \pm 328.4	3208 \pm 226.0
Parietal Cortex Thickness (μ m)	1380 \pm 54.2	1376 \pm 19.6	1368 \pm 80.3	1304 \pm 72.3
Caudate-Putamen Width (μ m)	2384 \pm 131.2	2224 \pm 116.3	2288 \pm 108.0	2152 \pm 133.8
Hippocampal Gyrus Thickness (μ m)	936 \pm 81.7	912 \pm 50.3	932 \pm 96.5	828 \pm 78.5

a Data extracted from the pathology report Tables 1 and 2, pages 666 through 669.

(*) Statistical findings are as follows (analysis included both PND 12 and 60): 1) Cerebellum (ant. to post.): main effect of [Group], $p=0.0029$; [Group X Time] interaction, $p=0.0249$; 2) Cerebellum (height): main effect of [Group], $p=0.0063$, [Group X Time] interaction, $p=0.0291$; 3) Parietal cortex thickness: main effect of [Group], $p=0.0199$; [Group X Time] interaction, $p=0.2596$; 4) Caudate-putamen width: main effect of [Group], $p=0.2792$, [Group X Time] interaction, $p=0.0528$; 5) Hippocampal gyrus thickness: main effect of [Group], $p=0.0011$, [Group X Time] interaction, $p=0.6434$. Further statistical analysis was not conducted.

C. F₁ GENERATION TOXICITY - ADULTS

1. Body Weights and Bodyweight Gains: Body weights (Table 13a) of the high-dose F₁ adult males (Subset 4) were decreased at PND 22 through 66 (\downarrow 11-17% vs controls; $p=0.0001$). High-dose F₁ adult females (Subset 4) also weighed less than controls at PND 22 (\downarrow 17% vs controls) and 40 (\downarrow 9%), but were of similar weight at PND 66. Bodyweight gains (Table 13b) were also decreased in the high-dose males for the PND 22-40 interval (\downarrow 13% vs controls) and PND 40-66 interval (\downarrow 7%). Mid- and low-dose males and females did not show significant differences in bodyweight gains.

Table 13a. Selected, mean bodyweights of F₁ adults (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Day Postpartum	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
22	54.3	55.8	55.2	45.1
40	186.7	191.2	190.5	160.3
66	388.9	385.4	389.8	348.0
Females				
22	51.8	53.4	51.6	42.8

40	146.5	148.8	145.4	133.3
66	228.7	238.1	228.8	220.3

a Data extracted from the study report Tables F3 and F4, pages 419 and 420.

(*) Rep-ANOVA, analysis included PND 1, 5, 12, 18, 22, 40, and 66; main effect of [Group], $p=0.0001$, [Group X Time], $p=0.0001$; analysis was no longer statistically significant when the high dose group was removed (see also Table 7b). No further statistical analysis was submitted.

Table 13b. Selected, mean bodyweight gains of F₁ adults (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Day Postpartum Interval	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
22-40	132.4	135.4	135.2	115.2
40-66	202.2	194.2	199.3	187.8
Females				
22-40	94.7	95.4	93.8	90.5
40-66	82.2	89.3	83.0	87.0

a Data extracted from the study report Tables F5 and F6, pages 421 and 422.

(*) Rep-ANOVA, analysis included days 1-5, 5-12, 12-18, 18-22, 22-40, and 40-66; significant main effect of [Group] ($p=0.0001$), significant [Group X Sex] interaction ($p=0.0307$), significant [Group X Time] interaction ($p=0.0001$). Analysis was no longer statistically significant when the high dose group was removed (see also Table 13b).

2. **Food Consumption:** Food consumption was decreased immediately after weaning (PND 23-30) in high-dose male and females ($\downarrow 13\%$ vs controls). In the later intervals (PND 40-47 and PND 59-66), food consumption was decreased only 3-10% versus controls. Data are shown in Table 14.

Table 14. Mean food consumption of F₁ adults (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Day Postpartum Interval	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males				
23-30	13.6	14.0	13.4	11.9
40-47	26.2	26.6	26.5	23.7
59-66	30.4	29.9	30.0	28.2
Females				
23-30	12.0	12.9	12.1	10.4
40-47	19.4	19.6	18.8	18.3
59-66	20.5	21.3	20.0	19.9

a Data extracted from the study report Tables F7 and F8, pages 423 and 424.

(*) Statistical analysis indicated a significant main effect of [Group] ($p=0.0001$); analysis was no longer significant when high dose group was removed.

3. **Mortality and Clinical Signs:** No treatment-related clinical signs were observed in adult F₁ rats. F₁ adult mortality was limited to 1 death in the mid-dose group (Subset 2), and 3 deaths in the high-dose group (Subsets 2 and 3).
4. **Sexual Maturation:** Sexual maturation was delayed as assessed by time to preputial separation (106% of controls; not significant [$p=0.0535$]) or vaginal patency (103% of controls; $p=0.0130$). Data are shown in Table 15. Such delays are not inconsistent with decreased body weights.

Table 15. Mean (\pm S.D.) day of sexual maturation observation in F₁ adult rats (Subset 4) from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5
Males				
Preputial Separation	44.2 \pm 1.9	43.4 \pm 1.9	45.2 \pm 3.2	47.0 \pm 5.9
Females				
Vaginal Patency	32.4 \pm 1.0	31.5 \pm 1.5	32.1 \pm 2.3	33.4 \pm 2.2*

a Data extracted from the study report Table F9, page 425.

* Significantly different from control group, $p<0.02$.

5. Sacrifice and Pathology at PND 66

Macroscopic Pathology - There were no treatment-related gross pathology lesions.

Terminal Body Weights and Brain Weights of F₁ Generation Adults: In the high-dose males, a decrease in terminal bodyweight (\downarrow 10% vs controls), decrease in brain weight (\downarrow 1%), and increase in the brain to body weight ratio (111% of controls) were observed. In the high-dose females, a slight decrease in brain weight (\downarrow 0.4%) and increase in brain to body weight ratio (\uparrow 2%) were also observed. The findings were considered to be marginal. Mid- and low-dose males and females did not show any treatment-related effect on body weight, brain weight, or brain to body weight ratio. Data are shown in Table 16.

Table 16. Terminal bodyweight, brain weight, and brain/bodyweight ratio of F₁ adult rats (Subset 4) at day postnatal day 66 from dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5

Males				
Terminal Body weight	410.7	384.8	408.2	368.7
Brain Weight	2.270	2.257	2.288	2.238
Brain/Body weight (%)	0.549	0.588	0.565	0.610
Females				
Terminal Body weight	234.5	254.2	233.1	230.4
Brain Weight	2.093	2.178	2.122	2.085
Brain/Body weight (%)	0.893	0.864	0.919	0.908

a Data extracted from the study report Tables F12 and F13, pages 428 and 429.

(*) Statistical findings (see also Table 11): Note that both day 12 and day 66 findings were included in the same statistical analysis; 1) Terminal body weight: main effect of [Group], $p=0.004$; [Group X Sex] interaction, $p=0.0149$; when high dose was excluded from analysis, [Group X Sex] interaction $p=0.0273$; 2) Brain weight: main effect of [Group], $p=0.0001$, [Group X Time] interaction, $p=0.0276$; when high dose was excluded from analysis, main effect of [Group], $p=0.0275$; 3) Brain/body weight (%): main effect of [Group], $p=0.0001$, [Group X Time] interaction, $p=0.001$; when high dose was excluded from analysis, there were no significant findings.

Microscopic Pathology - There were no microscopic neuropathological lesions.

Brain Morphometry - Morphometric measurements performed on PND 66 (Subset 4) revealed some slight intergroup differences (Table 17). There were no apparent treatment-related differences between control and high dose male brain measurements, but in high dose females, the parietal cortex measurement was decreased (5%) and the thickness of the hippocampal gyrus was decreased (7%). Results of the statistical analysis are discussed above and in the footnote for Table 12. Without an analysis excluding the high-dose group, statistical significance cannot be attributed at the high-dose only. Evaluation of mid-dose females indicated a possible dose-related trend in the findings, but the study pathologist dismissed them as representative of random variation. Although the pathologist concluded that the results demonstrated a recovery from any delays in brain development observed at PND 12, there remains some concern that a marginal deficit in brain morphometry (in particular for hippocampus and parietal cortex) still exists for the high-dose females at PND 66, even after body and brain weight are no longer significantly decreased as compared to control. Due to incompleteness of the statistical analysis, it is not clear whether there are significant effects at the mid-dose. If significant effects were found at that dose, brains from the low dose females would need to be evaluated at this time point.

Table 17. Selected, mean (\pm S.D.) morphometric data at PND 66 from F1 offspring (Subset 4) of dams dosed with chlorpyrifos from gestation day 6 to lactation day 11.^a

Observation	Dosage (mg/kg/day)			
	0	0.3	1	5 (*)
Males (n=6)				
N	6	0	0	6
Cerebellum (Ant. to Post.; mm)	5.683 \pm 0.232	--	--	5.667 \pm 0.216
Cerebellum (Height; μ m)	5152 \pm 218.2	--	--	5104 \pm 351.0

Parietal Cortex Thickness (μ m)	1756 \pm 79.5	--	--	1792 \pm 58.1
Caudate-Putamen Width (μ m)	2800 \pm 176	--	--	2744 \pm 98.0
Hippocampal Gyrus Thickness (μ m)	1640 \pm 91.9	--	--	1612 \pm 95.3
Females (n=6)				
N	6	0	6	6
Cerebellum (Ant. To Post.; mm)	5.517 \pm 0.232	--	5.500 \pm 0.261	5.383 \pm 0.098
Cerebellum (Height; μ m)	5016 \pm 120.5	--	4888 \pm 150.0	4968 \pm 207.6
Parietal Cortex Thickness (μ m)	1792 \pm 36.1	--	1716 \pm 36.4	1700 \pm 55.6
Caudate-Putamen Width (μ m)	2576 \pm 131.2	--	2552 \pm 178.1	2704 \pm 112.2
Hippocampal Gyrus Thickness (μ m)	1708 \pm 57.6	--	1644 \pm 129.5	1592 \pm 86.8

a Data extracted from the pathology report Tables 1 and 2, pages 32 through 33.

(*) Statistical findings are as follows (analysis included both PND 12 and 60; see also Table 12): 1) Cerebellum (ant. to post.): main effect of [Group], $p=0.0029$; [Group X Time] interaction, $p=0.0249$; 2) Cerebellum (height): main effect of [Group], $p=0.0063$, [Group X Time] interaction, $p=0.0291$; 3) Parietal cortex thickness: main effect of [Group], $p=0.0199$; [Group X Time] interaction, $p=0.2596$; 4) Caudate-putamen width: main effect of [Group], $p=0.2792$, [Group X Time] interaction, $p=0.0528$; 5) Hippocampal gyrus thickness: main effect of [Group], $p=0.0011$, [Group X Time] interaction, $p=0.6434$. Further statistical analysis was not conducted.

III. DISCUSSION

A. Investigator's Conclusions: No effects, other than cholinesterase inhibition in the dam, were seen in either the dam or pups at dosage levels of 0.3 or 1.0 mg/kg/day. The study report highlights brain cholinesterase inhibition at 5 mg/kg/day as the only adverse effect, while plasma and RBC cholinesterase inhibition at 1 and 0.3 mg/kg/day are not considered to be adverse. Administration of chlorpyrifos at 5 mg/kg/day produced clinical evidence of cholinergic toxicity in the dams, supported by non-statistically significant reductions in body weight change and food consumption. In the high-dose group (5 mg/kg/day), increased pup mortality in early lactation, decreased offspring body weight, related decreases in brain size and brain layer thickness on PND 12, transient changes in the auditory startle response (decreased response and increased latency to response, attributed by the study author to "motor slowing"), and delayed pinna detachment, vaginal opening and preputial separation were observed. Learning, memory, and habituation were not impaired in the pups at any time at any dosage. The study author concluded that chlorpyrifos is not a selective developmental neurotoxicant in the rat, and that the no-observed-adverse-effect-level (NOAEL) for maternal and pup toxicity in this study was 1 mg/kg/day.

B. Reviewer's Conclusions

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (DG 6) through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on DG 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F₁ generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated

vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.

The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F₁ generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); bodyweight gains were reduced in these animals during the same period (↓5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17-19%) and the Subset 4 (PND 66) high-dose males (↓10%). For the F₁ generation adults, body weights of the high-dose males were decreased at PND 22 through 66 (↓11-17% vs controls). High-dose F₁ adult females also weighed less than controls at PND 22 (↓17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (↓13% vs controls) and PND 40-66 interval (↓7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (↓13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/litter (↓27%) and live litter size at culling (↓16%), pup viability index (↓29%), and pups found dead or presumed cannibalized (day 1 - 7.2% treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high dose male and female pups on PND 14 (↓56% in males and ↓37% in females), and increased in high dose females on PNDs 18 and 22 (↑51% on both days). There was a statistically significant increase (↑16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose

animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑13% vs controls), reduced anterior to posterior measurement of the cerebellum (↓24% vs controls), reduced height of the cerebellum (↓14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), and decreased thickness of the hippocampal gyrus (↓9% vs controls). High-dose female pups had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑14% vs controls), thickness of the parietal cortex (↓6% vs controls), width of the caudate-putamen (↓10% vs controls), and thickness of the hippocampal gyrus (↓12% vs controls). In Subset 4 F1 animals, killed on PND 66, morphometric analysis revealed decreased parietal cortex measurements (↓5%) and decreased thickness of the hippocampal gyrus (↓7%) in high-dose females. These measurements were also decreased in mid-dose females (parietal cortex, ↓4%; hippocampal gyrus, ↓4%). The statistical significance of the differences in mid-dose females was not evaluated, and there was no evaluation of low dose females. Brain weight in high dose females was similar to control brain weight at day 66 (↓0.3%).

The tentative developmental neurotoxicity LOAEL is 5 mg/kg/day.

The tentative NOAEL is 1 mg/kg/day.

In setting this developmental NOAEL based upon all available data from this study, the following issues are acknowledged:

- 1) No cholinesterase inhibition data were generated for offspring (fetuses and pups) on this study. Therefore, this study in and of itself may not provide a definitive assessment of maternal vs fetal susceptibility following chlorpyrifos exposure. It is recognized that a separate study has been submitted by the Registrant to address pharmacokinetic issues and comparative cholinesterase inhibition in adult vs neonatal rats (MRID 44648102). The results of the developmental neurotoxicity study should be considered in context of the supplementary special study.
- 2) As discussed by the investigator, other confounding factors in the interpretation of these data include:
 - Appropriateness of the route of administration to the dams (gavage vs dietary) and pups (in utero and/or via the milk).
 - Time of exposure to the offspring (pre- vs postnatal) and its relevance to vulnerable periods of neurodevelopment in humans
 - Unknown pharmacokinetic and pharmacodynamic information in dams vs offspring and how such considerations may have affected study outcome or interpretation
 - The contribution of maternal toxicity to observation in the offspring (confounding interpretation of effects).

Study Deficiencies: The submitted stability data did not provide storage temperature.

The statistical analyses as presented in the text tables of the study report were in general difficult to interpret, due to lack of data on variations over time or due to differential responses by sex, e.g., body weight and motor activity data. Statistical analysis of some parameters was apparently not performed, e.g., cholinesterase inhibition data and PND 66 morphometric data. As a result, a definitive developmental neurotoxicity NOAEL and LOAEL could not be determined. Submission of more complete statistical analysis is required.

This study in the rat is classified **unacceptable (§83-6)** and does not satisfy the guideline requirements for a developmental neurotoxicity study. The study may be upgradable, following submission of more complete statistical analysis.

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