Thiamethoxam

Thiamethoxam – Developmental Neurotoxicity Study in Rats (RR0936). Response to Submitter Interpretations Related to EU Classification and Labelling.

Supporting Document for Submission

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1.0 INTRODUCTION

In January 2018 France submitted a CLH dossier to ECHA for the substance thiamethoxam (TMX). In the dossier France proposed a classification of H361 Reproductive Toxicity Category 2 based on effects in the testes in the multigeneration studies and dog studies as well as increased qualitative susceptibility of offspring in the developmental neurotoxicity (DNT) study (CLH report, 2018 and Annex to CLH report Thiamethoxam dRAR Volume 3 – B6 (AS)). This response document will address the comments relating to the DNT study, and the effects on the testes will be addressed in separate documents ([redacted] *et al*. 2019 and [redacted] et al. 2019).

2.0 SUBMITTER INTERPRETATIONS AND OVERALL SYNGENTA RESPONSE

Conclusions related to developmental neurotoxicity findings in the CLH proposal for thiamethoxam (CLH report, 2018) were as follows:

Delayed preputial separation was observed at the highest dose level in the presence of reduced body weight gain in dams during gestation and offspring decreased body weight (10% compared to controls).

AND

While the maternal and the offspring NOAELs were set at the same dose, it is considered that young animals exhibited increased susceptibility compared to adults since findings in the pups (reduced brain weight and significant changes in brain morphometric measurements) were more severe than those in the dams (decreased body weight gain and food consumption).

Syngenta's position on these findings is as follows:

- The F1 offspring effects in this study (lower absolute brain weights, smaller morphometry measurements, and delay in sexual maturation in males) **are all secondary to smaller bodyweights that were observed at birth (Day 1) and continued throughout the treatment period of the F1 offspring.**
- These smaller pup birth weights are a direct consequence of reduced maternal food consumption, also resulting in reduced maternal weight gain.
- Hence these observations in the offspring are not a specific effect on development, and do not show any evidence of increased susceptibility of the young compared to the adults.

3.0 DETAILED COMMENTS

3.1 Brain Weight and Bodyweight Association in Developing Rats

<u>Submitter comment:</u>

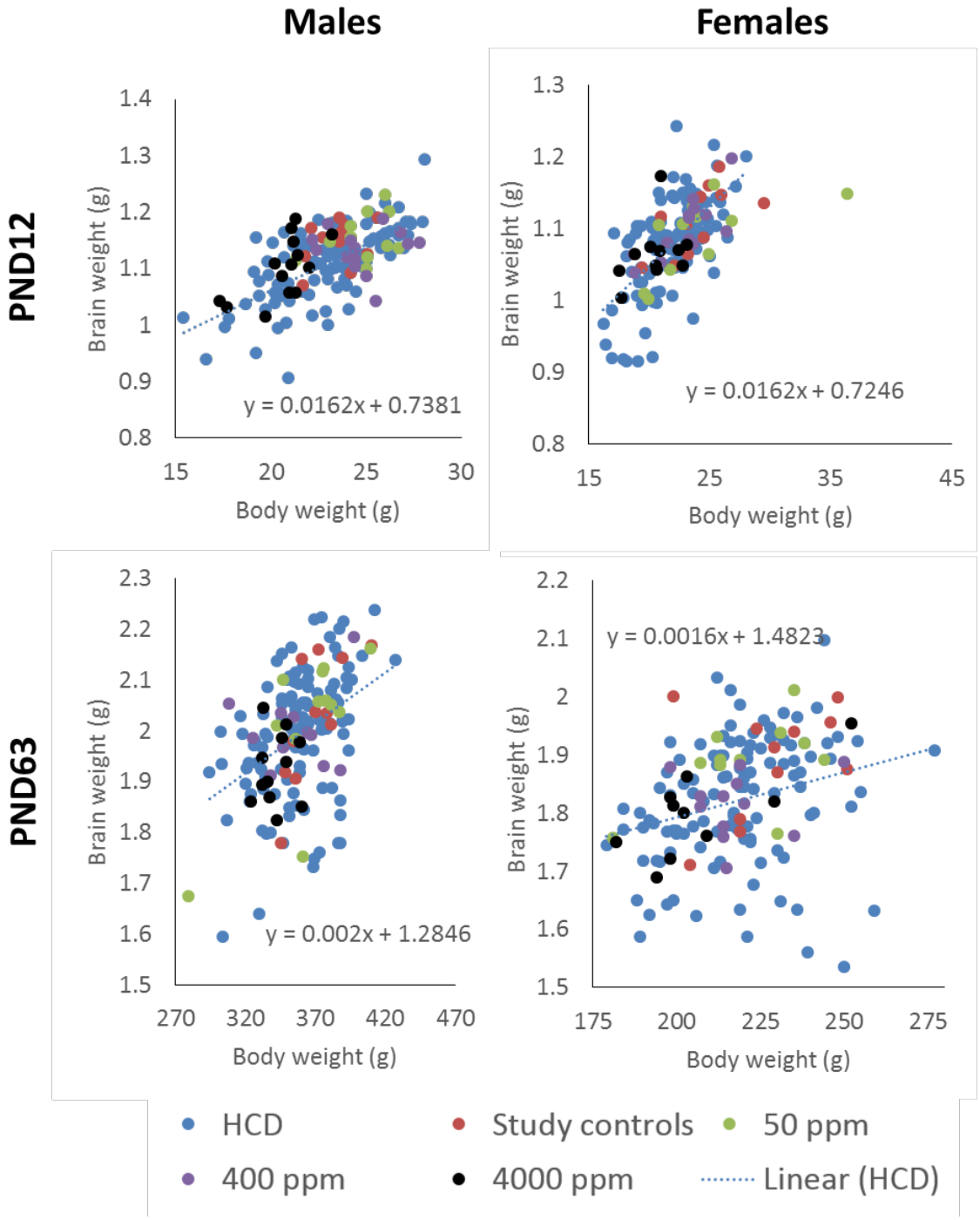
The bodyweights of male and female pups born of dams fed 4000 ppm TMX were statistically significantly lower than controls on day 12 (by approximately 12-15%) and on day 63 (by approximately 6-9%). However since brain weight is relatively insensitive to body weight change, the decreased absolute brain weight observed on Day 12 and Day 63 cannot be disregarded as only secondary to decreased bodyweight.

<u>Syngenta response:</u>
Syngenta disagrees with the submitter conclusion that the lower brain weights in thiamethoxam treated juvenile rats cannot be attributed to the lower body weight.

In order to demonstrate that the lower brain weights in thiamethoxam-treated rats are related to lower body weight, control data from 11 contemporaneous and historical DNT studies conducted at the same laboratory between 2001 and 2004 are provided (summarized in Appendix 3). Scatter graphs for the individual brain weights versus body weights for the historic control data (HCD) groups and the thiamethoxam DNT study (RR0936) groups are presented. These plots demonstrate that the thiamethoxam-treated rats at each dose level are clearly within the distribution of the control animal data.

This comparison demonstrates that the lower brain weights in thiamethoxam-treated F1 rats is attributable in extent and severity to the effect of thiamethoxam on the body weight of the F1 rats and therefore should not be considered evidence of developmental neurotoxicity.

Brain Weight Versus Body Weight in Control and RR0936 Groups for Post-Natal Day 12 and PND 63 rats.



Note truncated axes. Graphs without truncation are shown in Appendix 1 and 2.
Control data (HCD) collected from 11 studies conducted between 2001 and 2004

As a further illustration of this relationship, the table below provides mean brain weights per group, by three different methods:

- The absolute brain weight is shown, as discussed in the final report (RR0936).
- The actual percent of control values are calculated
- The derived percent of control values, by using the linear regression equation in the above figures for historic control data of brain weight vs. bodyweight, are also presented.

By these methods, the actual vs. derived percent of control values (mean per group) are virtually identical for the Day 12 assessments, and only slightly different for the Day 63 assessment. Thus, these comparisons indicate that the lower brain weights in the 4000 ppm male and female offspring are actually an expected outcome at all time points, given the significantly lower bodyweights in pups of this age. Based on this observation and comparison, it is logical that brain weight adjusted for bodyweight by ANCOVA showed no differences between control and treated groups (also shown in the table below, for completeness).

Absolute Brain Weights in F1 Offspring and Percent of Control Values

		Dose level of TMX (ppm)							
		Males				**Females**			
Dose (ppm)		**0**	**50**	**400**	**4000**	**0**	**50**	**400**	**4000**
Day 12									
Absolute brain weight (g)	Mean	1.15	1.16	1.13	1.10*	1.11	1.09	1.10	1.06*
Actual % of control		100.0	100.9	98.8	96.0	100.0	98.0	98.9	95.7
Derived % of control[a]		100.0	101.9	101.9	95.9	100.0	98.4	98.5	94.9
Adjusted brain weight (g) [b]		1.15	1.14	1.12	1.13	1.10	1.08	1.10	1.09
Day 63 (post perfusion)									
Absolute brain weight (g)	Mean	2.03	2.01	2.00	1.93*	1.89	1.89	1.82*	1.80*
Actual % of control		100.0	99.3	98.8	95.1	100.0	100.0	96.3	95.2
Derived % of control[a]		100.0	99.4	98.6	97.3	100.0	99.4	99.1	98.2
Adjusted brain weight (g) [b]		1.99	1.99	2.01	1.97	1.87	1.88	1.82	1.82

[a] Derived percent of control values were determined based on linear regression equations in graphs of absolute brain weight (g) vs. body weight (g) in pups from 11 historic control DNT studies (Appendix 1 – 2). Using the mean bodyweights of each treatment group/sex, the mean absolute brain weights were derived for control, 50, 400 and 4000 ppm groups. The derived values for each thiamethoxam-treated group were then divided by the control derived brain weight value, to determine the "Derived % of control".
[b]Adjusted brain weights after ANCOVA with terminal bodyweight as the covariate.

*$p<0.05$ vs. control, Student's t-test on absolute brain weight. After analysis by ANCOVA with body weight as covariate, none of the adjusted brain weight values were significantly different vs. control.

3.2 Effects of General Maternal Toxicity on Rat Fetal Development Parameters

Submitter comment:
"…in the DNT study brain effects were observed in offspring at the highest dose level in the presence of moderate maternal toxicity. There is therefore an increased qualitative susceptibility of developing organism since the effects in the pups (reduced brain weight and significant changes in brain morphometric measurements) are considered more severe than effects in the dams (decreased body weight gain and food consumption) observed at the same dose."

Syngenta response:
The adult brain is relatively insensitive to changes in body weight, as correctly stated by the submitter. However, multiple studies described in the literature (Carney et al., 2004; Garman et al., 2001; West and Kemper, 1976) have demonstrated that the developing brain is affected by deficits in maternal nutrition, food consumption and bodyweight. For thiamethoxam, maternal food consumption deficits of up to 15% at the 4000 ppm dose level was sufficient to cause deficits in body weight, absolute brain weight and developmental landmarks in the offspring, consistent with observations in these literature food restriction studies. As discussed previously, the scatter graphs presented in Section 3.1 demonstrate that the brain weights of offspring in all groups from the thiamethoxam DNT study are consistent with control animals of a similar body weight. Therefore, the absence of brain weight changes in the dams treated at 4000 ppm, and the presence of absolute brain weight changes in the F1 offspring, does not suggest increased susceptibility of the offspring to thiamethoxam.

3.3 Brain Morphometry Changes

Submitter Comments:
The applicant considered that changes in brain morphometry measurements in high dose male and female F1 offspring were not evidence of developmental neurotoxicity but rather secondary to decreased body weight (general toxicity). However it is questionable that such body weight reduction (less than 10%) would be responsible of the quite large brain morphometric changes observed at termination in high dose animals.

The morphological changes were not associated with neuro-histopathological finding or change in functional or neurobehavioral parameters. However, it should be noted that the Y-maze for learning and memory assessment is a low sensitivity assay of behavioural change unless associated with appropriate difficulty tasks, which was not the case in the present study.

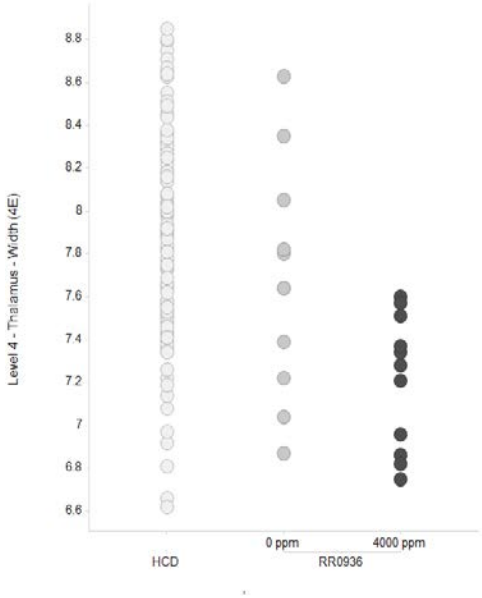
Syngenta response:
As a visual aid to the reviewers, and to help illustrate where numerical or statistical differences occurred in brain morphometry parameters, the original values are all displayed in Tables 1 and 2 (both as mean values, and as adjusted mean values after ANCOVA, with bodyweight as the covariate). The later re-analysis of parameters in the 50 ppm and 400 ppm groups are not included in Table 2, because (as captured by the submitter), the possible

artefact of tissue shrinking when stored in blocks may have affected these intermediate groups' values vs. the original morphometry values of the control group. The prior assessment of selected brain morphometry measurements in the intermediate dose groups confirmed that there were no effects of treatment on brain morphometry at 50 or 400 ppm, and this was also the conclusions of US EPA (2007) and the submitter (Annex to the CLH report dRAR, Volume 3 - B.6 (AS), 2018). In addition to providing a summary of the original morphometry values, Tables 1 and 2 contain several corrections to the historic control data values, compared to what was summarized in the tables of the CLH report and Annex (CLH report and Annex to the CLH report dRAR, Volume 3 - B.6 (AS), 2018).

In addition to the HCD range of mean values included in Tables 1-2, comparisons have been prepared for selected example morphometry parameters where all of the individual animal data are plotted together along with HCD individual values. Some illustrative patterns visible from these Figures are as follows:

- Thalamus width in females (Level 4, Day 12): both the range of 0 ppm concurrent control animals and 4000 ppm thiamethoxam animals were within the wider HCD range. However, the 4000 ppm individual values were at the lower end of this HCD range, while 0 ppm individual values were across a wider range. Difference vs. control was -6% ($p<0.01$).

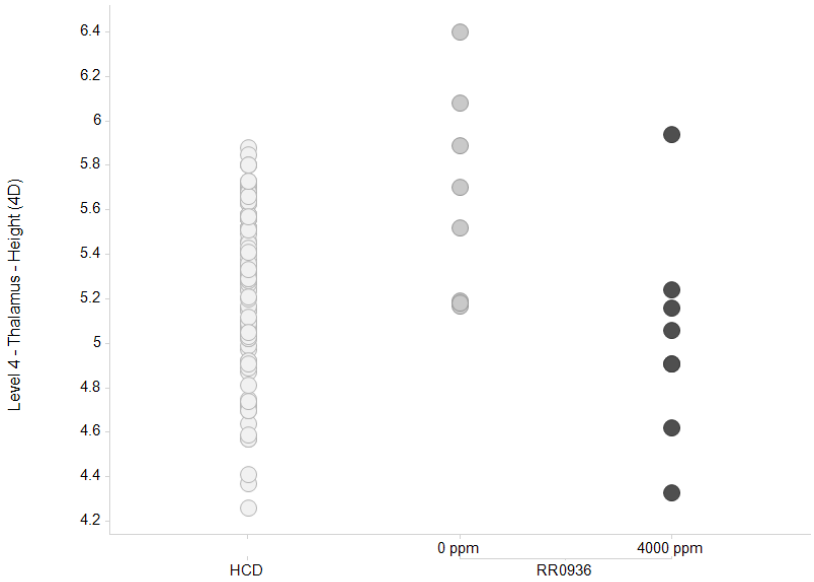
Individual Animal Data: Level 4 Thalamus Width in Females at PND12



HCD = combined individual values from 11 studies, same laboratory (Appendix 3).

- Thalamus height in males (Level 4, Day 63): The 4000 ppm individual values were in the same range as the HCD values. In contrast, the concurrent 0 ppm control group had values notably higher than the HCD. This suggests the concurrent control group had values that were unusually high, and this may be the primary reason for a statistical difference vs. the 4000 ppm group mean (-11%, $p<0.01$).

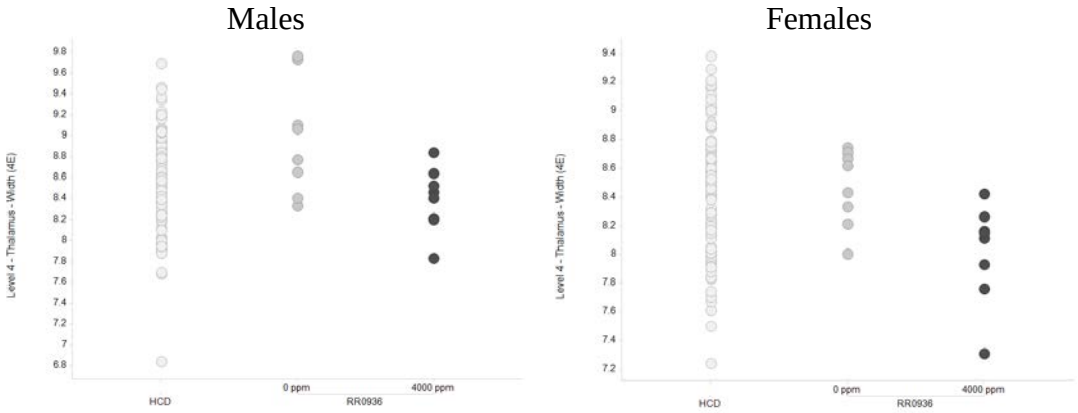
Individual Animal Data: Thalamus Height (level 4) in Males on Day 63



HCD = combined individual values from 11 studies, same laboratory (Appendix 3).

- Thalamus width (Level 4, Day 63): both for males and for females, the range of individual values at 4000 ppm was in the lower part of the HCD range, and the concurrent 0 ppm control values were at the higher end (males) or in the middle (females). Differences in means vs. control was -7% (males, $p<0.01$) and -5% (females, $p<0.01$).

Individual Animal Data: Thalamus Width (Level 4) in Males (A) and Females (B) on Day 63

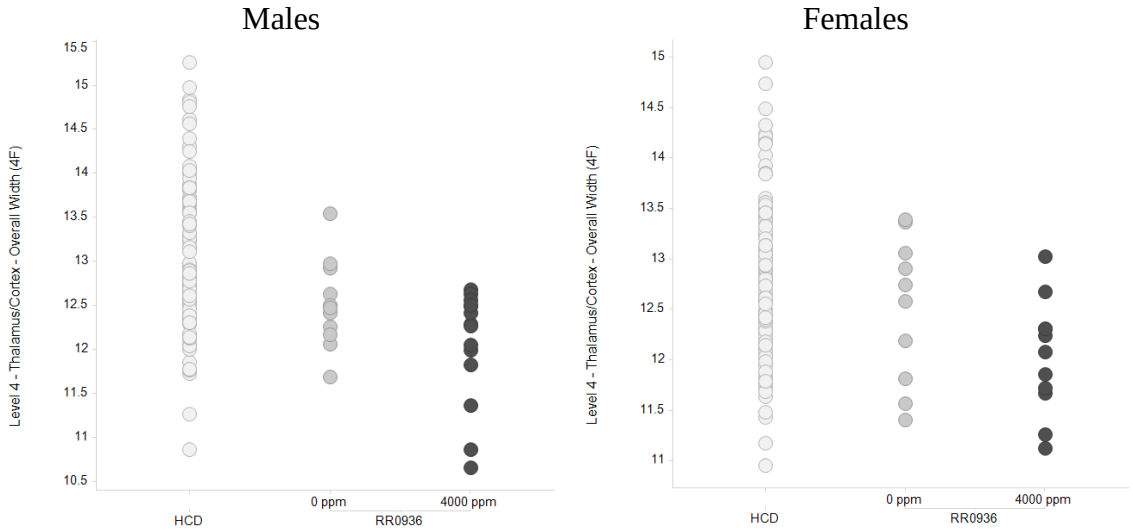


- HCD = combined individual values from 11 studies, same laboratory (Appendix 3).

- Thalamus/cortex overall width (Level 4, Day 63): both for males and for females, the range of individual values at 4000 ppm were toward the lower end of the HCD range, and the concurrent 0 ppm control values had a similar variation but toward the middle

range of the HCD values. Differences in means vs. control were -5% (males, $p<0.05$) and -7% *females, $p<0.01$).

Individual Animal Data: Level 4 Thalamus / cortex Overall Width in Males (A) and Females (B) on Day 63



HCD = combined individual values from 11 studies, same laboratory (Appendix 3).

Analysis of brain morphometry measurements using the absolute measurements (by ANOVA) showed a variety of parameters that were statistically significantly lower than control on Day 12 and Day 63 (Tables 1 and 2). However, when adjusted for bodyweight via analysis of covariance, only a small number of brain morphometry measurements were statistically significantly lower than controls. The comparisons to individual animal values in the HCD dataset vs. 0 and 4000 ppm thiamethoxam groups has shown that for certain parameters (e.g. thalamus width (PND12 females, PND63 males/females) and thalamus / cortex overall width (PND63 males/females)), the 0 ppm and 4000 ppm groups were within the range of the HCD values, but the 4000 ppm animals were at the lower end of the distribution. The percent difference in these circumstances (-5% to -7%) was quite comparable to the difference in brain weight (-4% to -5%) and in bodyweight (e.g. -7 to -8% on Day 63) in males and females. In contrast, certain parameters that had a larger percent difference in the 4000 ppm group (e.g. thalamus height in PND 63 males; -11%) occurred only in one sex and showed a range of concurrent control individual values that do not seem reflective of the normal HCD range; thus the true magnitude of difference in certain structures may be less than the calculated values.

The use of adjusted brain weights and morphometry measurements is discussed in more detail in Appendix 4.

These comparisons and statistical analyses indicate a high probability that smaller brain weights and correspondingly smaller brain morphometry measurements were a consequence of the reduced birth weight and lower body weights (and brain weights) seen at 4000 ppm,

and not a sign of developmental neurotoxicity attributable to thiamethoxam. Other weight of evidence also supports this conclusion, including the following:

- There were no micropathology changes seen in any structures of the brain or nervous system.
- Also, no changes in functional or neurobehavioural parameters, as assessed by the functional observational battery, locomotor activity, auditory startle response or performance in the learning and memory tests, were observed in any treatment group.
- In a retrospective review of guideline DNT studies for six neonicotinoid insecticides including thiamethoxam (Sheets et al., 2016), there was no consistent findings across the class for brain morphometry changes, or neuropathology findings.

As a result, these small changes in a limited number of brain morphometry parameters, coupled with body weight related decreases in brain weight, are not considered evidence of developmental neurotoxicity.

3.4 Preputial Separation

The age at preputial separation is to some extent dependent upon bodyweight (Hood 2012; Cameron 1991). In the absence of body weight changes, a less than two day change in the timing of preputial separation is considered to be of no toxicological significance (Ashby & Lefevre 2000; Clark 1998). In the developmental neurotoxicity study with thiamethoxam, there was a delay by an average of 1.5 days in the top dose (4000 ppm) group versus the control group, which was also in conjunction with a significant reduction in body weight. Therefore, based on the small magnitude of the difference together with the bodyweight effects at 4000 ppm, this delay was considered to be of no toxicological relevance. The delay in preputial separation in the multi-generation study was of even smaller magnitude, and is discussed in [redacted] et al. (2018).

Effects on dams and the F1 offspring following food restriction studies (Carney et al., 2004) also showed that the day of preputial separation in male F1 offspring was delayed by 1.0 days (n.s.) following 30% food restriction, and was further delayed by 6.8 days ($p<0.05$) for a 50% food restriction group. This supports a position that the delay in preputial separation is a consequence of lower pup weight, secondary to maternal effects.

4.0 CONCLUSIONS

Syngenta concludes that the results from the developmental neurotoxicity study with thiamethoxam do not support a Reproductive Toxicity Category 2 (H361) classification.

The reduction in brain weight and the related assessment of brain morphometry measurements are attributable in extent and severity to the bodyweight reductions observed in F1 animals. Published literature has demonstrated that effects on maternal food consumption and bodyweight due to dietary restriction, has produced the same effects seen in the 4000 ppm thiamethoxam group, including a delay in the developmental landmark of preputial separation. Further HCD from 11 studies are now provided, that confirm that lower

brain weights are directly correlated to lower F1 offspring bodyweights, and are thus an expected outcome at the high dose where bodyweights were up to 13% lower than controls.

5.0 REFERENCES

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TABLES SECTION

TABLE 1 Selected Brain Morphometry Findings Investigated at Day 12

Parameters		Control	4000 ppm	HCD range
Males Day 12				
Level 4				
Thalamus – Width (mm)	Mean ± SD	7.54±0.43	7.37±0.47 (↓ 3.2%)	7.48 ± 0.36 to 8.37 ± 0.38
	Adjusted Mean	7.43	7.46	
Cerebellum				
Cerebellum Length (mm)	Mean ± SD	4.41±0.32	4.11±0.29* (↓ 6.8%)	3.71± 0.31 to 4.45 ± 0.14
	Adjusted Mean	4.26	4.24	
Pre-pyramidal Fissure - Thickness of Molecular Layer (µm) PPF	Mean ± SD	63.8±9.0	56.0±6.1** (↓ 12%)	45.41± 10.8 to 79.9 ± 12.8
	Adjusted Mean	59.8	59.1	
Females Day 12				
Level 4				
Thalamus – Width (mm)	Mean ± SD	7.68 ± 0.54	7.21 ± 0.31** (↓ 6%)	7.48 ± 0.28 to 8.30± 0.2
	Adjusted Mean	7.58	7.21	
Cerebellum				
Cerebellum Length (mm)	Mean ± SD	4.23 ± 0.39	4.26 ± 0.17	3.63 ± 0.58 to 4.37 ± 0.4
	Adjusted Mean	4.11	4.36	
Pre-pyramidal Fissure - Thickness of Molecular Layer (µm) PPF	Mean ± SD	60.9 ± 15.5	58.9 ± 7.9	48.1 ± 14.7 to 79.9 ± 12.8
	Adjusted Mean	59.2	60.5	

* Statistically significant difference from control group mean, $p<0.05$ (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, $p<0.01$ (Student's t-test, 2-sided)
Adjusted mean: after ANCOVA, with bodyweight as covariate.
HCD: Historical control data from 11 studies. Concurrent control from this study not included as part of the range.
Note: data generated as part of the 2007 supplementary study report is not included in this table

Incorrect values in Table 6.7.1.3-12 in the CLH dossier (Annex to CLH report Thiamethoxam dRAR Volume 3-B6 (AS) for HCD ranges have been corrected for: Level 4: Thalamus – Width (males).

TABLE 2 Selected Brain Morphometry Findings Investigated at Day 63

Parameters		Control	50 ppm@	400 ppm@	4000 ppm	HCD range
Males Day 63						
Level 3						
Dorsal Cortex 1 - Thickness	Mean ± SD	1.58 ± 0.12	1.35 ± 0.11** (↓ 15%)	1.37 ± 0.12** (↓ 13%)	1.40 ± 0.11** (↓ 11%)	1.22 ± 0.11 to 1.53 ± 0.11
	Adjusted Mean	1.58	1.35**	1.37**	1.40**	
Dorsal Cortex 2 - Thickness	Mean ± SD	1.88 ± 0.14			1.74 ±0.16* (↓ 7%)	1.48 ±0.19 to 1.77 ± 0.11
	Adjusted Mean	1.89			1.73	
Piriform Cortex - Thickness	Mean ± SD	1.52 ± 0.18			1.38± 0.12** (↓ 9%)	1.05 ±0.11 to 1.38 ± 0.09
	Adjusted Mean	1.51			1.38	
Level 4						
Dorsal Cortex - Thickness	Mean ± SD	1.53 ± 0.16			1.36 ± 0.09** (↓ 11%)	1.11± 0.17 to 1.53 ± 0.16
	Adjusted Mean	1.54			1.35	
Corpus Callosum - Thickness	Mean ± SD	0.46 ± 0.06			0.37 ± 0.09** (↓ 20%)	0.31±0.08 to 0.46 ± 0.11
	Adjusted Mean	0.42			0.40	
Thalamus - Height	Mean ± SD	5.64 ± 0.46			5.02 ± 0.47** (↓ 11%)	5.03±0.26 to 5.42 ± 0.34
	Adjusted Mean	5.41			5.26	
Thalamus - Width	Mean ± SD	8.98 ± 0.55			8.39 ± 0.31** (↓ 7%)	8.27±0.63 to 8.86 ±0.58
	Adjusted Mean	8.83			8.53	
Thalamus/Cortex – Overall width	Mean ± SD	14.82 ± 0.66			14.08 ± 0.60* (↓ 5%)	14.2±0.5 to 14.9± 0.4
	Adjusted Mean	14.79			14.12	
Hippocampus – Width Dentate Gyrus	Mean ± SD	0.64 ± 0.05			0.58 ± 0.05** (↓ 9%)	0.54 ± 0.05 to 0.64± 0.07
	Adjusted Mean	0.62			0.60	
Level 5						
Dorsal Cortex - Thickness	Mean ± SD	1.40±0.07			1.32±0.12 (↓6%)	1.19 ± 0.1 to 1.41 ± 0.1
	Adjusted Mean	1.41			1.31	
Thalamus - width	Mean ± SD	8.11 ± 0.51	8.04 ± 0.26	7.93 ± 0.18	7.49 ± 0.39** (↓ 8%)	7.41 ± 0.39 to 7.98 ± 0.25
	Adjusted Mean	8.10	8.03	7.93	7.51**	
Hippocampus width overall	Mean ± SD	1.55 ± 0.12	1.54 ± 0.10	1.61 ± 0.07	1.45 ± 0.15* (↓ 6%)	1.31 ± 0.12 to 1.54 ± 0.008
	Adjusted Mean	1.56	1.54	1.61	1.45*	

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)
Adjusted mean: after ANCOVA, with bodyweight as covariate.
HCD: Historical control data from 11 studies. Concurrent control from this study not included as part of the range.
Note: data generated as part of the 2007 supplementary study report is not included in this table

Incorrect values in Table 6.7.1.3-12 in the Annex to the CLH report (Volume 3 – B.6, 2018) for HCD ranges have been corrected for: Level 4: thalamus – Width (males). Thalamus - Height (females). Thalamus/Cortex – Overall width (males). Level 5: Hippocampus width overall (males). Thalamus – width (females).

TABLE 2 Selected Brain Morphometry Findings Investigated at Day 63 (continued)

Parameters		Control	50 ppm@	400 ppm@	4000 ppm	HCD range
Females Day 63						
Level 3						
Dorsal Cortex 1 - Thickness	Mean ± SD	1.51 ± 0.10	1.50 ± 0.10	1.48 ± 0.09	1.46 ± 0.09	1.22 ± 0.10 to 1.46 ± 0.11
	Adjusted Mean	1.54	1.51	1.48	1.41*	
Dorsal Cortex 2 - Thickness	Mean ± SD	1.78 ± 0.13			1.71 ± 0.11	1.47 ± 0.06 to 1.73 ± 0.12
	Adjusted Mean	1.79			1.70	
Piriform Cortex - Thickness	Mean ± SD	1.41 ± 0.14			1.40± 0.14	1.09 ± 0.12 to 1.37 ± 0.15
	Adjusted Mean	1.40			1.42	
Level 4						
Dorsal Cortex - Thickness	Mean ± SD	1.41 ± 0.12			1.29 ± 0.11 (↓ 9%)	1.16 ± 0.09 to 1.43 ± 0.09
	Adjusted Mean	1.40			1.30	
Corpus Callosum - Thickness	Mean ± SD	0.39 ± 0.08			0.42 ± 0.09	0.30 ± 0.05 to 0.45 ± 0.10
	Adjusted Mean	0.39			0.42	
Thalamus - Height	Mean ± SD	5.27 ± 0.38			5.17 ± 0.53	4.88 ± 0.43 to 5.5 ± 0.27
	Adjusted Mean	5.18			5.26	
Thalamus - Width	Mean ± SD	8.46 ± 0.27	8.51 ± 0.26	8.73 ± 0.20* (↑ 3%)	8.01 ± 0.32** (↓ 5%)	8.19 ± 0.48 to 8.71 ± 0.40
	Adjusted Mean	8.44	8.50	8.73*	8.04**	
Thalamus/Cortex – Overall width	Mean ± SD	14.49 ± 0.50	14.41 ± 0.59	14.72 ± 0.72	13.5 ± 0.53** (↓ 7%)	13.6 ± 0.8 to 14.6 ± 0.2
	Adjusted Mean	14.44	14.40	14.73	13.55**	
Hippocampus – Width Dentate Gyrus	Mean ± SD	0.61 ± 0.06			0.58 ± 0.07	0.49 ± 0.04 to 0.62 ± 0.02
	Adjusted Mean	0.60			0.60	
Level 5						
Dorsal Cortex - Thickness	Mean ± SD	1.41 ± 0.06	1.39 ± 0.07	1.35 ± 0.07	1.33 ± 0.08** (↓ 6%)	1.19 ± 0.09 to 1.41 ±0.09
	Adjusted Mean	1.40	1.39	1.35	1.33	
Thalamus - width	Mean ± SD	7.88 ± 0.34	7.65±0.32	7.74 ± 0.41	7.28 ± 0.31** (↓8%)	7.18 ± 0.35 to 7.72 ± 0.36
	Adjusted Mean	7.86	7.64	7.74	7.31**	
Hippocampus width overall	Mean ± SD	1.55 ± 0.08			1.46 ± 0.08** (↓ 6%)	1.34 ± 0.06 to 1.58 ± 0.09
	Adjusted Mean	1.53			1.49	

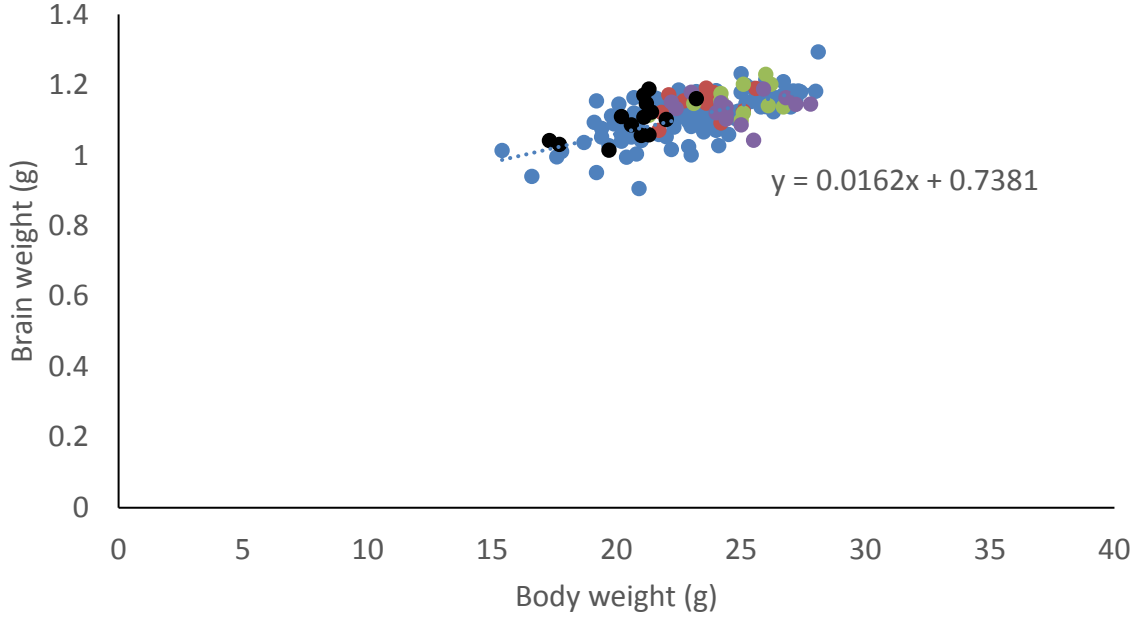
* Statistically significant difference from control group mean, $p<0.05$ (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, $p<0.01$ (Student's t-test, 2-sided)
Adjusted mean: after ANCOVA, with bodyweight as covariate.
HCD: Historical control data from 11 studies. Concurrent control from this study not included as part of the range.
Note: data generated as part of the 2007 supplementary study report is not included in this table

Incorrect values in Table 6.7.1.3-12 in CLH dossier (Annex to CLH report Thiamethoxam dRAR Volume 3-B6 (AS) for HCD ranges have been corrected for: Level 4: thalamus – Width (males). Thalamus - Height (females). Thalamus/Cortex – Overall width (males). Level 5: Hippocampus width overall (males). Thalamus – width (females).

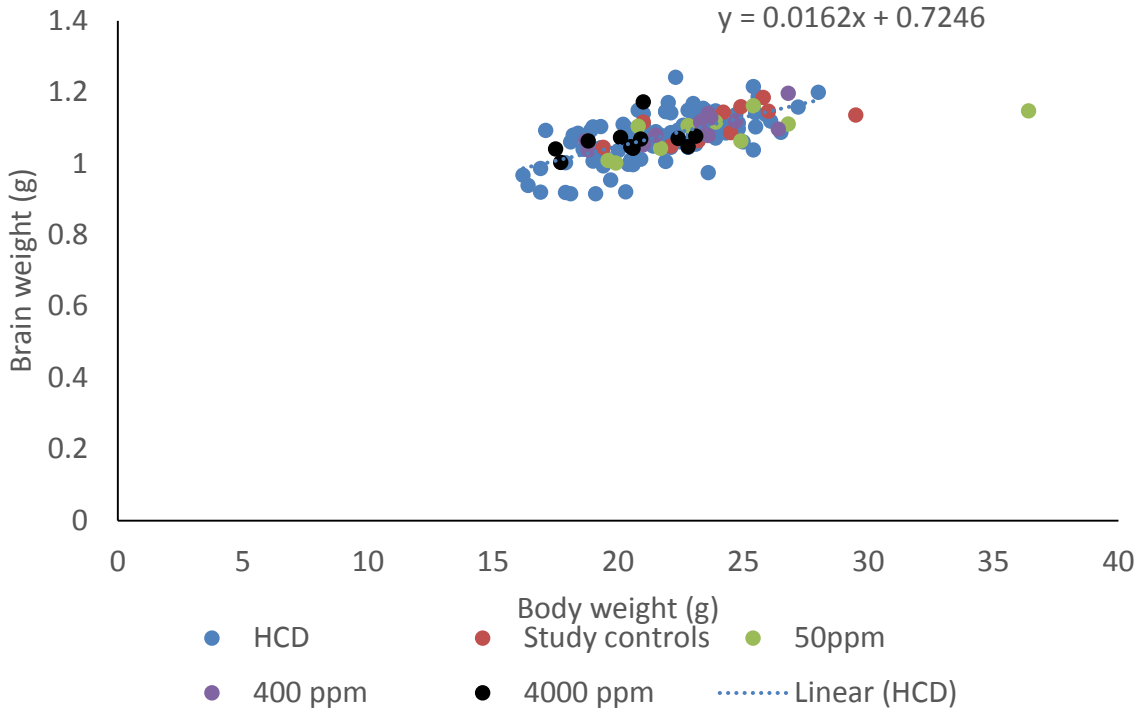
APPENDICES SECTION

APPENDIX 1 Brain Weight versus Body Weight in Control and RR0936 groups for Day 12 Post-partum Rats with Non-truncated Axes

A) Males

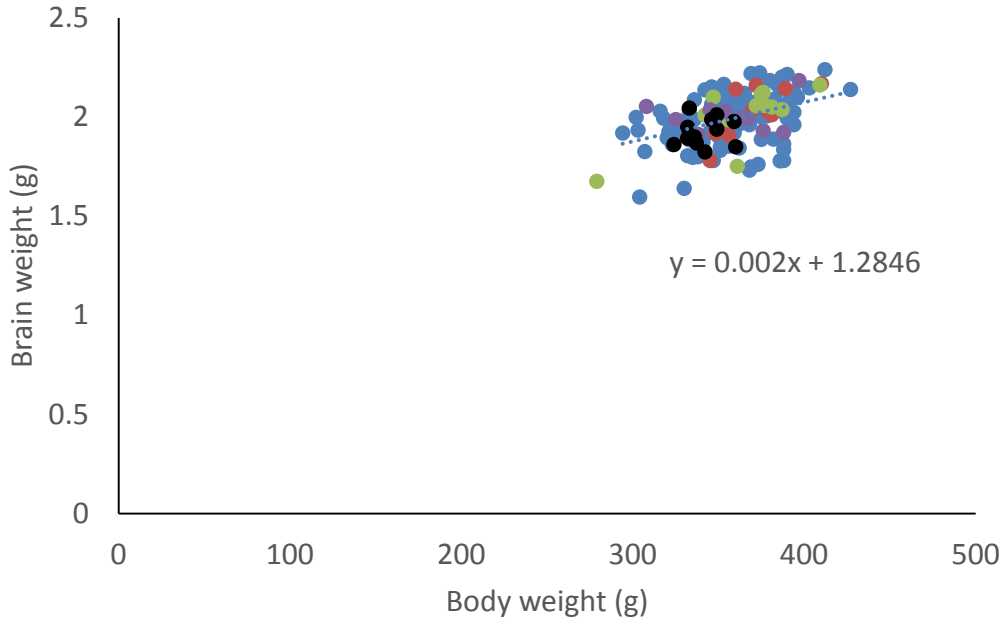


B) Females

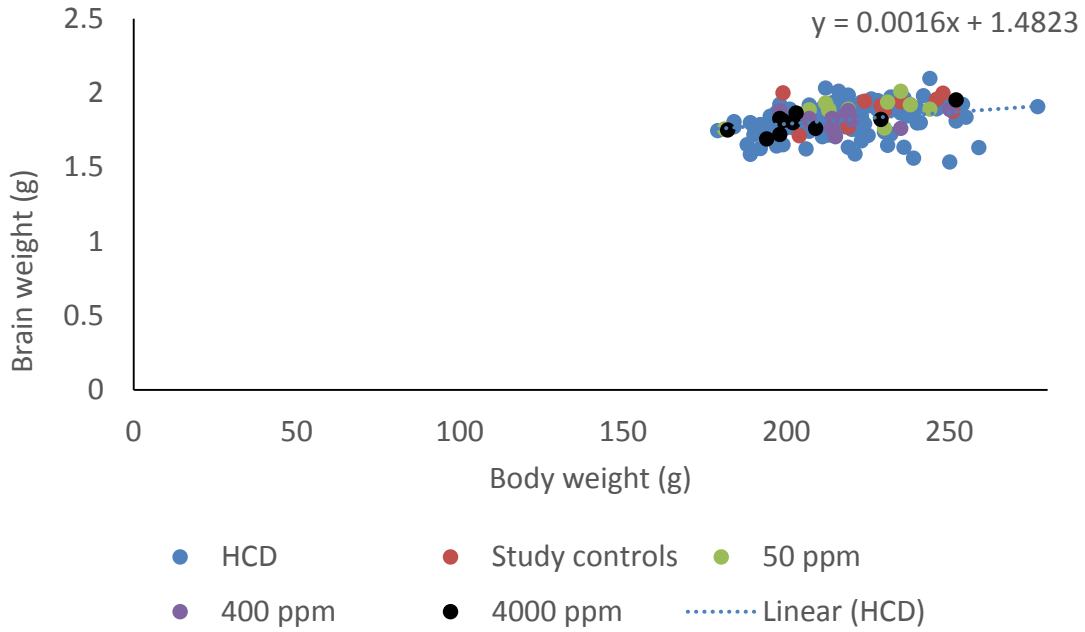


APPENDIX 2 Brain Weight versus Body Weight in Control and RR0936 groups for Day 63 Post-partum Rats (Post perfused) – Non-truncated Axes

A) Males



B) Females



APPENDIX 3 Historical Control Data

Table 3.1 Studies used for Historical Control Data

Study	Start Date	Route	Strain	Diet	Vehicle
RR0930	2001/10	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0928	2002/07	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0938	2002/07	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0882	2002/08	ORAL	Alpk:APfsd Wistar BABU Rat	CT1	corn oil
RR0884	2002/10	ORAL	Alpk:APfsd Wistar BABU Rat	CT1	water
RR0922	2002/11	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0886	2002/12	ORAL	Alpk:APfsd Wistar BABU Rat	CT1	water
RR0926	2003/03	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0969	2003/04	FEEDING	Alpk:APfsd Wistar BABU Rat	CT1	n/a
RR0988	2003/04	ORAL	Alpk:APfsd Wistar BABU Rat	CT1	water
RR1048	2004/10	ORAL	Alpk:APfsd Wistar BABU Rat	CT1	sesame oil

Table 3.2 Absolute Brain Weight at Day 12 – Historic Control Data from 11 Studies

Study identification	Start date	Sex	n	Brain weight – minimum (g)	Brain weight – maximum (g)	Brain weight – Mean (g)
RR0930	2001/10	M	12	1.017	1.215	1.129
		F	12	1.045	1.169	1.089
RR0928	2002/07	M	14	0.996	1.199	1.133
		F	13	0.994	1.242	1.105
RR0938	2002/07	M	11	1.025	1.209	1.130
		F	10	1.006	1.200	1.103
RR0882	2002/08	M	10	1.059	1.161	1.118
		F	9	0.954	1.159	1.078
RR0884	2002/10	M	10	1.037	1.145	1.098
		F	11	0.916	1.143	1.027
RR0922	2002/11	M	10	0.940	1.184	1.096
		F	10	0.968	1.147	1.065
RR0886	2002/12	M	11	1.011	1.179	1.099
		F	11	1.003	1.146	1.071
RR0926	2003/03	M	10	1.014	1.164	1.096
		F	10	0.987	1.171	1.101
RR0969	2003/04	M	10	1.042	1.294	1.158
		F	10	1.073	1.216	1.123
RR0988	2003/04	M	8	0.906	1.181	1.026
		F	8	0.916	1.148	1.014
RR1048	2004/10	M	10	1.034	1.130	1.081
		F	10	0.994	1.229	1.095

Table 3.3 Absolute Brain Weight at Day 63 (post perfusion) – Historic Control Data from 11 Studies

Study identification	Start date	Sex	n	Brain weight – minimum (g)	Brain weight – maximum (g)	Brain weight – Mean (g)
RR0930	2001/10	M	10	2.015	2.202	2.105
		F	10	1.846	1.973	1.909
RR0928	2002/07	M	10	1.835	2.138	2.017
		F	10	1.715	2.011	1.863
RR0938	2002/07	M	10	1.888	2.239	2.066
		F	10	1.755	1.966	1.887
RR0882	2002/08	M	10	1.780	2.041	1.927
		F	10	1.561	1.924	1.752
RR0884	2002/10	M	10	1.833	2.103	2.006
		F	10	1.587	1.865	1.758
RR0922	2002/11	M	10	1.732	2.024	1.888
		F	10	1.643	1.871	1.767
RR0886	2002/12	M	11	1.779	2.101	1.951
		F	12	1.626	1.921	1.783
RR0926	2003/03	M	10	1.825	2.119	1.961
		F	10	1.623	1.948	1.791
RR0969	2003/04	M	10	1.849	2.087	2.017
		F	10	1.723	1.929	1.841
RR0988	2003/04	M	11	1.596	2.223	1.927
		F	11	1.535	2.033	1.761
RR1048	2004/10	M	10	1.761	2.220	2.056
		F	10	1.82	2.098	1.924

APPENDIX 4 Additional Responses to Comments from Submitter Included in the Annex to the CLH Report

Use of ANCOVA – for Brain Weight, Brain Morphometry, and F1 Offspring Bodyweights

Submitter Comments:

In the main study, morphometric data were analysed by ANOVA and ANCOVA using final body weight as a covariate. In the applicant summary, only adjusted data were reported. Since the adjustment for body weight, as a covariate is not recommended in OPPTS 870.6300 (1998) or OECD 426 (2007) and for transparency sake the unadjusted morphometric data are reported.

Furthermore, upon request from US-EPA, supplemental brain morphometry measurements were made on the offspring of intermediate dose groups killed on day 12 or 63 in the main developmental neurotoxicity study ([redacted], 2003). Brains had been processed to block or slides during the initial conduct of the study. Specified levels in the brain and, where necessary, a section of the cerebellum were examined from each animal and selected morphological measurements made. It should be noted that variations between groups processed early (high-dose and control in main study 2003) versus those processed much later (low-dose and mid-dose in the supplemental study 2007) could reflect a processing artefact (Garman, R.H., 2015).

The applicant considered that changes in brain morphometry measurements in high dose male and female F1 offspring were not evidence of developmental neurotoxicity but rather secondary to decreased body weight (general toxicity). However it is questionable that such body weight reduction (less than 10%) would be responsible of the quite large brain morphometric changes observed at termination in high dose animals.

The morphological changes were not associated with neuro-histopathological finding or change in functional or neurobehavioral parameters. However, it should be noted that the Y-maze for learning and memory assessment is a low sensitivity assay of behavioural change unless associated with appropriate difficulty tasks, which was not the case in the present study.

Syngenta response:

More Recent Guidance on DNT Studies, Based on Wider Experience

While use of ANCOVA with bodyweight as the covariate is not recognized within the guidelines for DNT studies cited by the submitter, it should be recognized that these guidelines were written prior to the availability of large numbers of GLP-compliant DNT studies, and therefore could not have anticipated all circumstances that might arise and might require additional scientifically-appropriate statistical treatments. Now that a larger number of studies have been conducted, recent literature is an important additional source of information and guidance.

Holson et al. (2008) published a report of an international panel of researchers (ILSI) from academia, government and industry that discussed statistical issues and techniques appropriate for DNT studies, based on experience with multiple studies across six different laboratories. This paper highlighted the fact that because of the very large number of parameters that are tested in DNT studies, in particular in the F1 offspring, a recognized concern in DNT studies based on experience is a high likelihood of Type I errors. Type I errors (i.e. false positive conclusions) can and will occur based on the likelihood that with a large number of p-values being generated (1306 in their example), the occurrence of the expected 5% of the p-values achieving statistical significance due to chance (based on a p-value of 0.05) can incorrectly be construed to represent a real effect.

Holson et al. (2008) also discuss the possible use of ANCOVA as part of the statistical analysis of certain parameters in a DNT study. Their even-handed discussion reflects differing previous published views of statisticians about use of ANCOVA, and advises that it can be the method of choice for some parameters such as relative organ weights, but they advise expert statistical advice when using ANCOVA. For this DNT study with thiamethoxam, a career statistician who was employed by the Syngenta Central Toxicology Laboratory ([redacted]) helped design the appropriate use of ANCOVA in the DNT study. In his professional opinion as a statistician, use of ANCOVA on brain morphometry measurements with individual animal bodyweight as the covariate was logical and appropriate. In this way, the comparisons to the control group for brain morphometry parameters can be assessed after adjusting for the known differences in bodyweight (and its known impact on pup brain weight). Further details on this use of ANCOVA for analysis of brain weights, brain morphometry, and bodyweights are provided in the sections below, along with the results for these parameters.

Use of Analysis of Covariance in the Thiamethoxam DNT Study

Differences in terminal bodyweight can affect the weight and size of the brain. This is particularly the case at Day 12 when the brain is growing rapidly. In order to investigate whether observed differences in brain size and weight are influenced by terminal bodyweight, Syngenta uses analysis of covariance.

The analysis of covariance (ANCOVA) is a basic statistical technique covered in most statistics textbooks (e.g. Snedecor and Cochran, 1989). It is readily available for use in all major statistical computing packages e.g. SAS (2004). ANCOVA with terminal body weight as the covariate is the preferred method of analyzing relative organ weights (Shirley, 1996).

The output of the analysis of covariance for a particular parameter, with terminal body weight as the covariate, is a set of adjusted group means which estimate what the parameter group means would have been had all the groups displayed identical group mean terminal bodyweights. The magnitude of the adjustments depends on the strength of the relationship between the parameter and terminal weight and the magnitude of the inter-group differences in terminal weight. Importantly, analysis of covariance is more than a simple adjustment based on the % difference in the covariate (e.g. a 6.8% bodyweight difference).

In the final report for this DNT study with thiamethoxam ([redacted], 2003), this analysis of covariance based on terminal body weight was used for:

- statistical evaluation of brain weights at Day 12 and Day 63
- statistical evaluation of brain morphometry measurements at Day 12 and Day 63.

In addition, analysis of covariance was used to adjust body weights in the F1 males and females, based on the group mean body weights on Day 5, when all groups were culled to form the F1 generation. Use of ANCOVA for body weights in the selected F1 animals provided a statistical comparison of adjusted group means, after adjusting for the intergroup differences seen on Day 5 upon culling.

For all parameters examined initially by analysis of variance (e.g. absolute brain weights), or by analysis of covariance (e.g. adjusted brain weights), differences from control were further compared by a two-sided Student's t-test, based on the error mean square in the analysis. Statistical methods were described in detail in Appendix G of the study report for RR0936.

Comparisons of Group Mean Bodyweights in F1 Offspring

In the 4000 ppm group, pup bodyweights were lower than control at birth (Day 1) and at Day 5.

In DNT studies, this is relatively uncommon for pup bodyweights to be affected at birth; high doses more frequently begin producing reduced bodyweights later in lactation or when pups begin feeding on test diet. For the selected male and female animals in the F1 generation, statistically significantly lower bodyweights, adjusted for initial day 5 bodyweight, persisted throughout the remainder of the study, and the adjusted bodyweights were a maximum of 13% lower than control (males, day 18) ([redacted], 2003; Section 6.5.2).

Comparisons of Absolute and Adjusted Brain Weights

Analysis of brain weights indicated that the absolute brain weights (unadjusted for terminal body weight) were significantly lower in the 4000 ppm group on Days 12 and 63 (Section 3.1). However, after adjustment for bodyweight by ANCOVA, there were no differences from control. Thus, smaller birth weights and persistently smaller bodyweights resulted in lower brain weights at 4000 ppm.