EPA Analysis

of the

Avian Dosing Study

Performed by

Battelle
505 King Avenue
Columbus, Ohio

Under EPA Contract Number 68-W-01-023
Work Assignments 2-17 and 5-7

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In addition to the statistical analyses performed by the contractor, EPA staff performed analyses using a statistical package (CHICKS 3_1) developed by the EPA's Environmental Fate and Effects Division (EFED) for analyzing the Avian Reproduction Test. The program was written in SAS 8.1, updating the basic structure of a previous version to include graphical output. In addition to the basic endpoints measured by the CHICKS 3_1 SAS macro, OSCP staff added endpoints pertinent to the two-generation test design.

The following variables were analyzed for all generational groups. All analyses are conducted on a 'per pen' basis.

EL - Eggs Laid
NEG_EC - Eggs Cracked
ENC_EL - (EL-EC)/EL (%)
ES - Eggs Set
ES_EL – Eggs Set/Eggs Laid (%)
VE - Viable Embryo(d8)
VE_ES – Viable Embryo/Eggs Set (%)
LE Live - Embryo(d15)
LE_VE – Live Embryo/Viable Embryo (%)
NH - Number Hatched
NH_EL – Number Hatched/Eggs Laid (%)
NH_ES – Number Hatched/Eggs Set (%)
NH_LE – Number Hatched/Live Embryo (%)
HS - Hatchling Survival(d14)
HS_ES – Hatchling Survival/Eggs Set (%)
HS_NH Hatchling Survival/Number Hatched (%)
THICK Eggshell thickness
HATWT Hatchling Weight
SURVWT Survivor Wt (d14)
FOOD Food Consumption (adults)
WTGAINM Male wt gain (adults)
WTGAINF Female wt gain (adults)

Endpoints not pertinent for the present analysis include ES, ES_EL, VE, LE, NH, NH_EL, and HS due to differences in protocols between the Avian Reproduction Study and the Avian Dosing Study, and are not reported here.

Endpoints that were added for the P1 generations include:

EGGLAY – Days to onset of egg laying
FTIBL – Tibial length of females
FTARL – Tarsal length of females
FTIBD – Tibial diameter of females
FTIBW – Tibial weight of females
MTIBL – Tibial length of males
MTARL – Tarsal length of males
MTIBD – Tibial diameter of males
MTIBW – Tibial weight of males

CLOACA – Cloacal area at necropsy
FOAM – Age at first foam
PLUMF – Female-type plumage length of females
PLUMM – Female-type plumage length of males
ADRBWF – Adrenal to body weight ratio in females
ADRBWM – Adrenal to body weight ratio in males
FFUEST – Fecal-urate estrogen content in females
FFUTEST – Fecal-urate testosterone content in females
MFUEST – Fecal-urate estrogen content in males
MFUTEST – Fecal-urate testosterone content in males

In the F1 generations, fecal-urate steroid hormone analyses were not performed. An additional endpoint analyzed for the F1 generations is:

SEXRC – Sex ratio of F2 chicks

Tests of assumptions for parametric analyses

Parametric data analyses require the assumptions of normality of the residuals and homogeneity of variance for all treatment groups. To test these assumptions, Shapiro-Wilk's test and Levene's test are performed. For both tests the residuals (from an Analysis of Variance) are calculated. Shapiro-Wilk's test for normality (Shapiro and Wilk, 1965) compares the distribution of the residuals to a normal distribution and a test statistic and p-value are calculated. For this test, the null hypothesis is that the data follow a normal distribution.

Levene's test for equality of variance (Levene, 1960) is performed by conducting an analysis of variance on the absolute values of the residuals. For this test, the null hypothesis is that the variance of each of the groups is equal, and the alternative hypothesis is that at the variances are not homogeneous.

Parametric analyses

The first of the parametric analyses is the Analysis of Variance (ANOVA, overall F-test). This tests the null hypothesis that all of the treatment means are equal verses the alternative that there is at least one difference in the set of treatment means. This test is a non-directional test – no assumptions are made regarding the direction of any differences.

Dunnett's mean-comparison procedure (Dunnett, 1955) is designed to compare a set of treated groups to a control group. The tests conducted by this SAS program are one-sided tests, specifically testing for a treatment mean significantly less than the control mean. The LOEC (Lowest Observed Effects Concentration) is set equal to the lowest dose level for which there was a significant effect (using an "-level=0.05). The NOEC (No Observed Effects Concentration) is set one dose lower than the LOEC.

Tukey's mean-comparison procedure (Sokal and Rohlf, 1995) is also performed. This test conducts all possible pairwise comparisons among the means (including the control). All comparisons are two-sided, and no dose-response relationship is implied.

Non-parametric analyses

The Kruskal-Wallis test (Daniel, 1990) is performed. This is a non-parametric analog to the ANOVA. It tests the null hypothesis that all of the treatment medians are equal verses the alternative that there is at least one difference in the set of treatment medians. This test is a non-directional test - no assumptions are made regarding the direction of any differences. For this test, the "-level is set at 0.05; all tests resulting in p-values <0.05 would reject the null hypothesis in favor of the alternative hypothesis.

Next, a series of mean-comparison procedures are performed. Mann-Whitney test with Bonferroni adjustment is conducted to compare each of the dosed treatment medians to the control median. This non-parametric test is analogous to Dunnett's test (used in the parametric setting). In this SAS program, the test is implemented as a one-sided test for determining if the treated group median is significantly less than the control group median. The LOEC is set equal to the lowest dose level for which there is a significant treatment effect (using an "-level=0.05). The NOEC is set one dose lower than the LOEC.

Jonckheere's test (Jonckheere 1954, Daniel 1990) is a non-parametric test used to compare responses for a series of dose levels to a control group. It assumes that the effect of the chemical shows a negative dose-response relationship. If the test is statistically significant, this implies that there is a negative trend across all the groups. The LOEC is set equal to the lowest dose level for which there is a significant dose effect (level = 0.05) using the step-down process. The NOEC is set one dose lower than the LOEC.

Special case: Eggs cracked

For all response variables, it is assumed that if there is an effect due to the chemical, it is a negative effect (i.e., mean or median response will be reduced). The one exception to this assumption is with the response variable, EggsCracked (EC). If there is an effect of the chemical, the expectation is that the response to shows a positive effect (an increase) for dosed treatments. Therefore with Dunnett's or Mann-Whitney's tests, the null hypothesis is that the dosed mean (or median) is not different from the control verses the alternative that the dosed mean (or median) is greater then the control. And with Williams' or Jonckheere's tests, the null hypothesis is that there is no dose-response relationship verses the alternative that there is a positive dose response relationship.

Summary

The majority of endpoints addressed were not significantly affected based on the analysis performed with CHICKS. These analyses did not corroborate a significant male to female sex ratio response as concluded in the "Draft Final Report on the Avian Dosing Study". Additionally, an effect on sexual maturation of males in the F1 generation offspring of P1A parents as measured by age to first foam and cloacal gland size was not confirmed. However, hatchling 14-day survival of P1A birds was determined to be significantly reduced with a NOEC of 1.25 ppm and a LOEC of 5 ppm. The length of feminized plumage was the most apparent endpoint affected as also concluded in the "Draft Final Report on the Avian Dosing Study", with a stronger response seen in the P1AF1A (pre-breeding, P1 treated and F1 treated group) than post-breeding P1 or untreated F1 groups.

Based on these analyses, the P1AF1A dosing regimen would be the preferred testing design to use in a two-generation test.

The following is a graphical representation of the results from the CHICKS analysis. For clarity, the P1A and P1B generations are graphed together, and the F1 groups (P1AF1A, P1AF1B, P1BF1A, and P1BF1B) are graphed together.

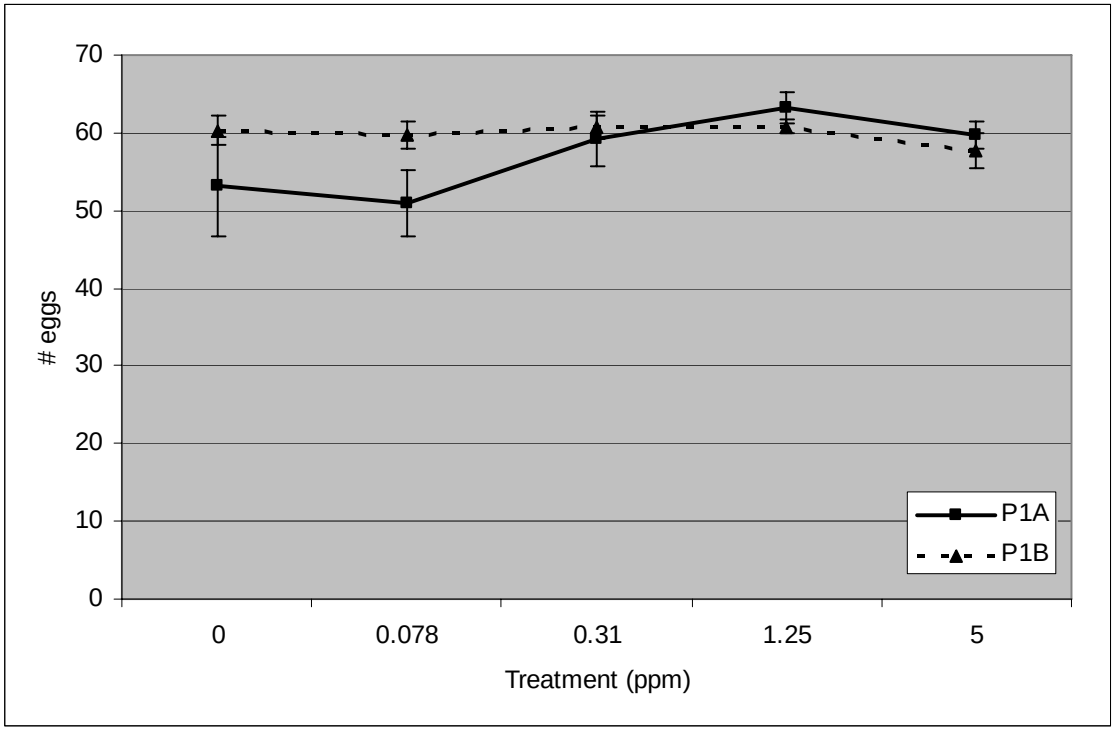

Figure 1. Total eggs laid by P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

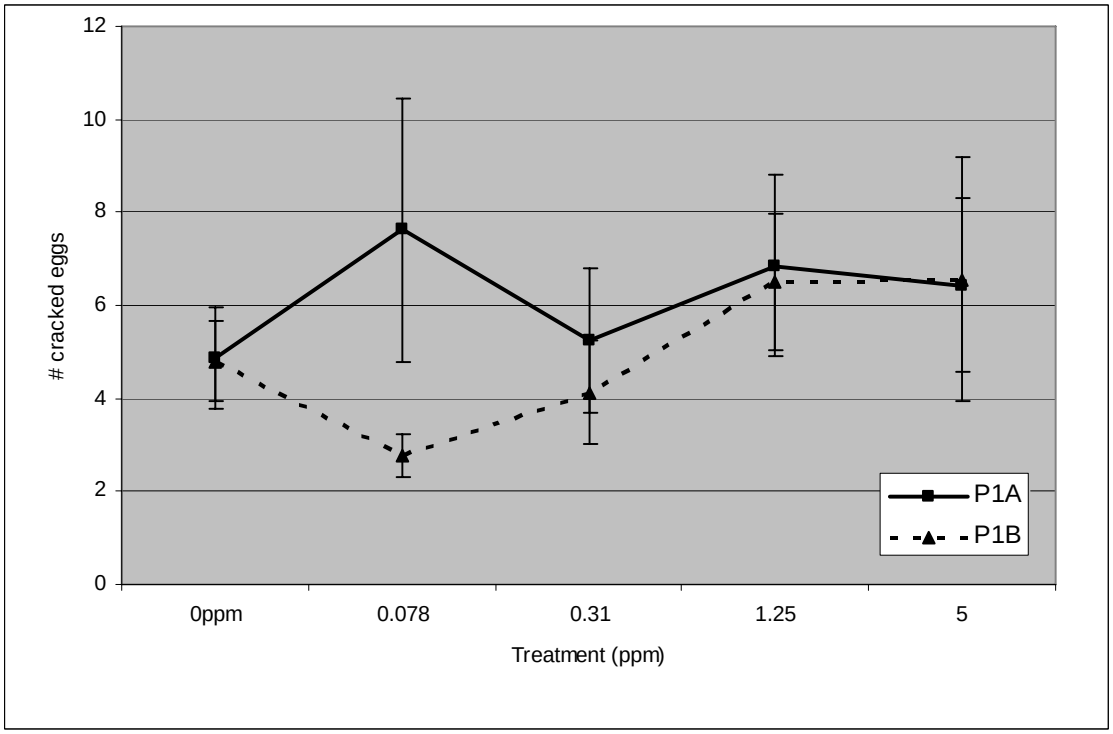

Figure 2. Total eggs cracked P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

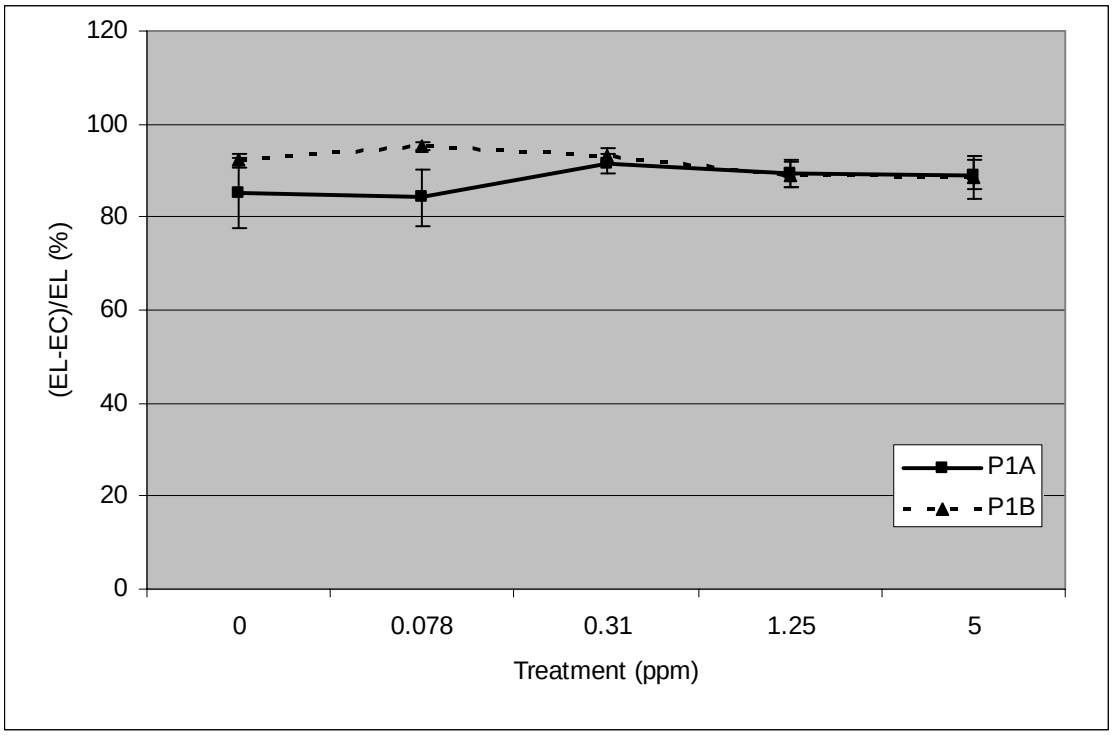

Figure 3. Eggs cracked out of eggs laid in P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

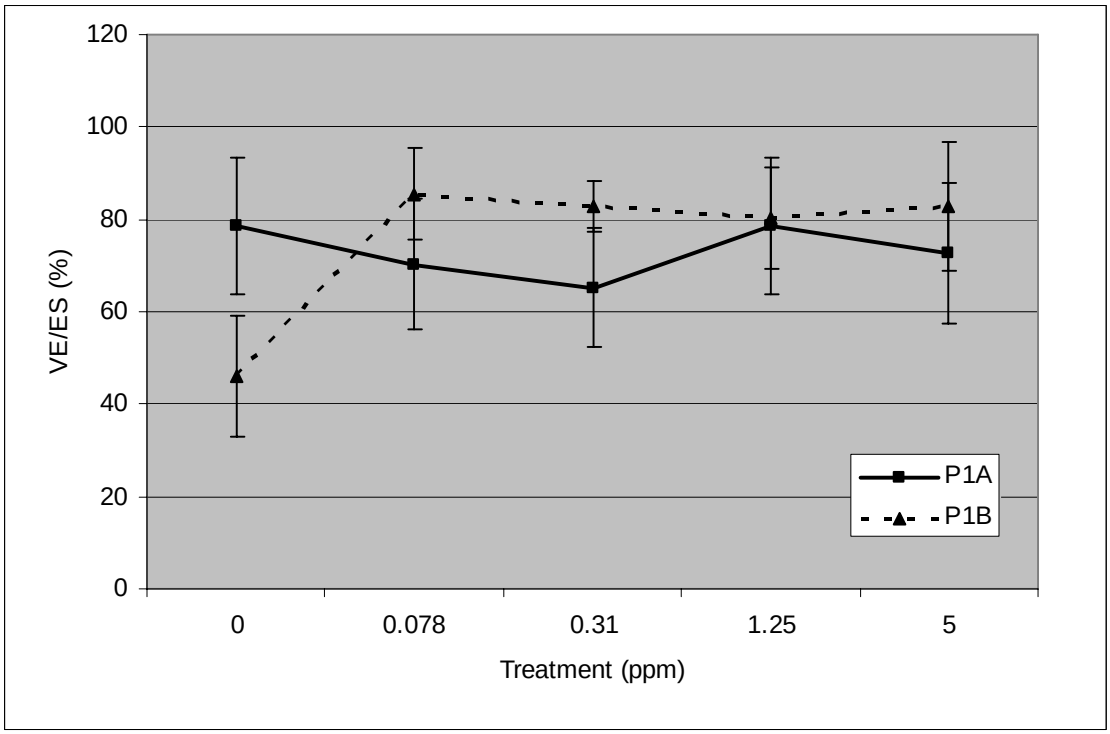

Figure 4. Viable embryos (day 8) out of eggs set for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

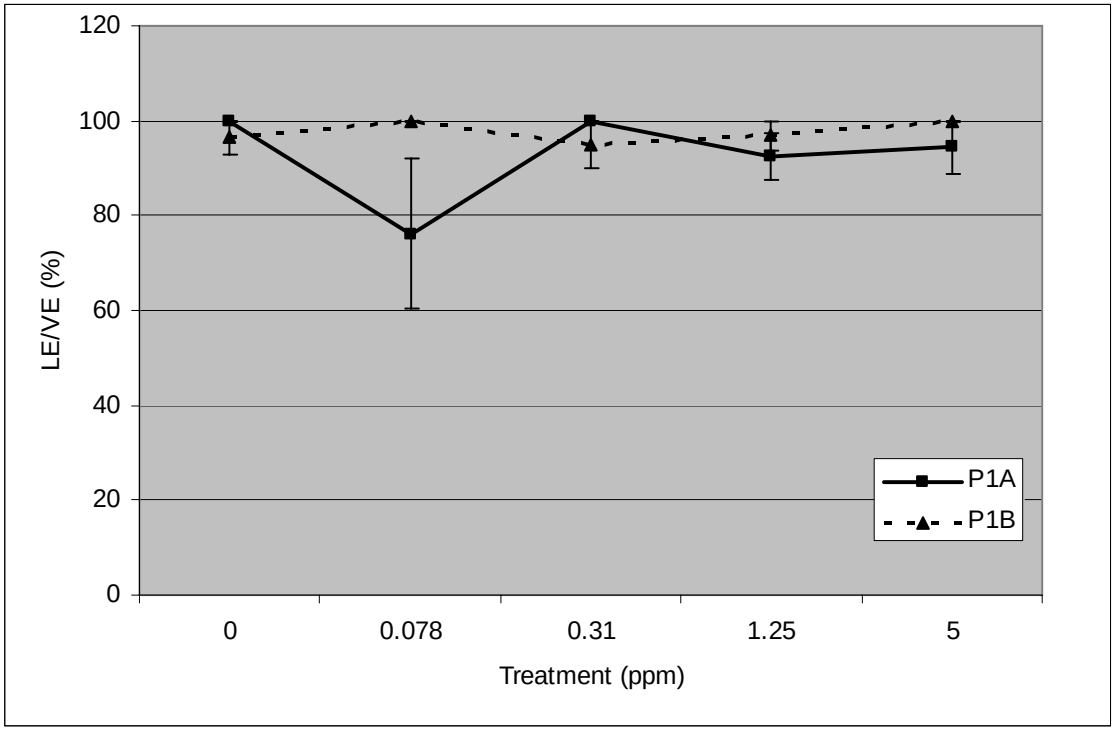

Figure 5. Live embryos (Day 15)/Viable embryos (Day 8) for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

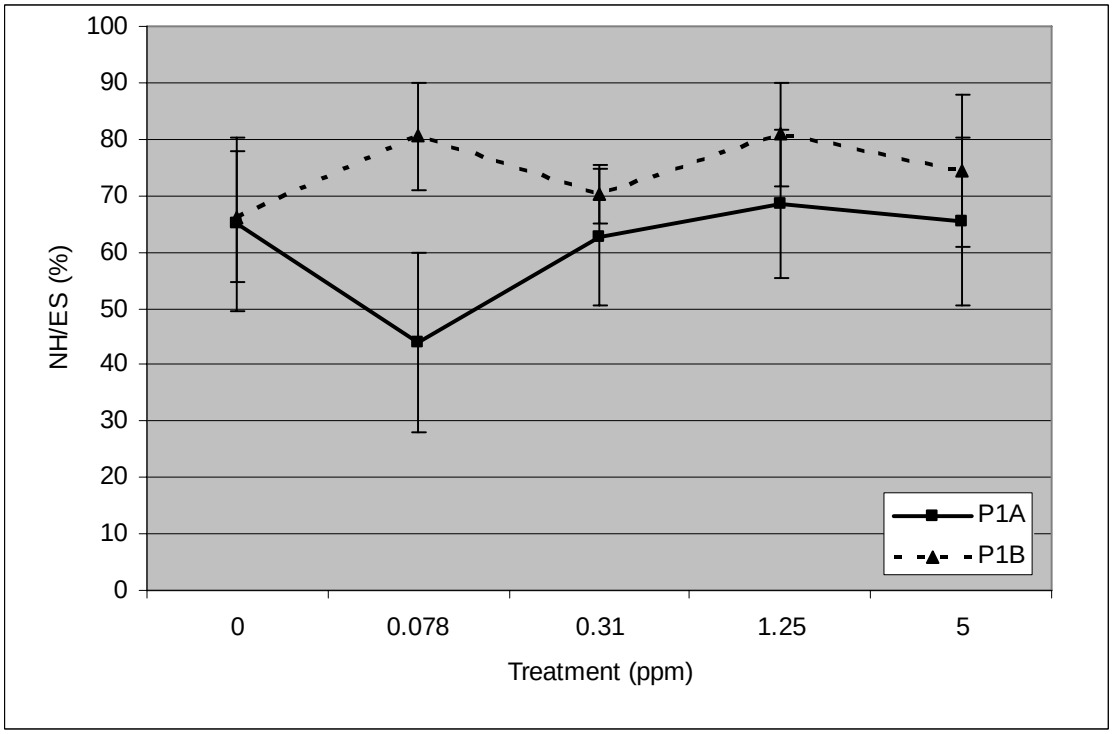

Figure 6. Eggs hatched/Eggs set for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

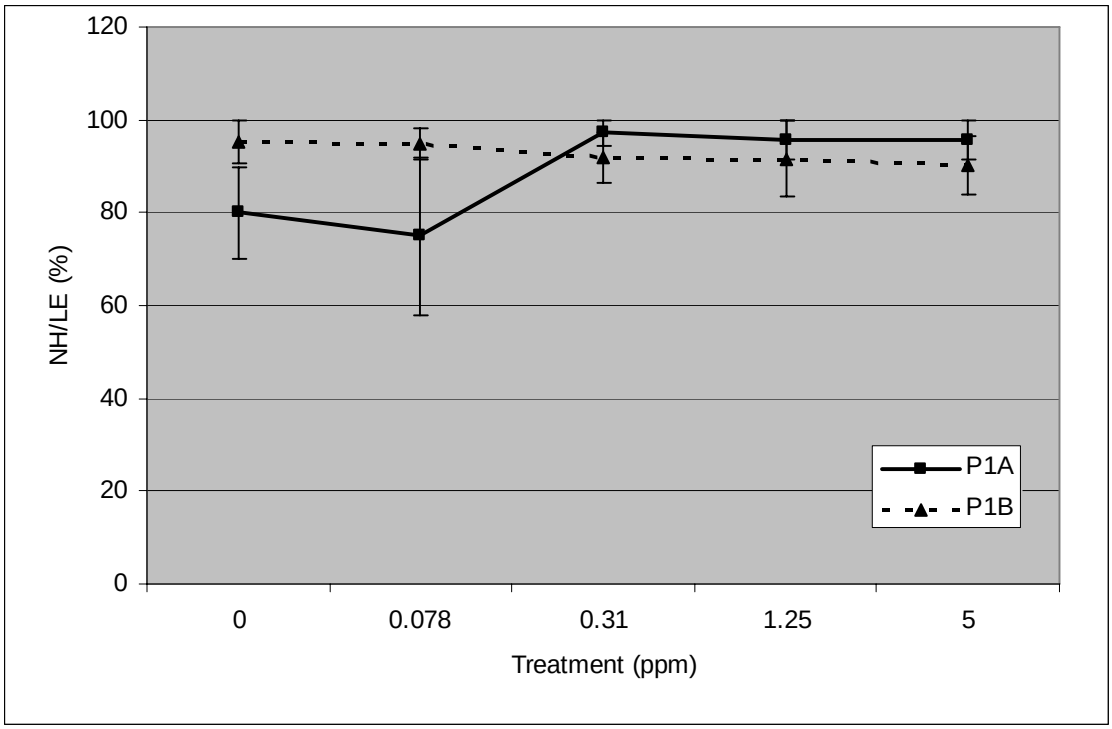

Figure 7. Number hatched/Live embryos (Day 15) of P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

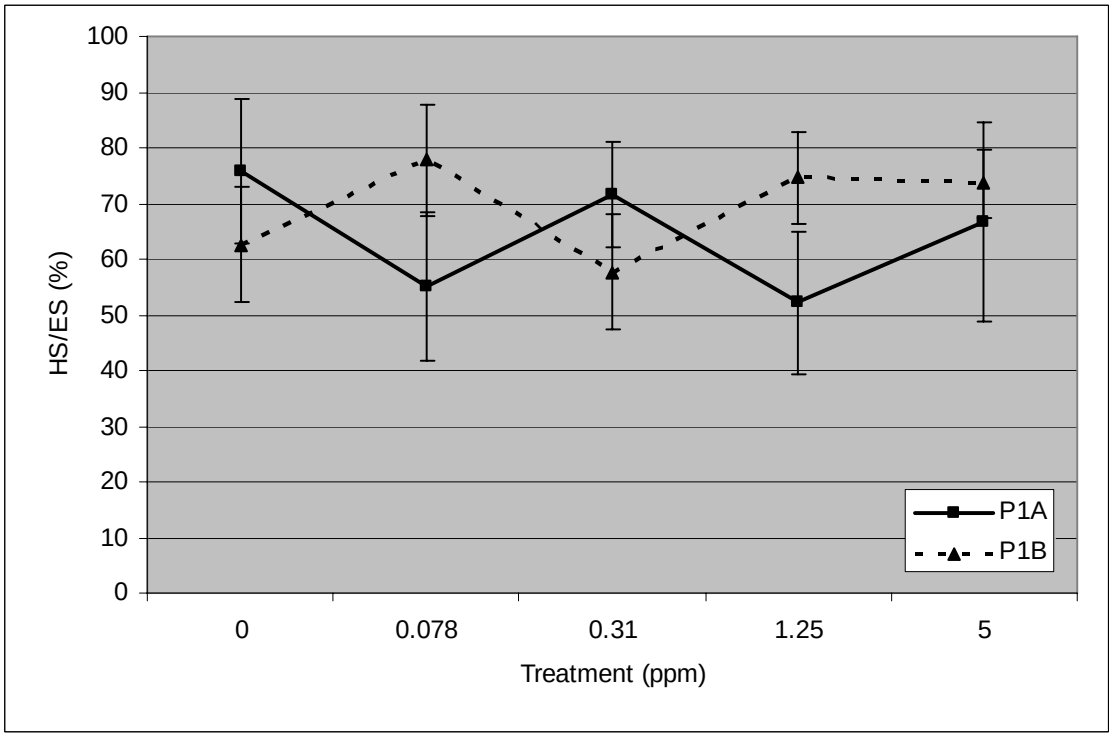

Figure 8. Hatchling survival/Eggs set for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both exposure groups.

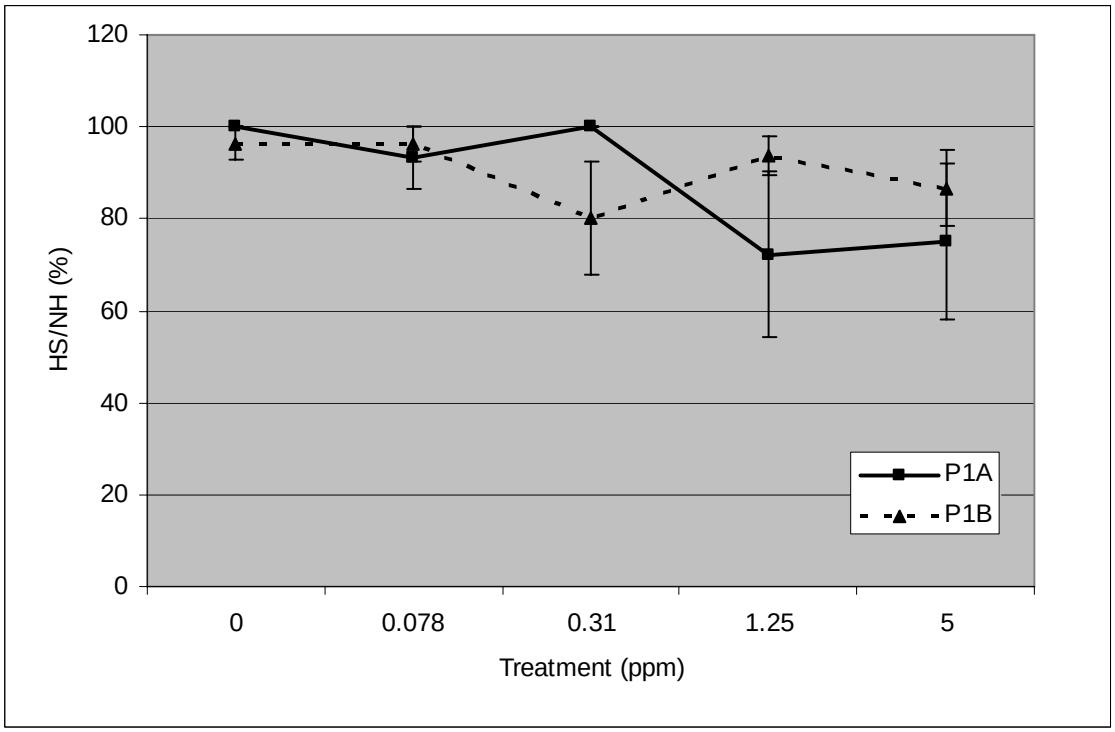

Figure 9. Hatchling survival/Number hatched for P1 generations. According to a Jonckheere's test, the NOEC = 1.25 and LOEC = 5 for the P1A group. For the P1B group, the LOEC is above 5 ppm.

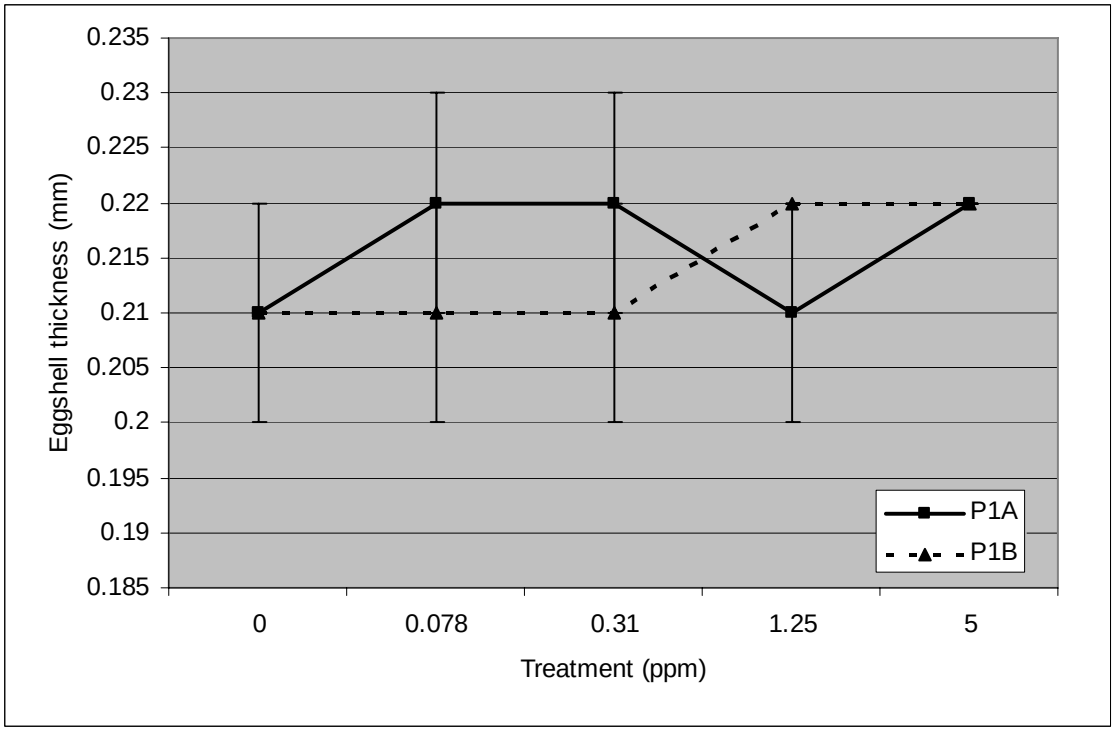

Figure 10. Eggshell thickness for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

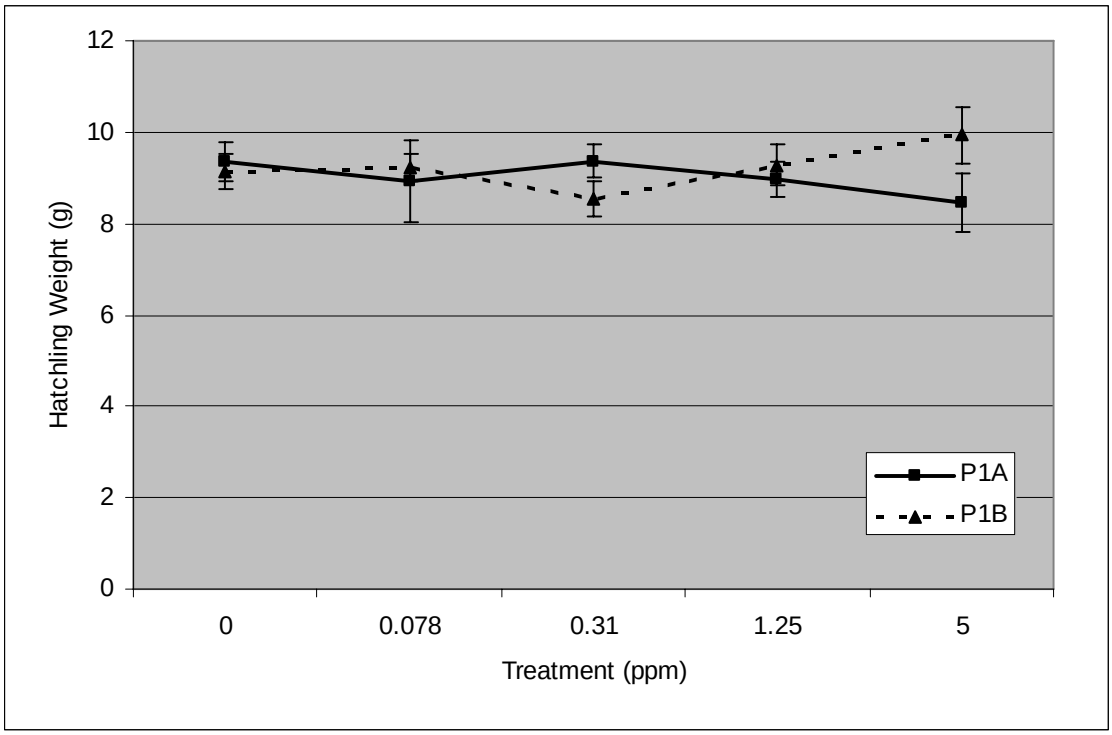

Figure 11. Hatchling weight per pen for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

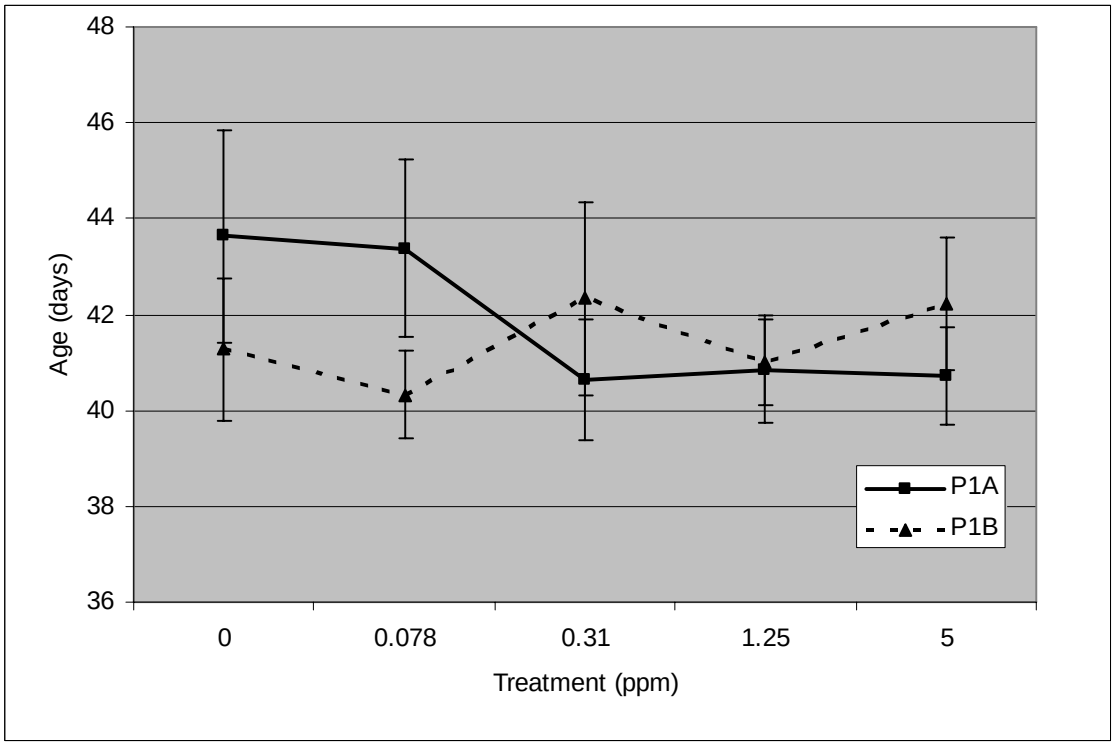

Figure 12. Days to onset of egg laying in P1 generations. Onset of egglaying occurred prior to dosing in the P1B generation. In the P1A generation, the LOEC is above 5 ppm.

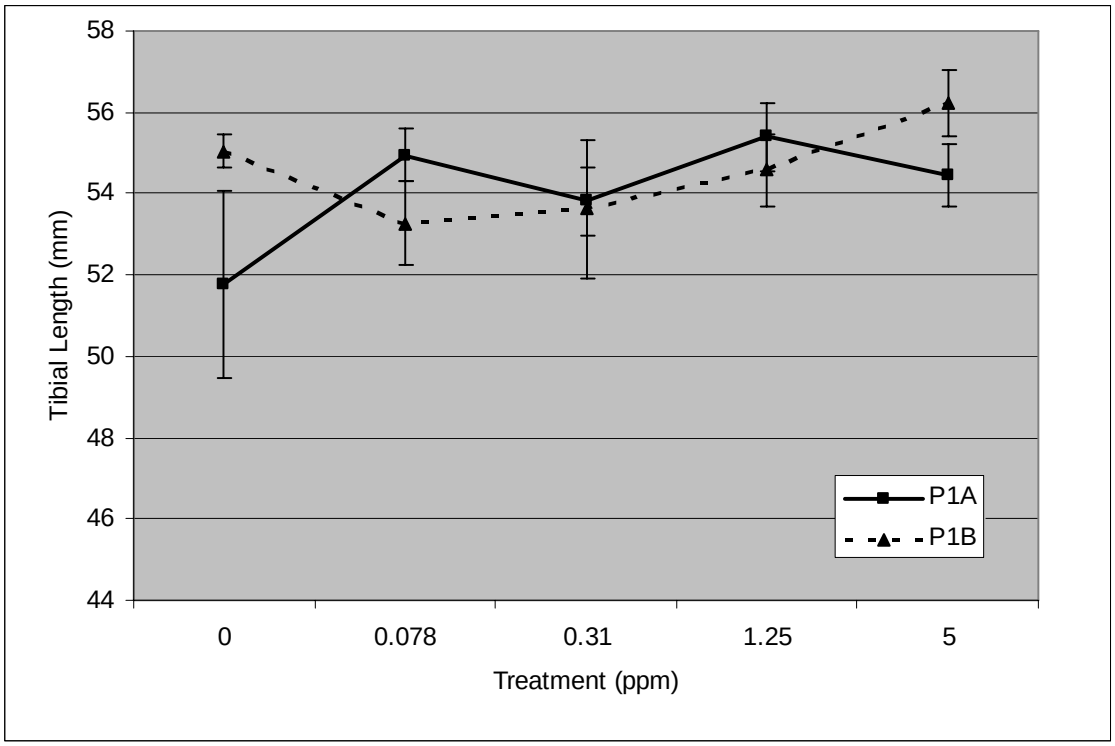

Figure 13. Female tibial length at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm in both groups.

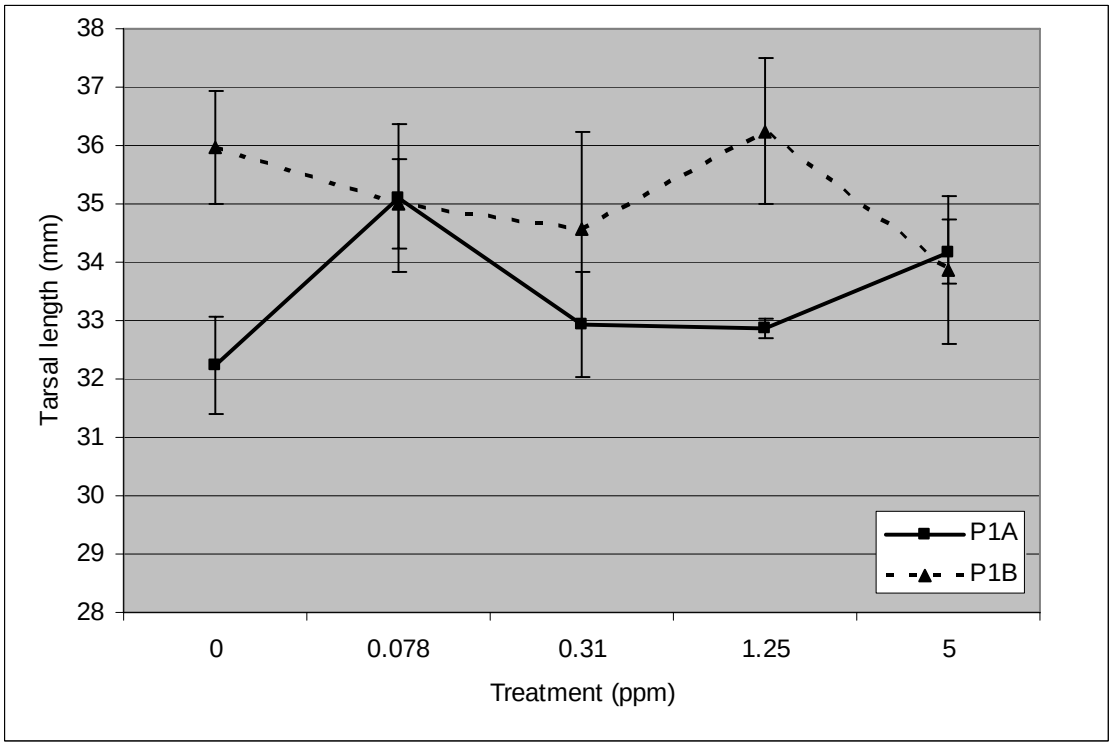

Figure 14. Female tarsal length at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm in both groups.

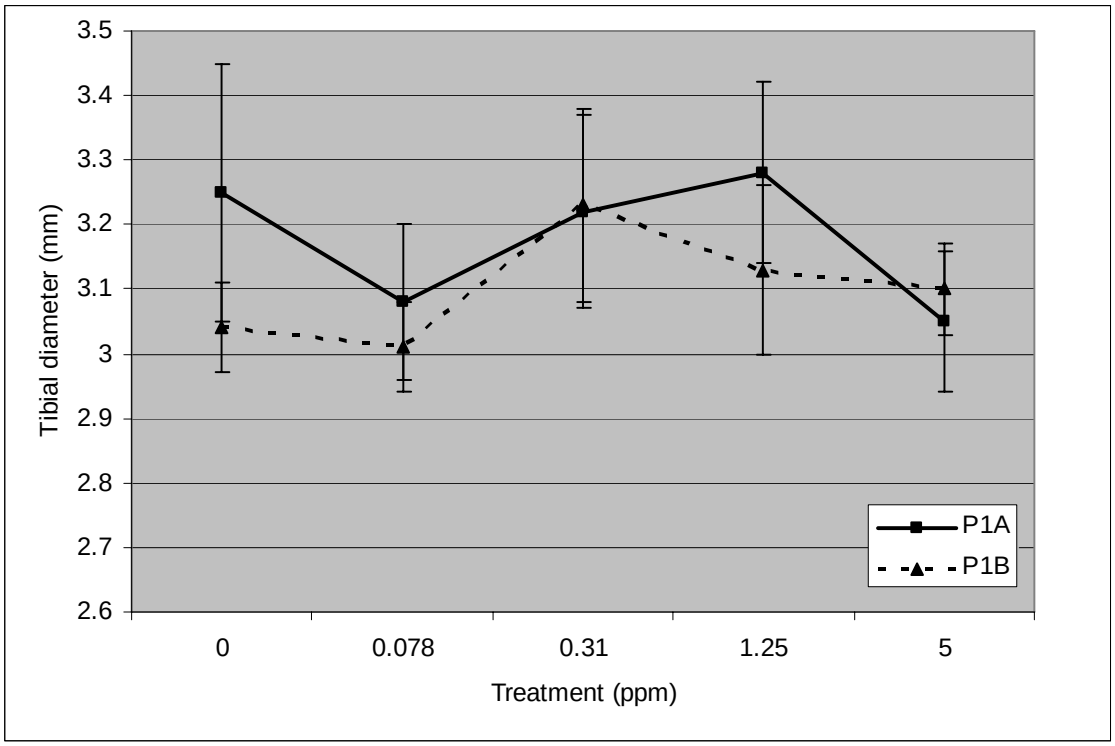

Figure 15. Female tibial diameter at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

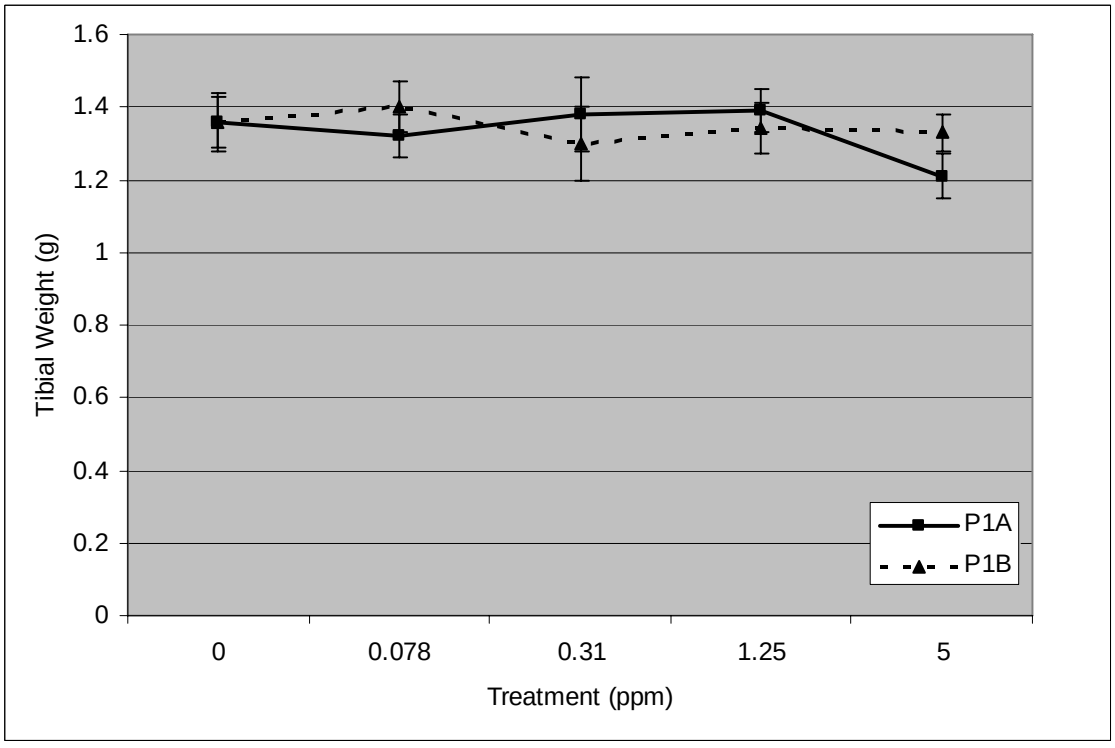

Figure 16. Female tibial weight at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

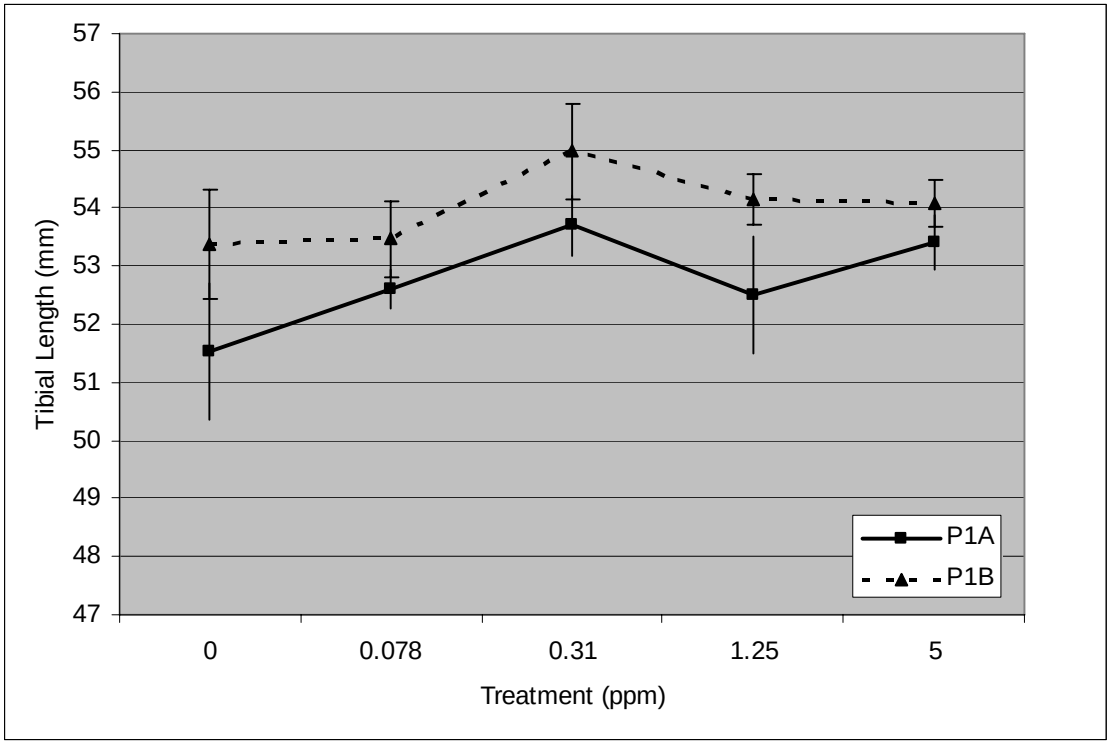

Figure 17. Male tibial length at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

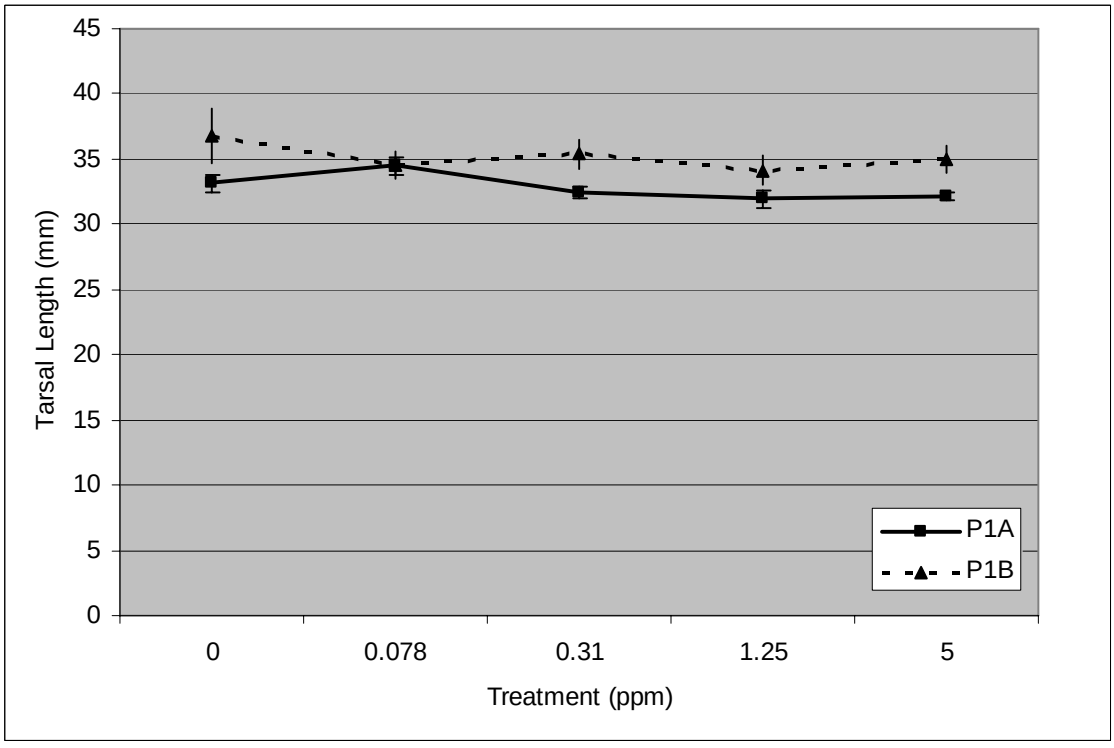

Figure 18. Male tarsal length at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

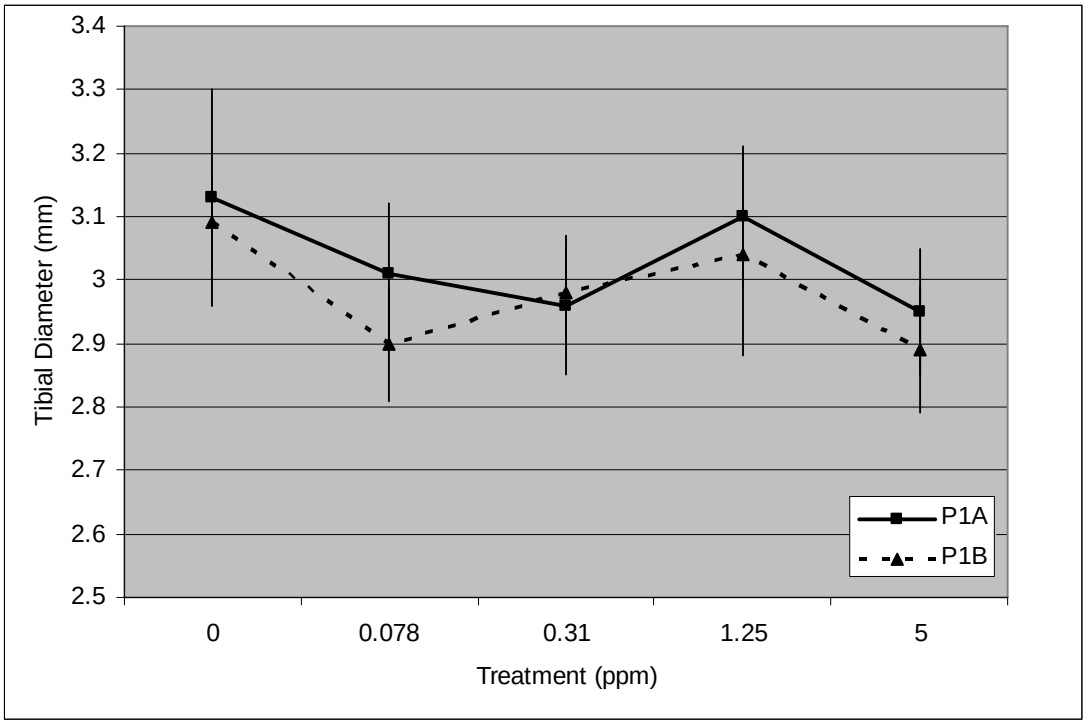

Figure 19. Male tibial diameter at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

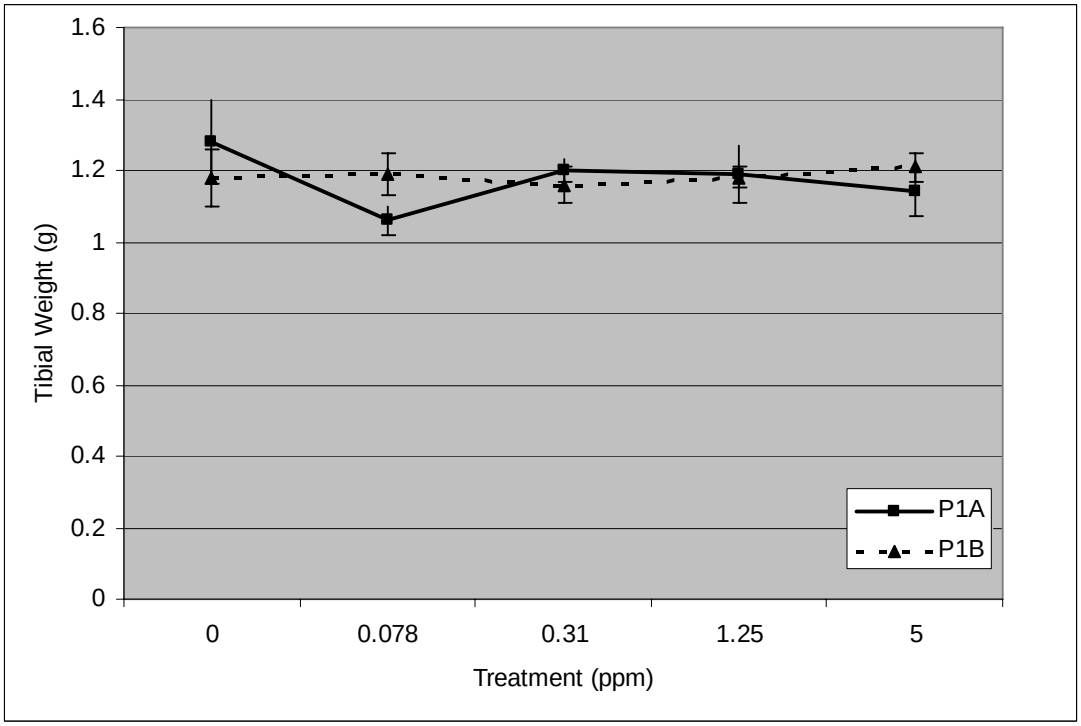

Figure 20. Male tibial weight at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

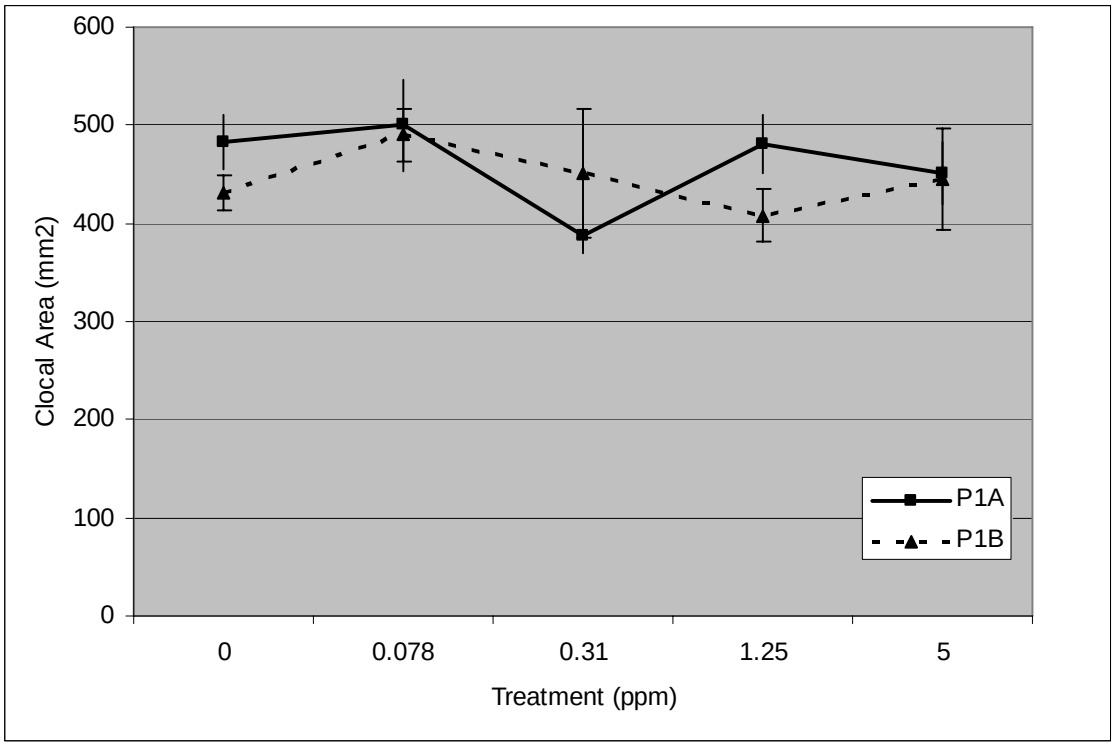

Figure 21. Cloacal area at necropsy for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

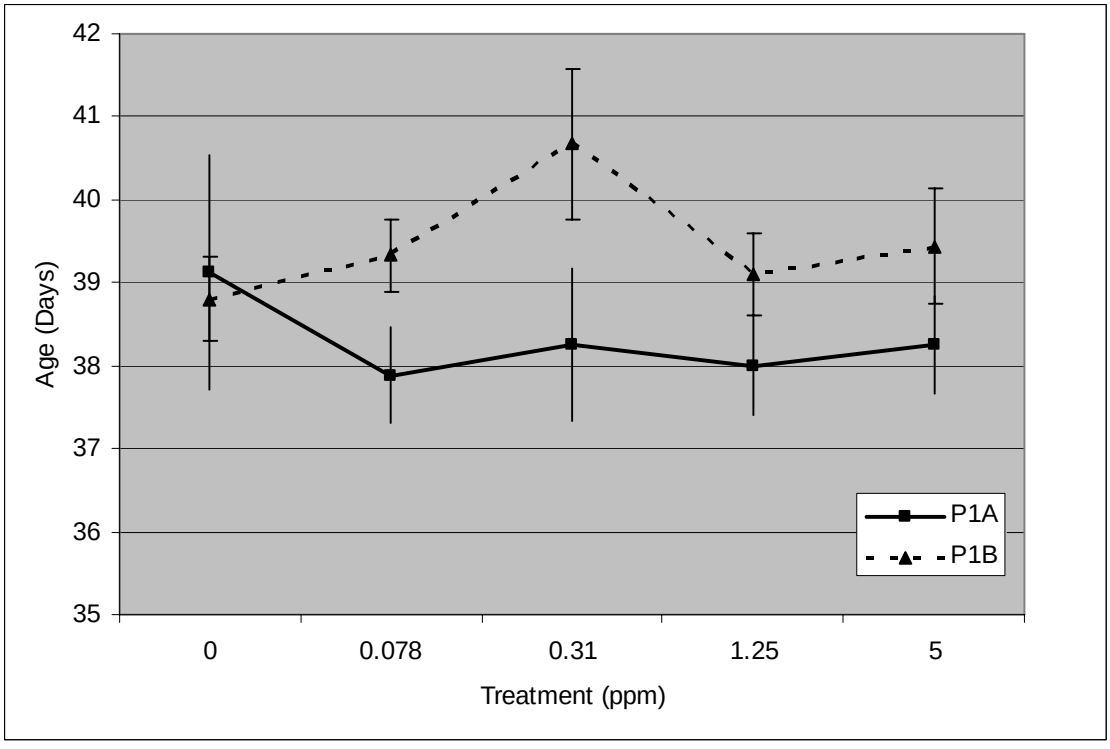

Figure 22. Age at first foam for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

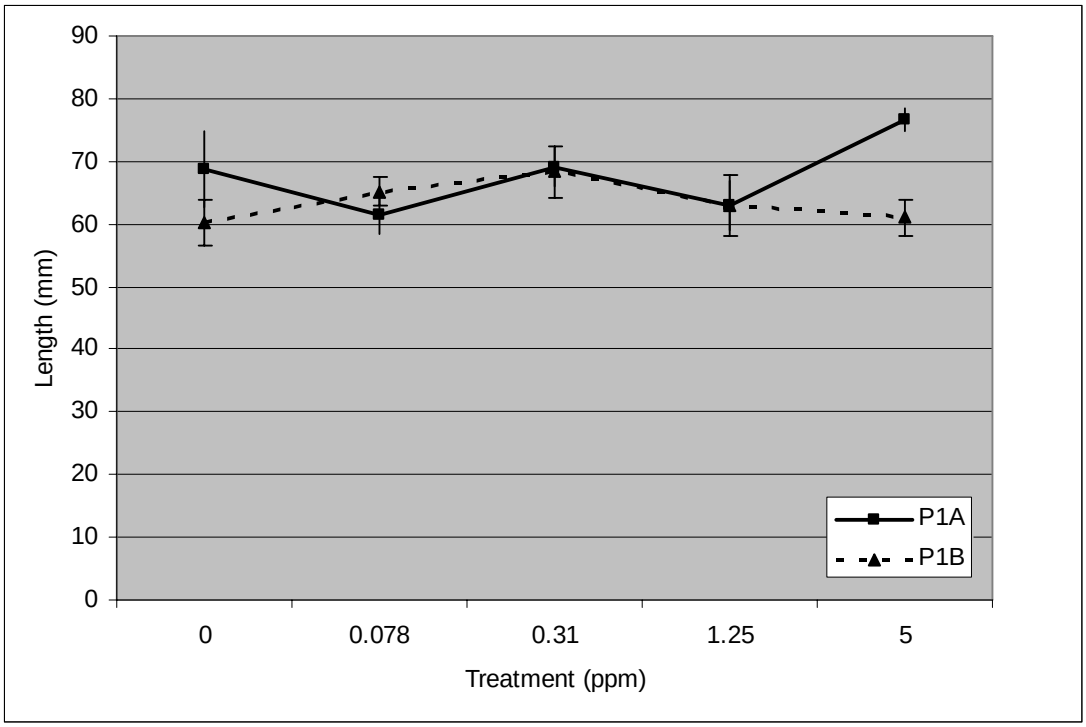

Figure 23. Length of female-type plumage in females at necropsy for P1 generations. No significant difference is detected in either group.

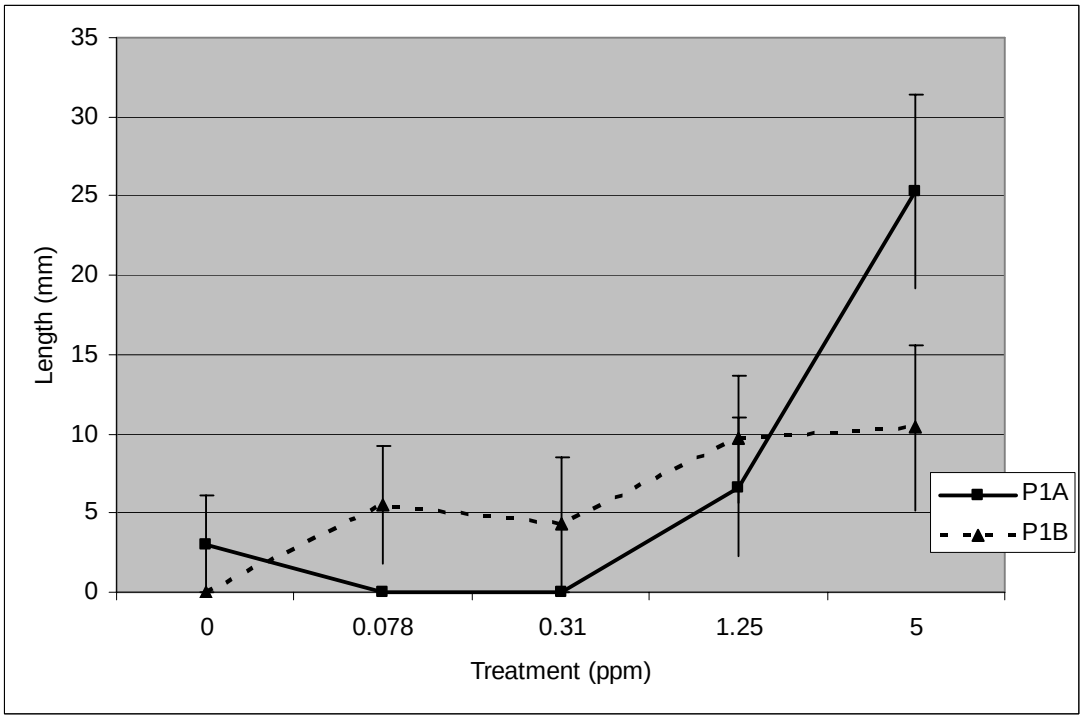

Figure 24. Length of female-type plumage in males at necropsy for P1 generations. According to a Kruskal-Wallis test, there is a significant difference between groups in the P1A generation, which is most apparent at the 5 ppm level. No significant effect is detected in the P1B group.

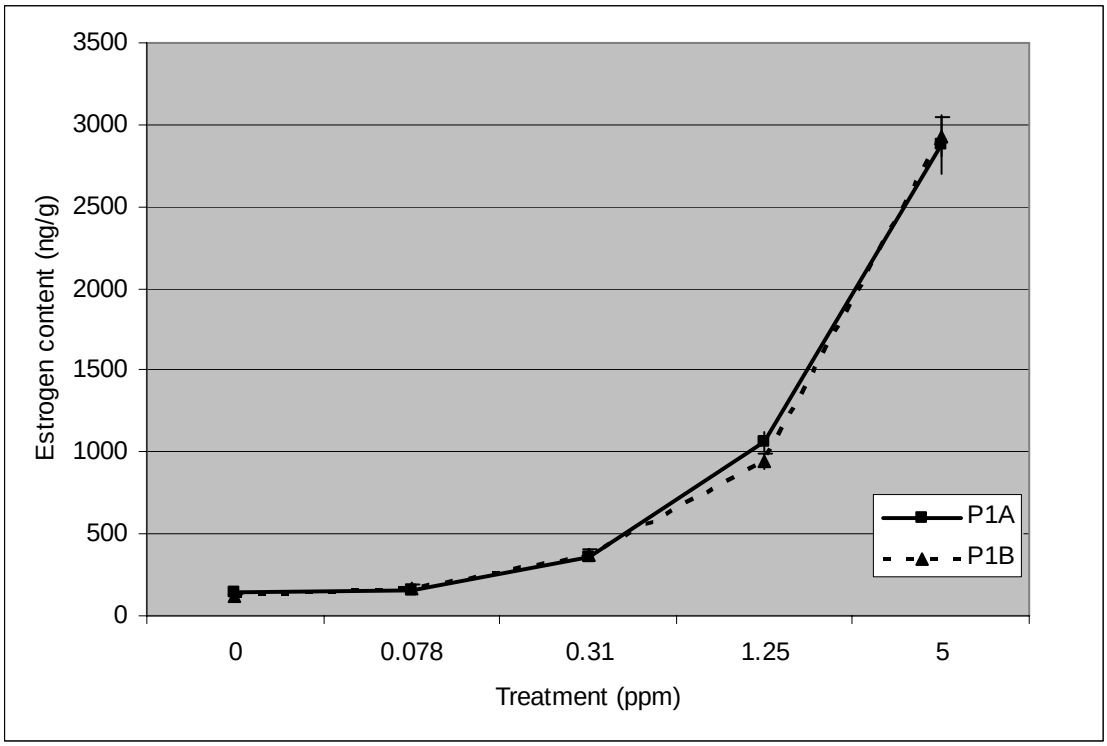

Figure 25. Fecal-urate estrogen content in females for P1 generations. According to a Kruskal-Wallis test, there is a significant difference between groups in both P1A and P1B generations.

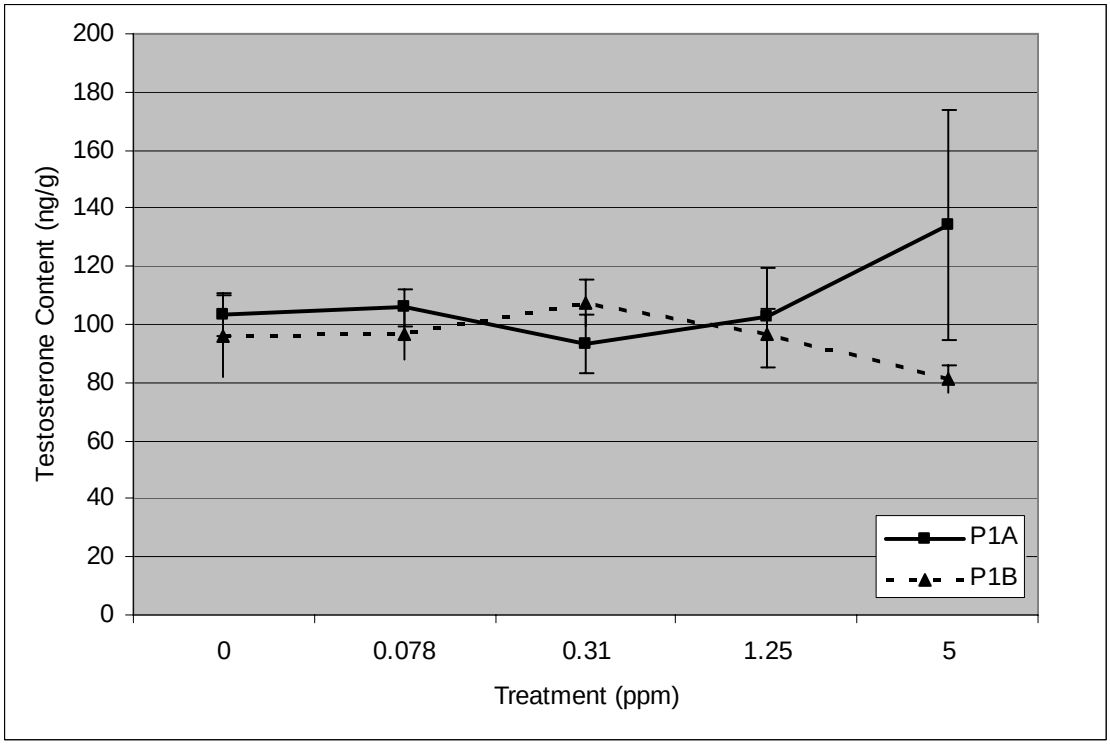

Figure 26. Fecal-urate testosterone content in females for P1 generations. No significant differences were determined, therefore the LOEC is above 5 ppm for both groups.

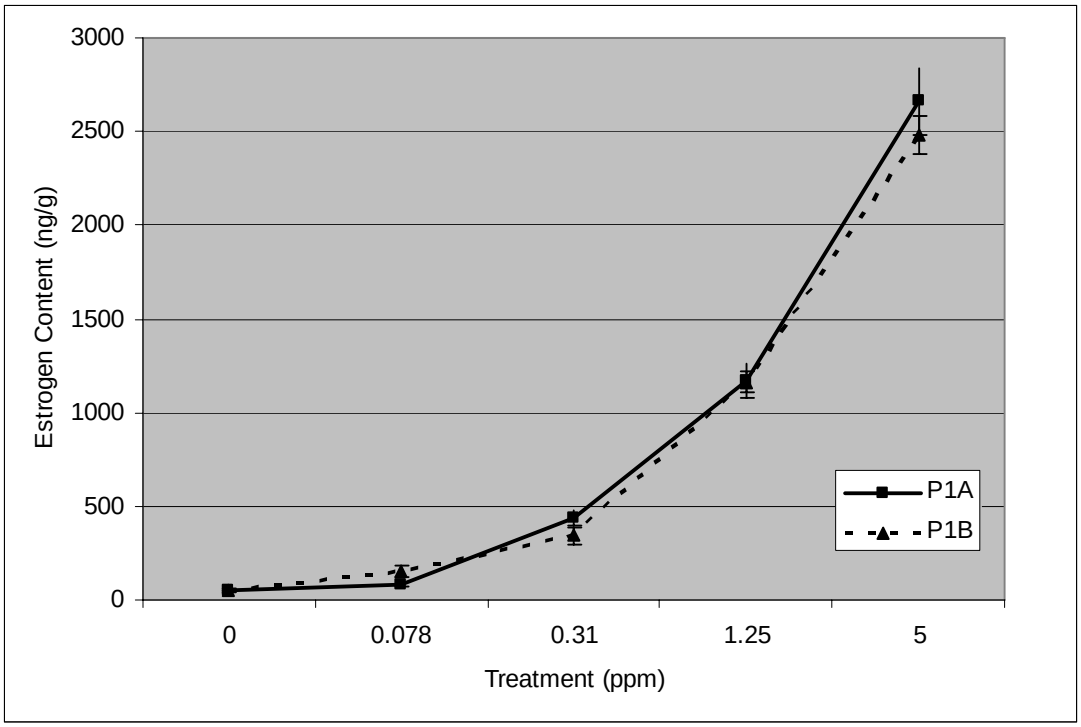

Figure 27. Fecal-urate estrogen content in males for P1 generations. According to a Kruskal-Wallis test, there is a significant difference between groups in both P1A and P1B generations.

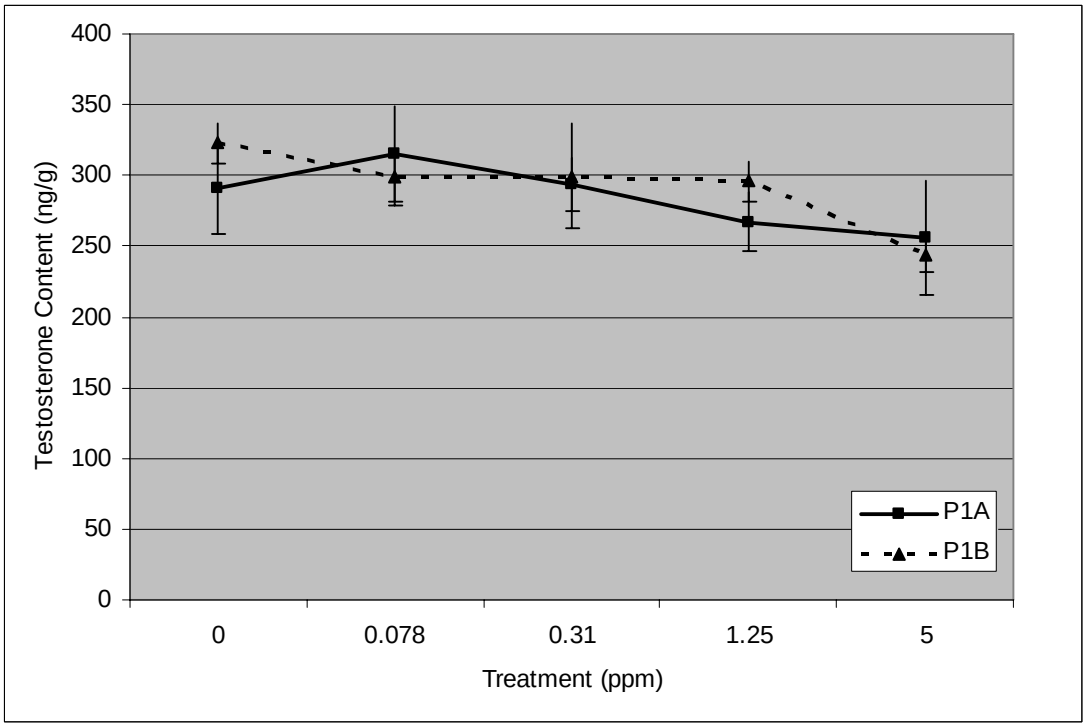

Figure 28. Fecal-urate testosterone content in males for P1 generations. In the P1A group, no significant difference between treatments is detected. In the P1B group, both a Jonckheere test and Mann Whitney test indicate that the LOEC = 5, and the NOEC = 1.25.

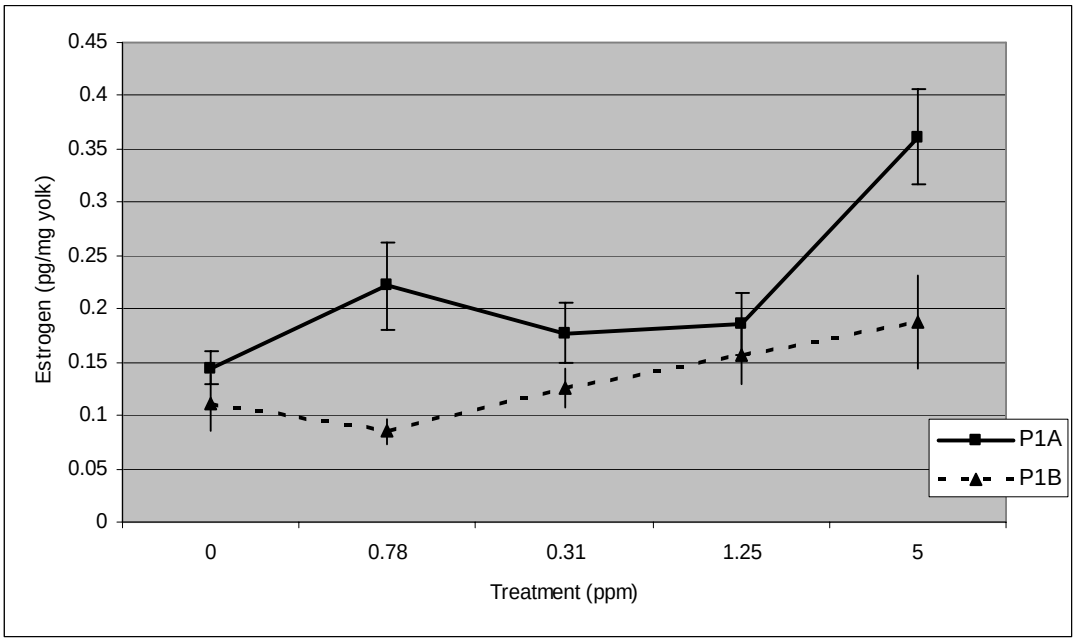

Figure 29. Egg estrogen content in P1 generations. No additional statistical analysis was performed.

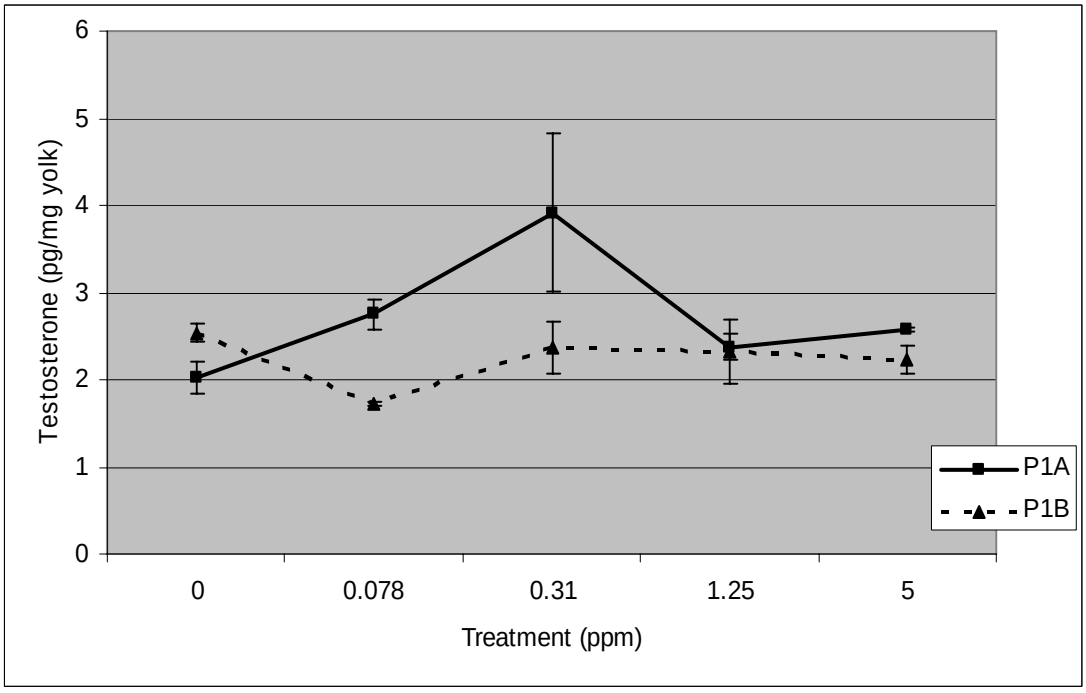

Figure 30. Egg testosterone content of P1 generations. No additional statistical analysis was performed.

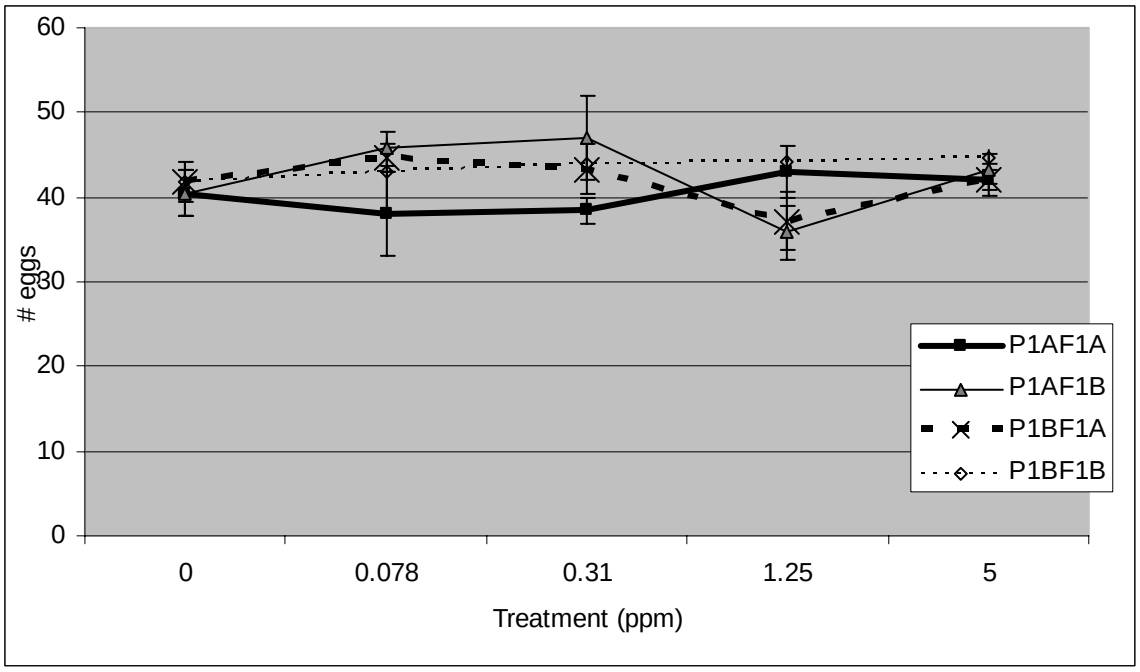

Figure 31. Number of eggs laid by F1 generation of quail. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

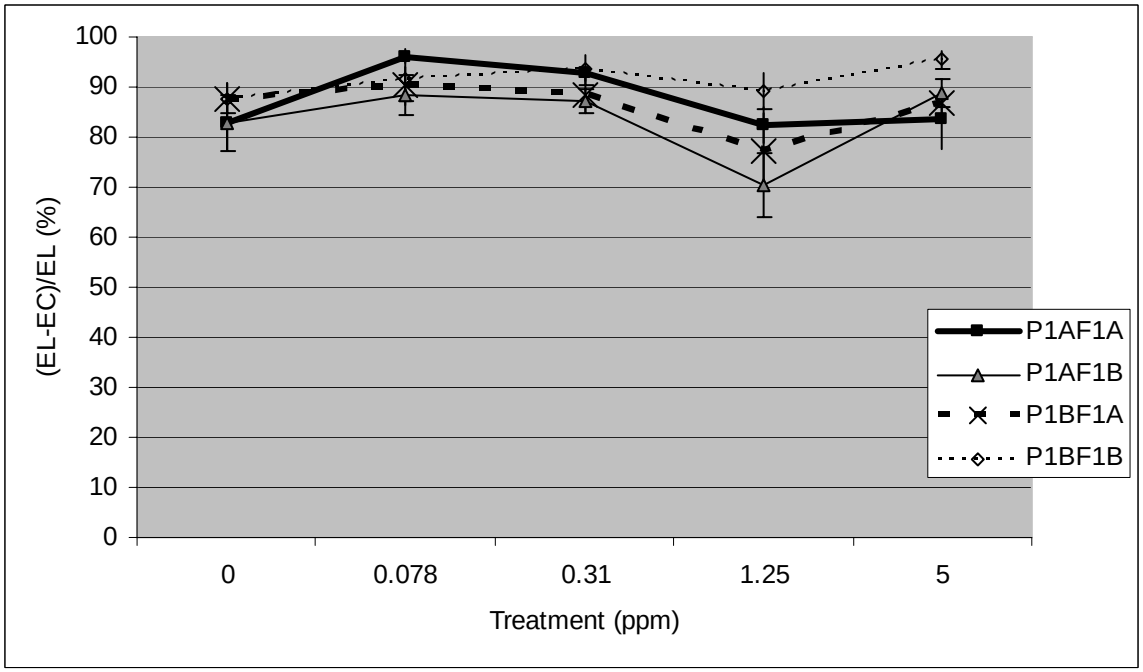

Figure 32. Percent intact eggs (not cracked): (Eggs laid - Eggs cracked)/Eggs laid (%). No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

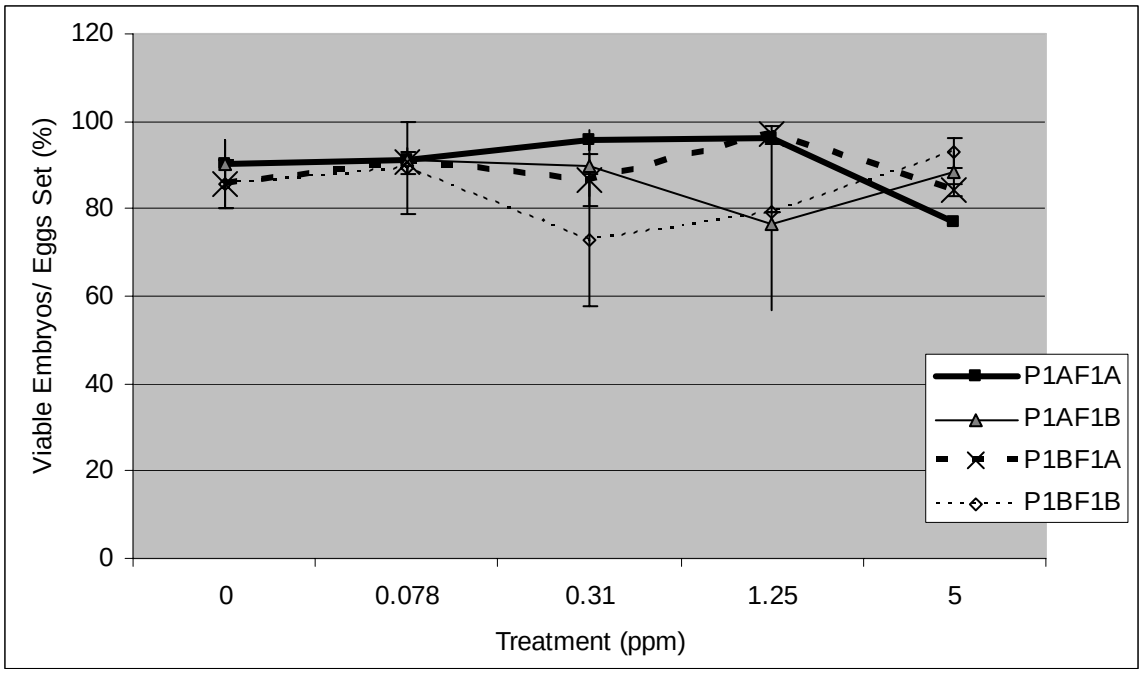

Figure 43. Viable embryos at day 8 per eggs set (%) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

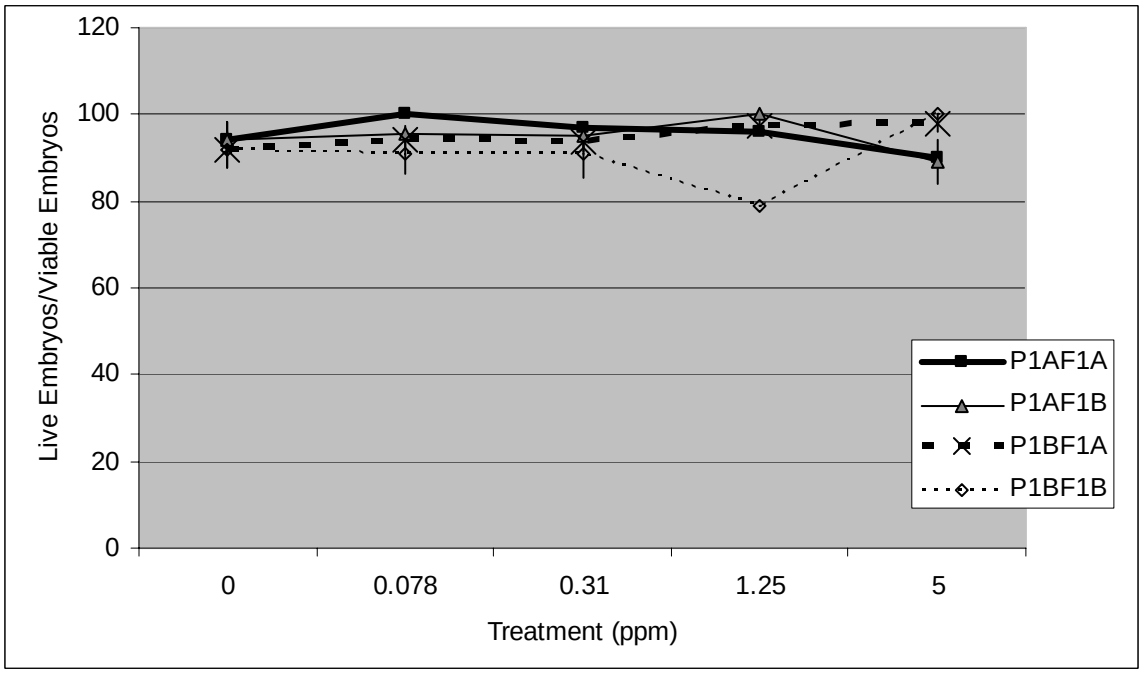

Figure 34. Live embryos (@ day 15)/Viable embryos (@ day 8) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

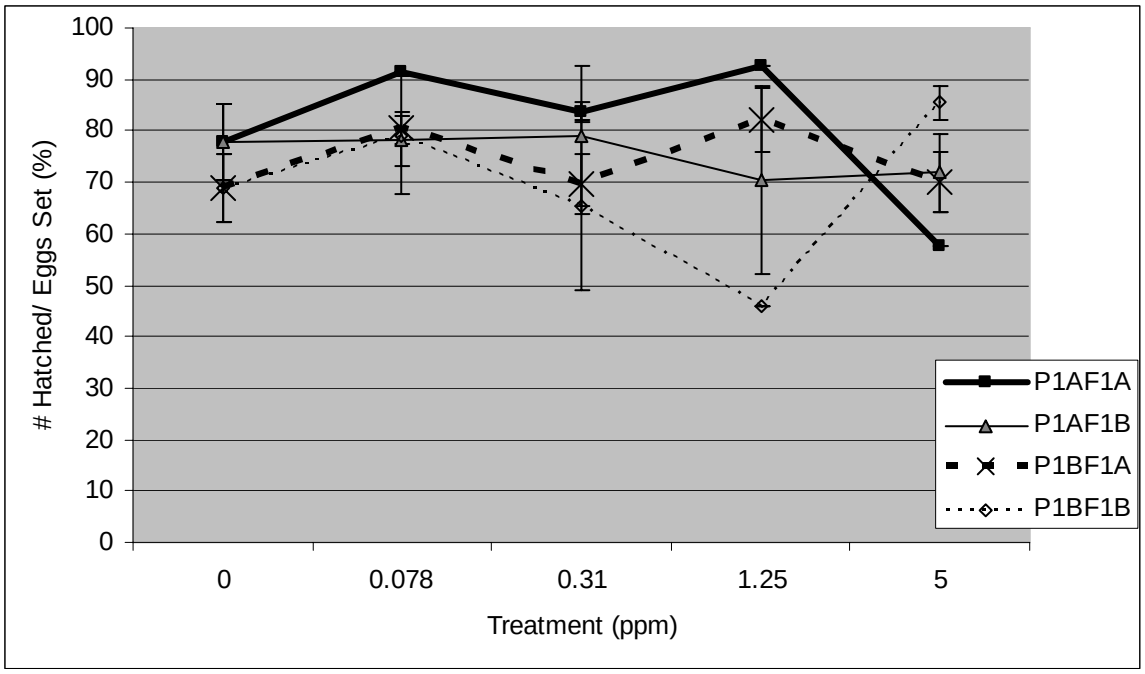

Figure 35. Number of eggs hatched/ Eggs set (%) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

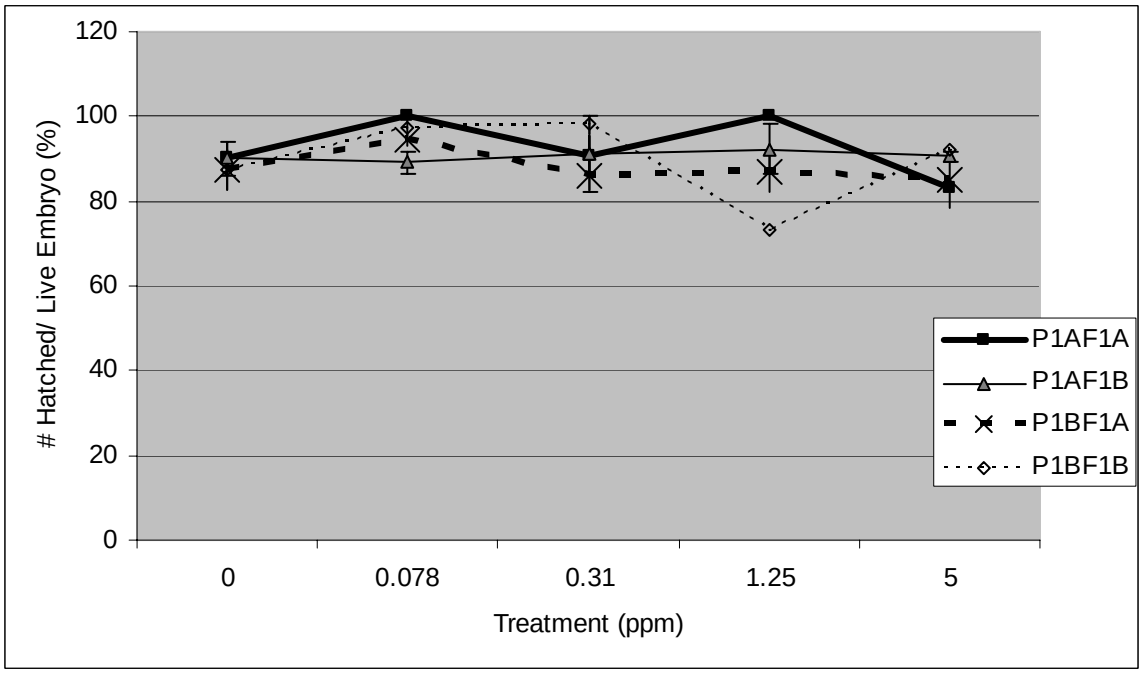

Figure 5. # Hatched/ Live embryo @ day 15 (%) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

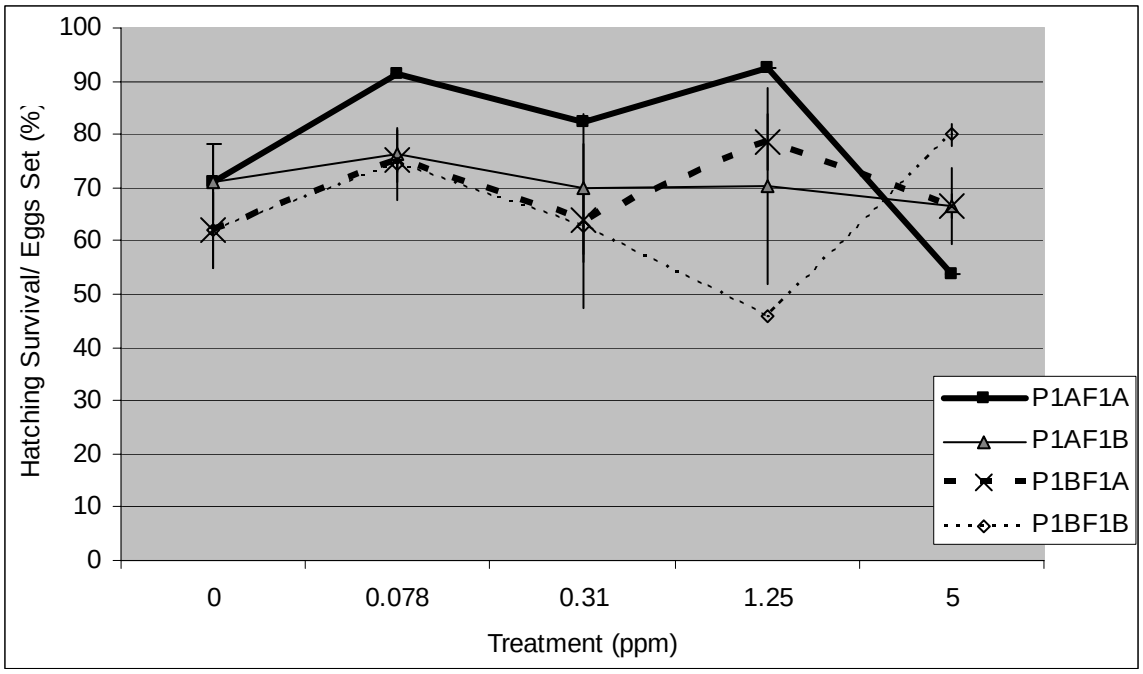

Figure 37. Hatchling survival/ Eggs set (%) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

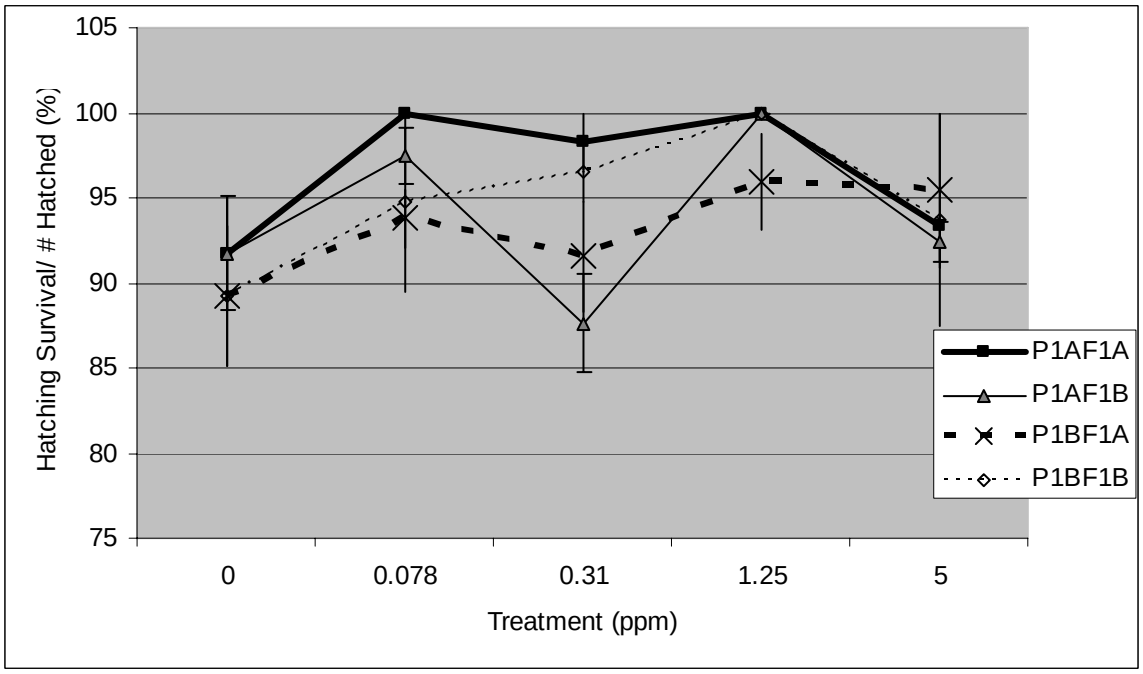

Figure 38. Hatchling survival/ # Hatched (%) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

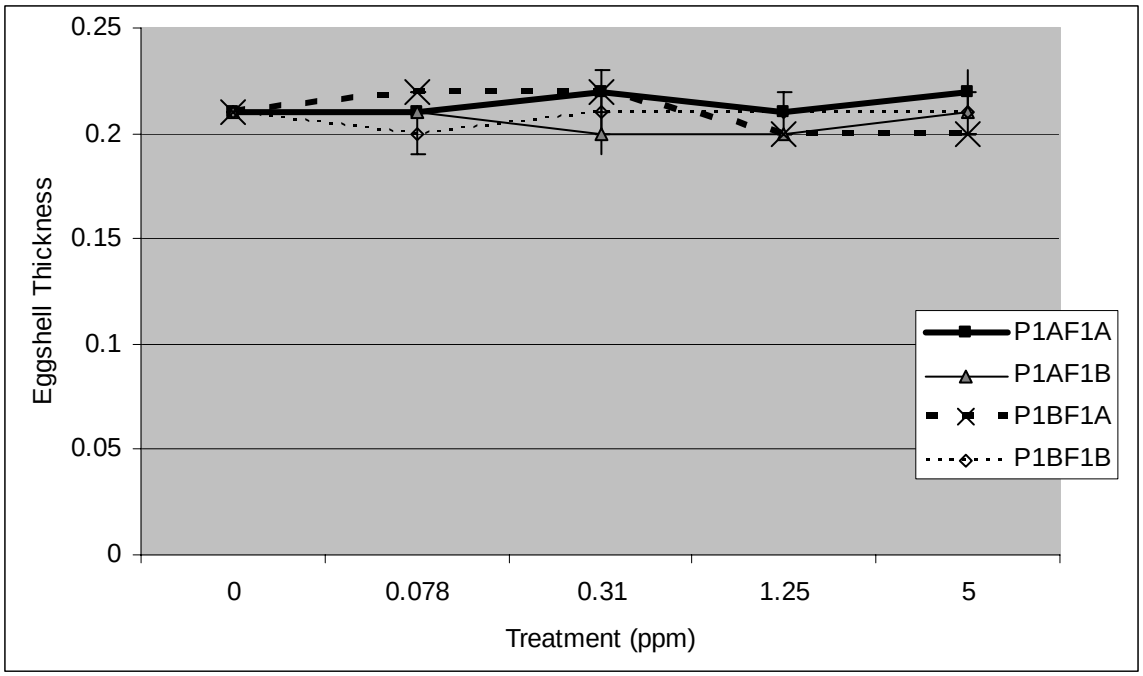

Figure 39. Eggshell thickness of eggs laid by F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

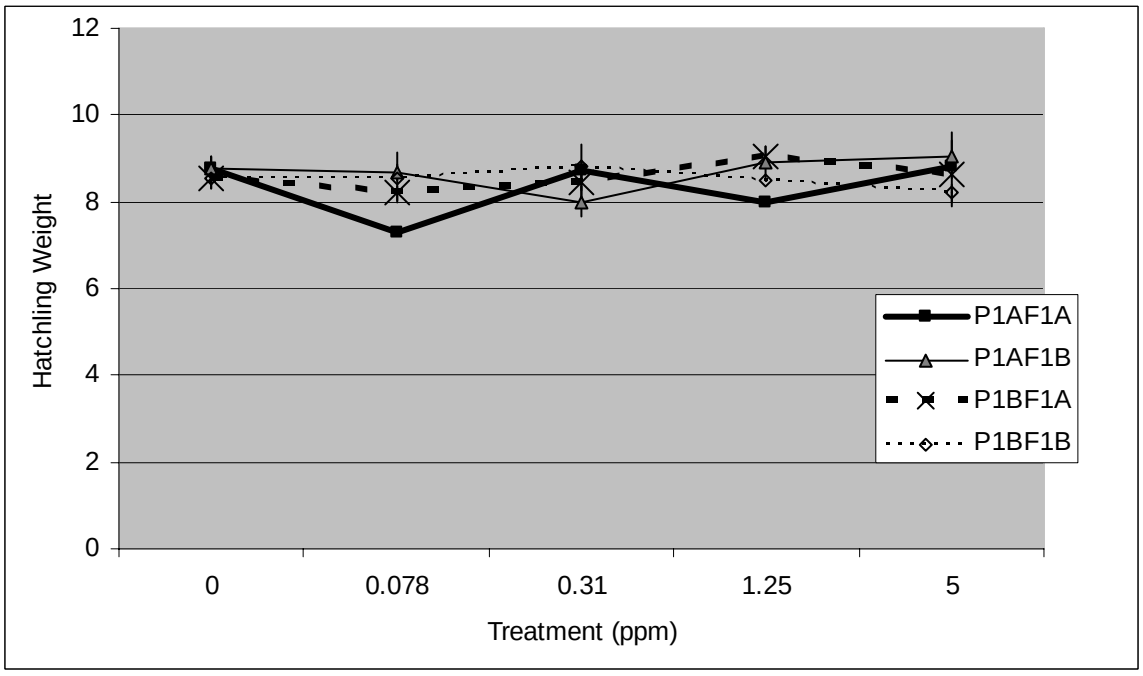

Figure 40. Hatchling weight for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

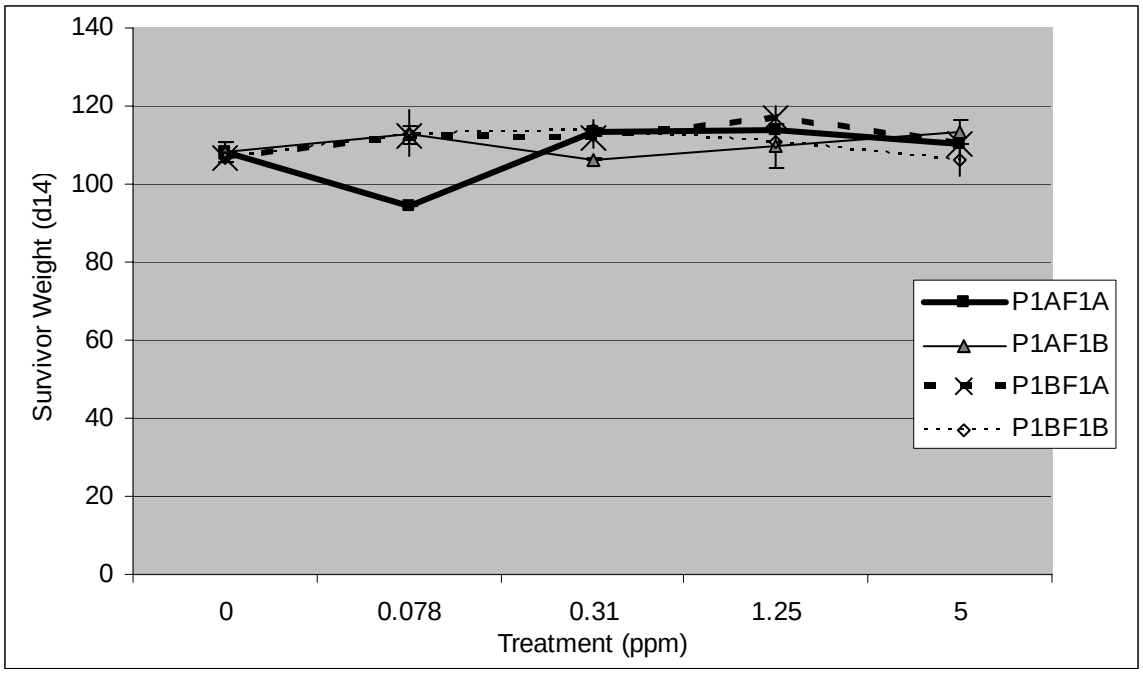

Figure 46. Survivor weight (day 14) for F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

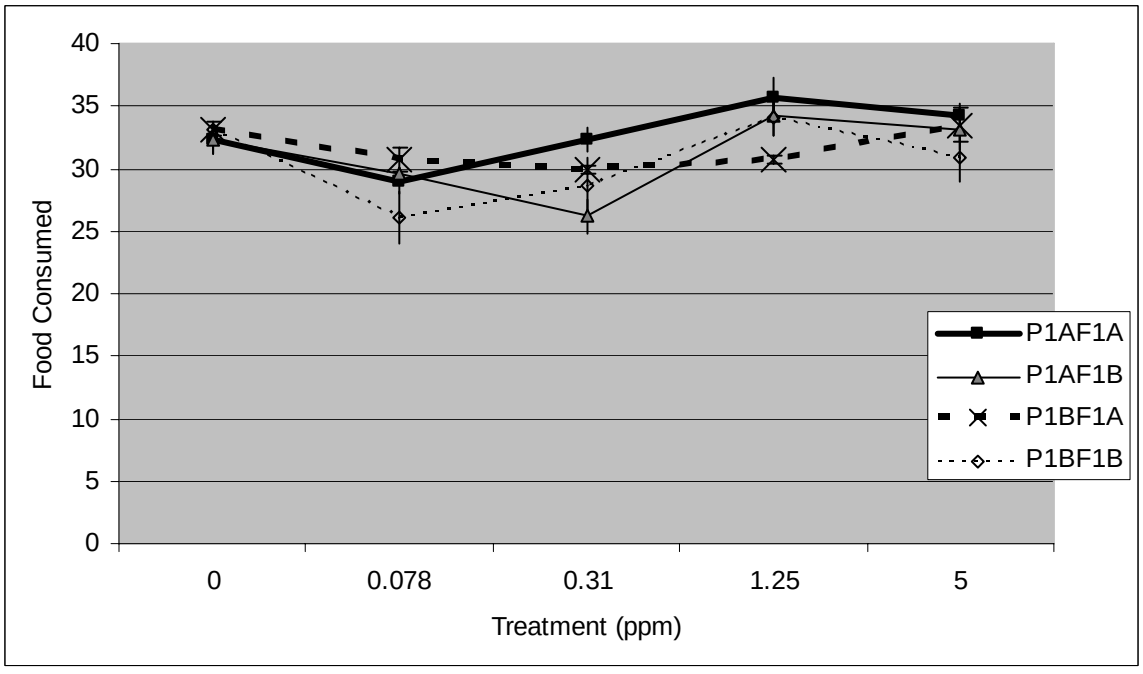

Figure 42. Food consumption by F1 generation. No significant differences compared to control were observed at any level for the P1AF1A exposure group. A significant difference from control was observed only at the 0.31 ppm level in both the P1AF1B and the P1BF1A groups. For the P1BF1B exposure group, a significant difference from control was observed only at the 0.078 ppm level. No dose response trend was observed in any group.

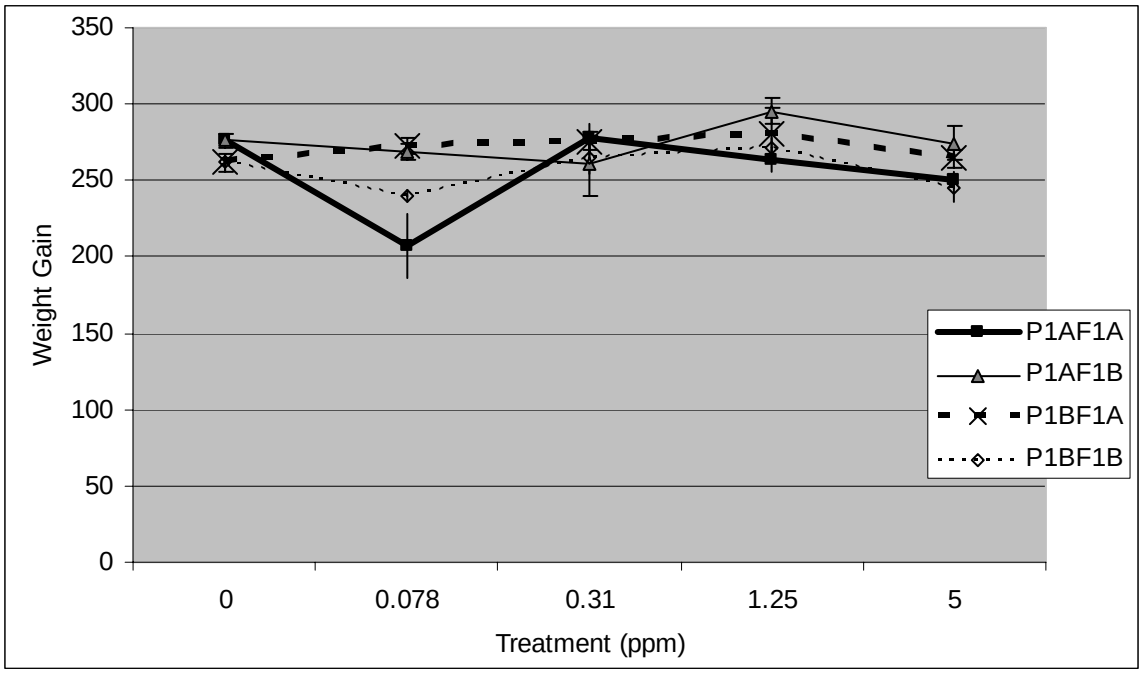

Figure 43. Weight gain of F1 females. For the P1AF1A exposure group, a significant difference from control was observed only at the 0.078 ppm level. With no apparent treatment related trend. No significant differences were observed for the other three exposure groups; thus the LOEC was greater than 5 ppm for the three other exposure groups.

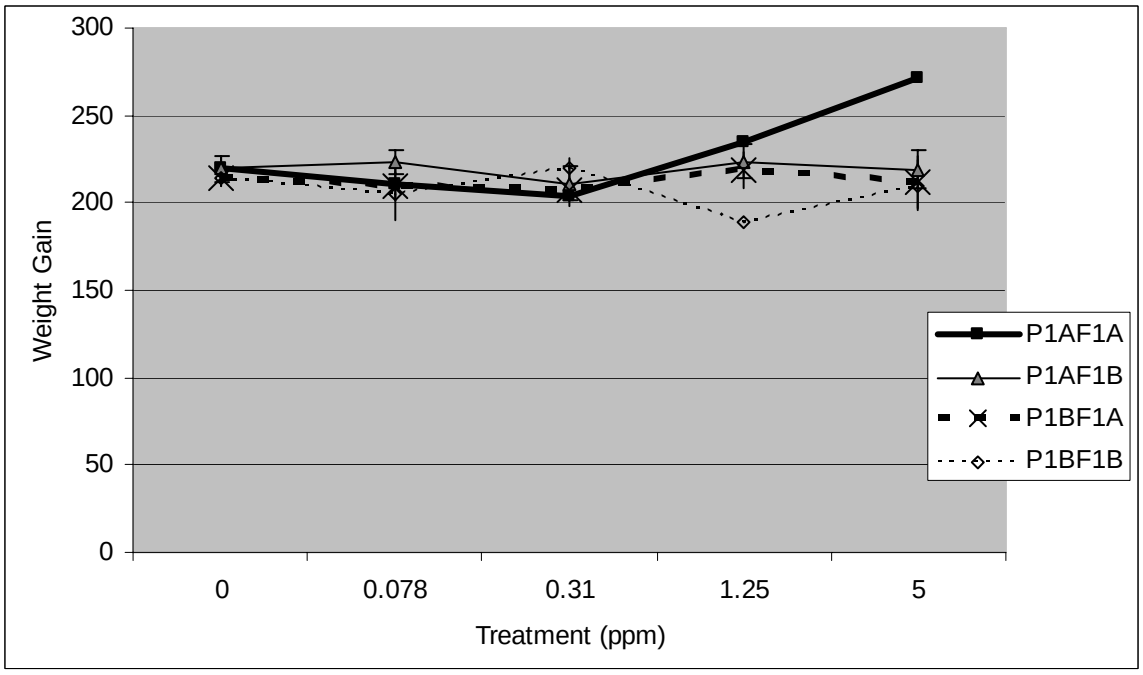

Figure 44. Weight gain of F1 males. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

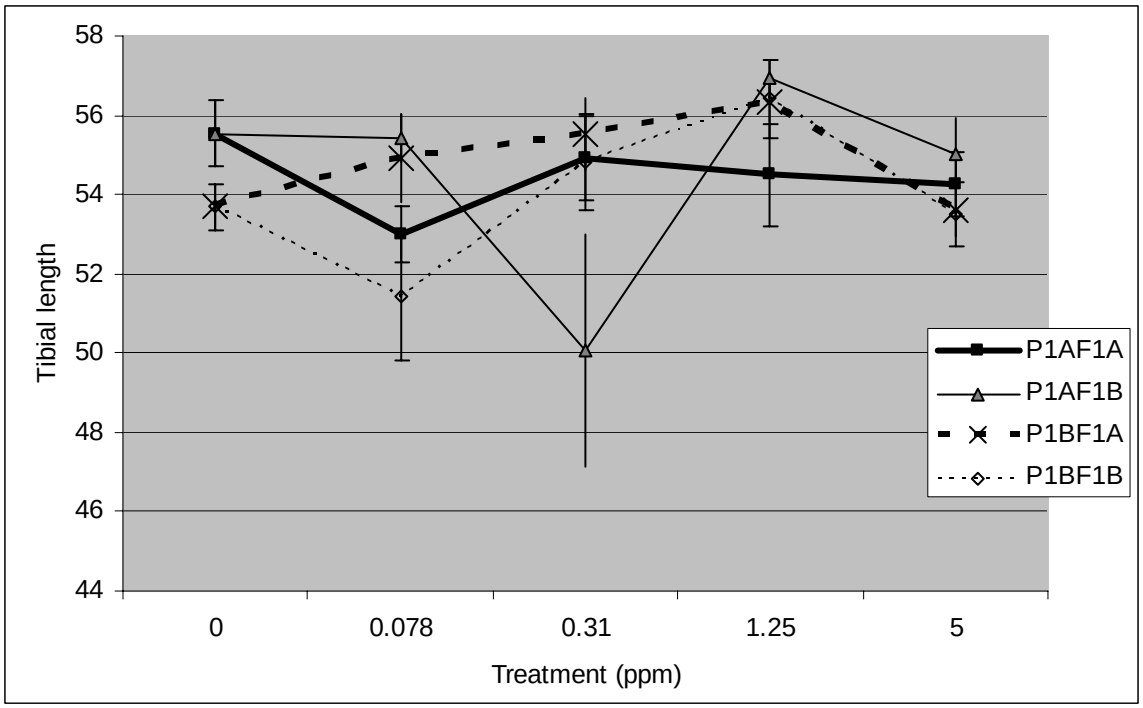

Figure 45. Tibial length of females of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

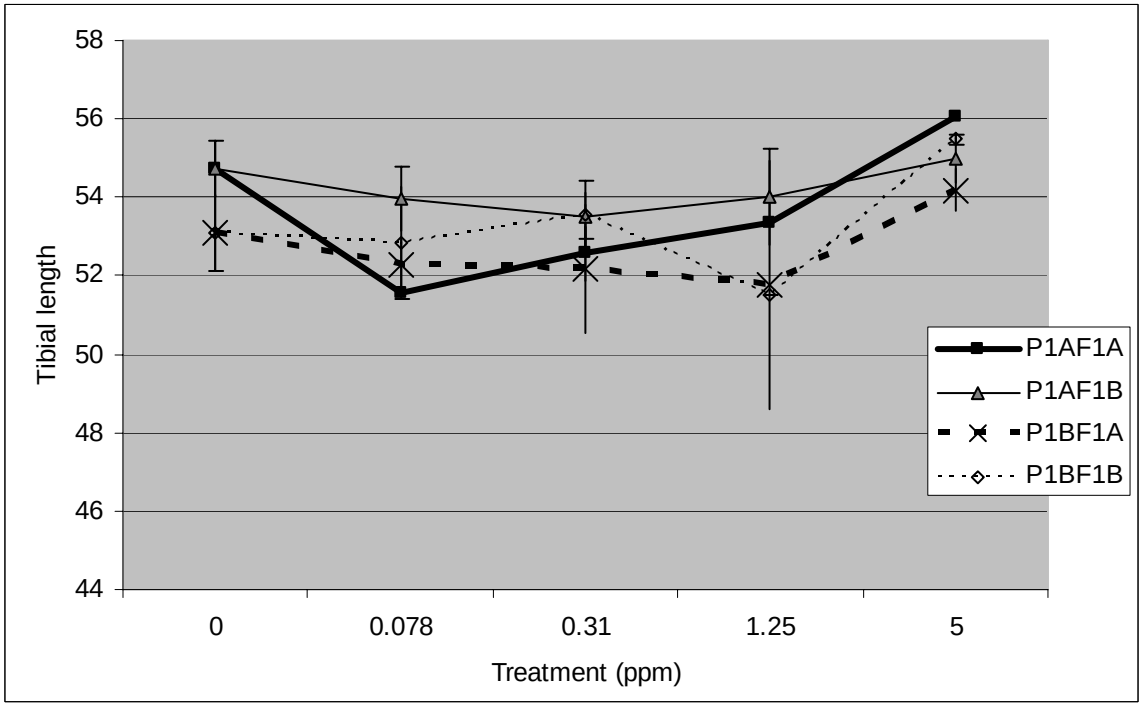

Figure 46. Tibial length of males of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

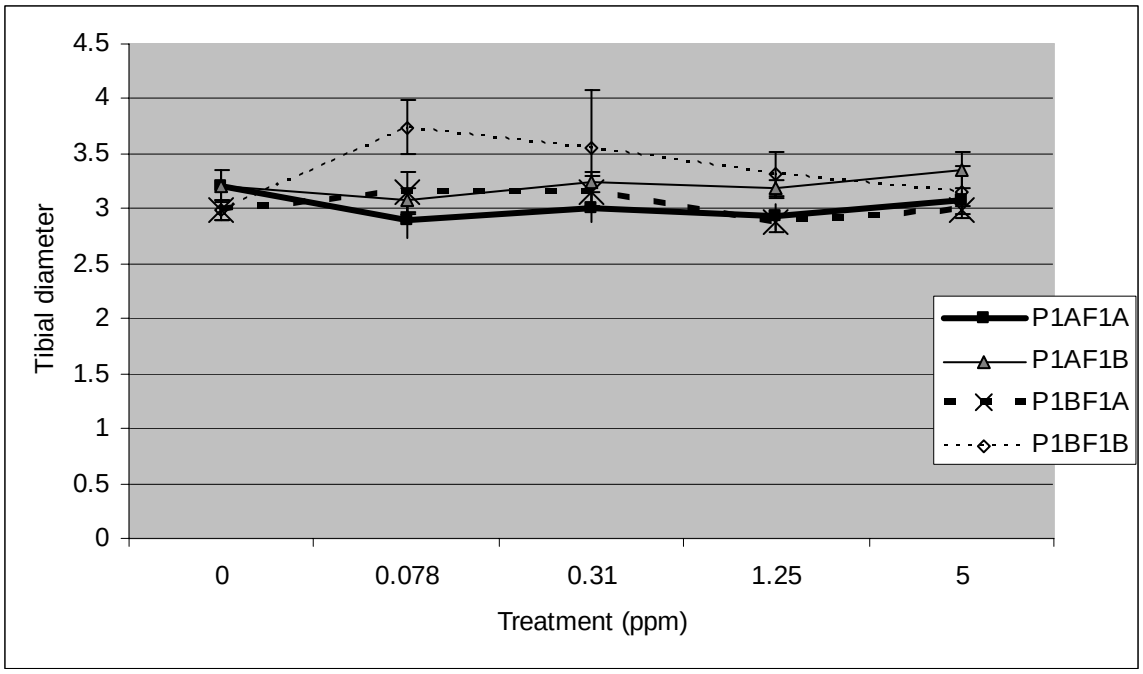

Figure 77. Tibial diameter of females of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

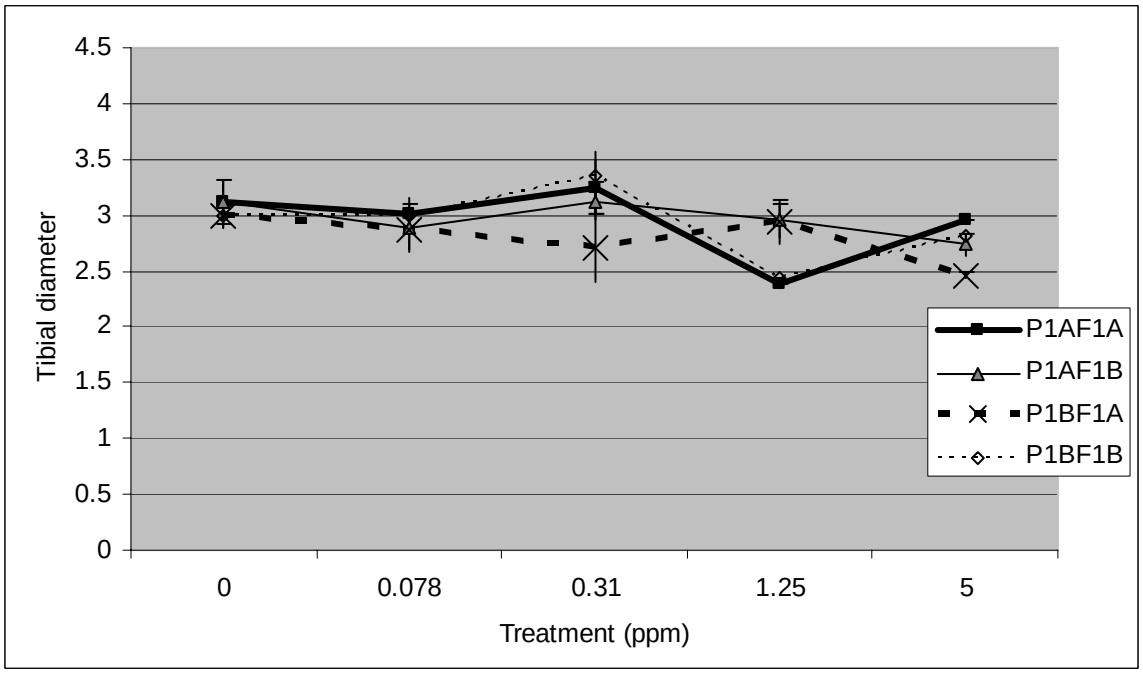

Figure 48. Tibial diameter of males of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

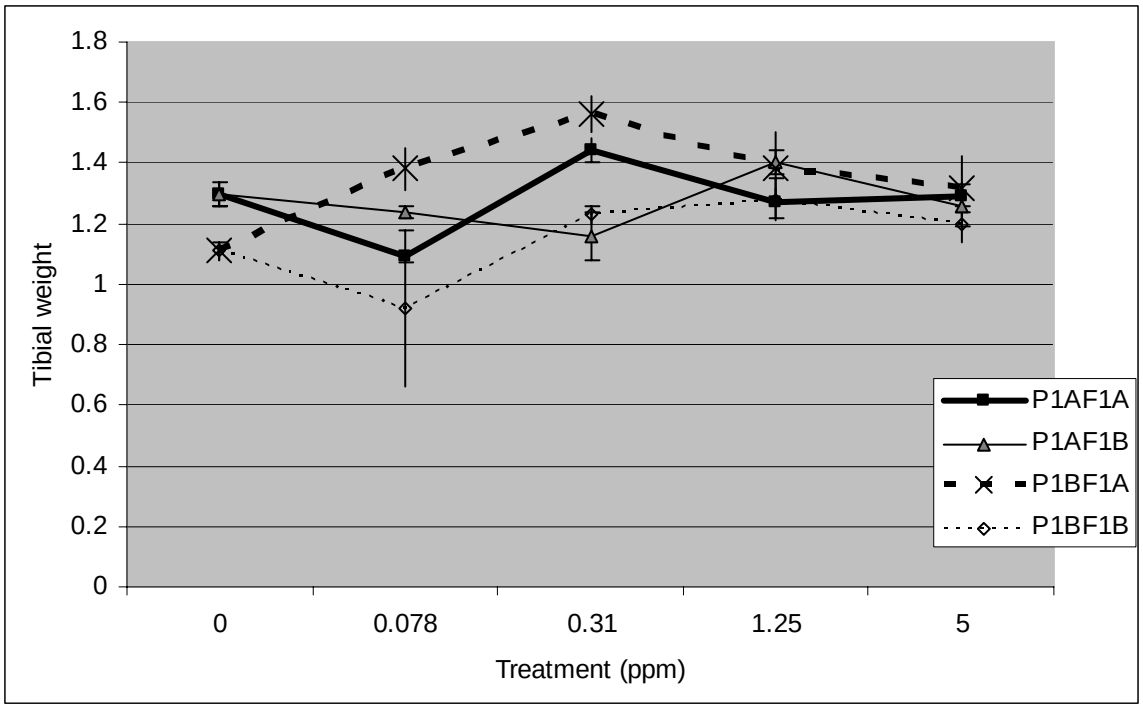

Figure 49. Tibial weight of females of the F1 generation. A Kruskal-Wallis test detected a significant difference for the P1BF1A exposure group, apparently between the control and all treatment levels. No significant differences were observed for the other three exposure groups; thus the LOEC was greater than 5 ppm for those three exposure groups.

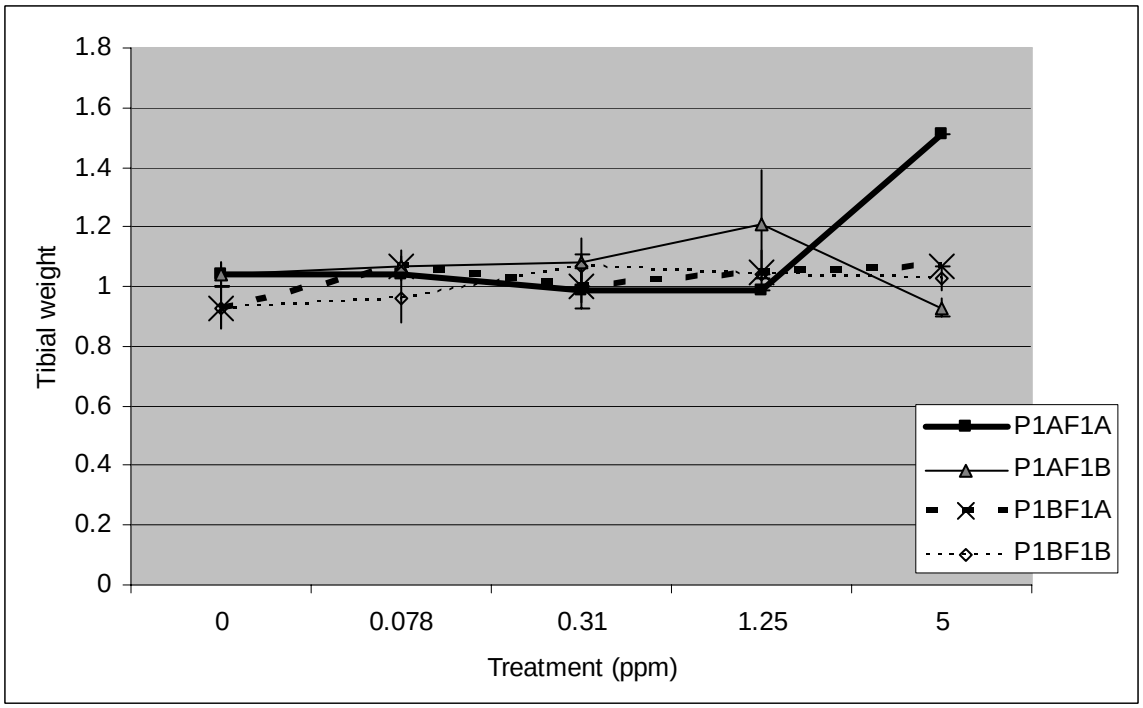

Figure 508. Tibial weight of males of the F1 generation. An Analysis of Variance detected a significant difference in the P1AF1A exposure group at 5 ppm. For the other three exposure groups, no significant differences were observed; thus the LOEC was greater than 5 ppm.

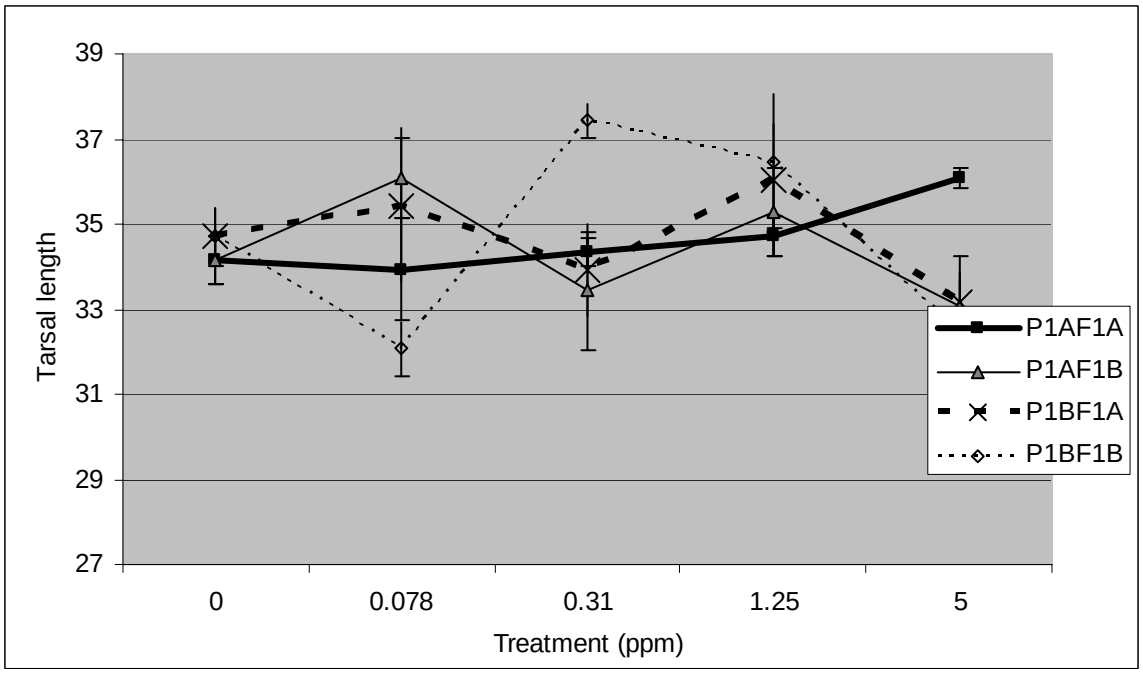

Figure 51. Tarsal length of females of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

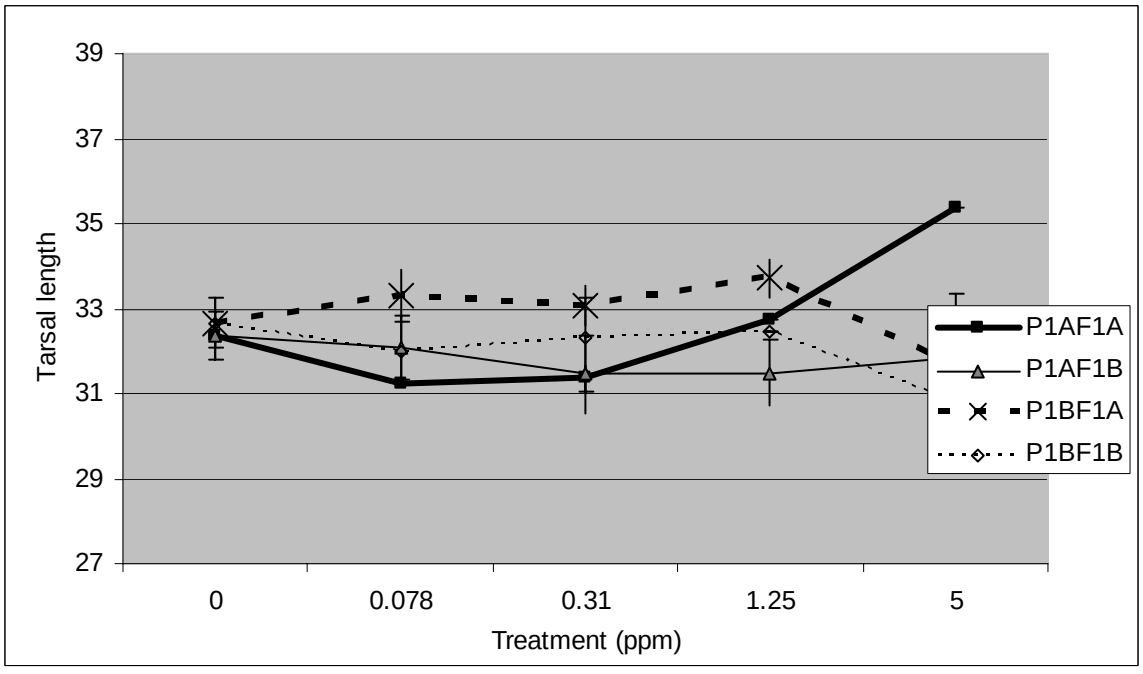

Figure 52. Tarsal length of males of the F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

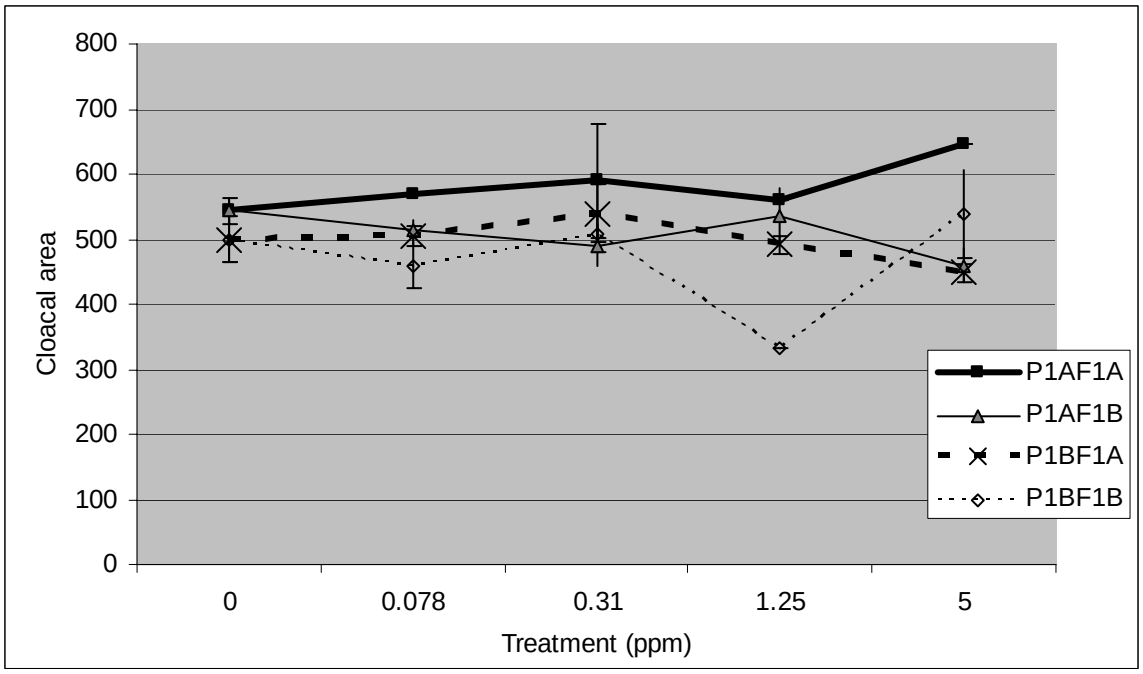

Figure 53. Cloacal area at necropsy (males) in F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

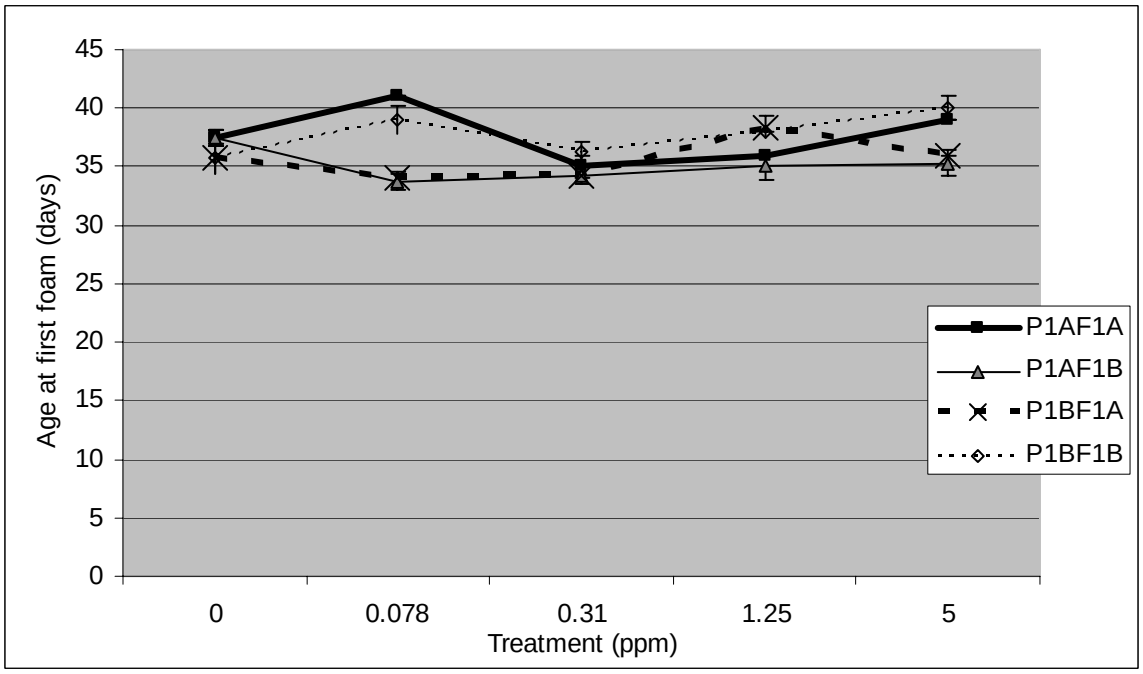

Figure 54. Age at first foam in F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

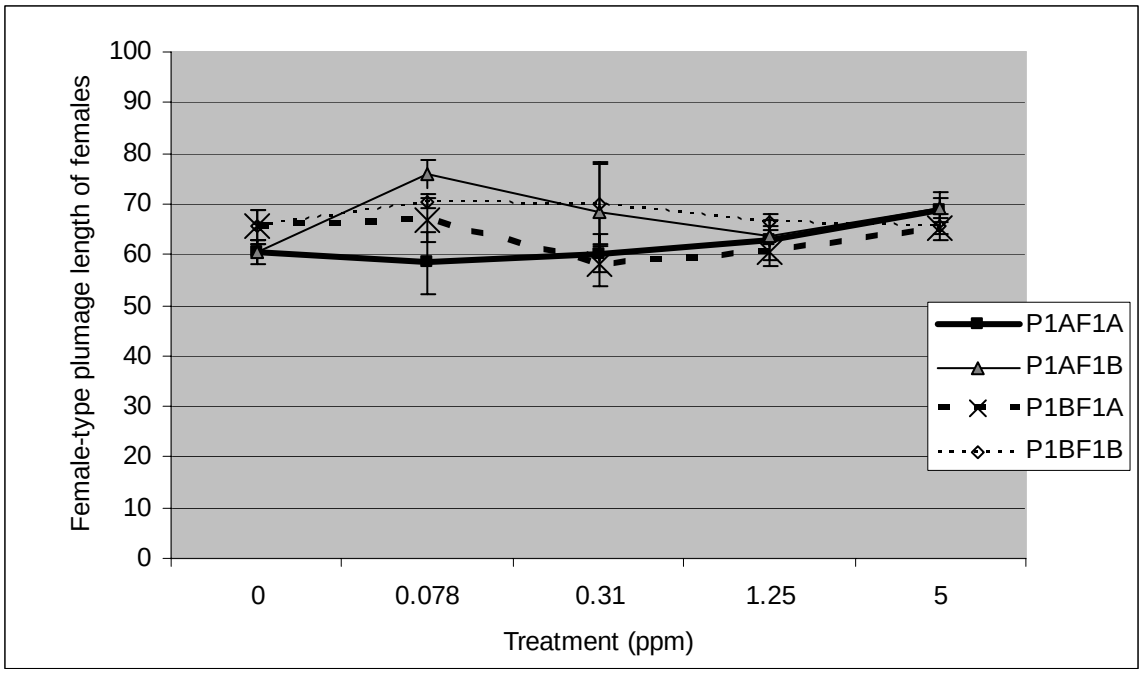

Figure 55. Female-type plumage length of females in F1 generation. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.

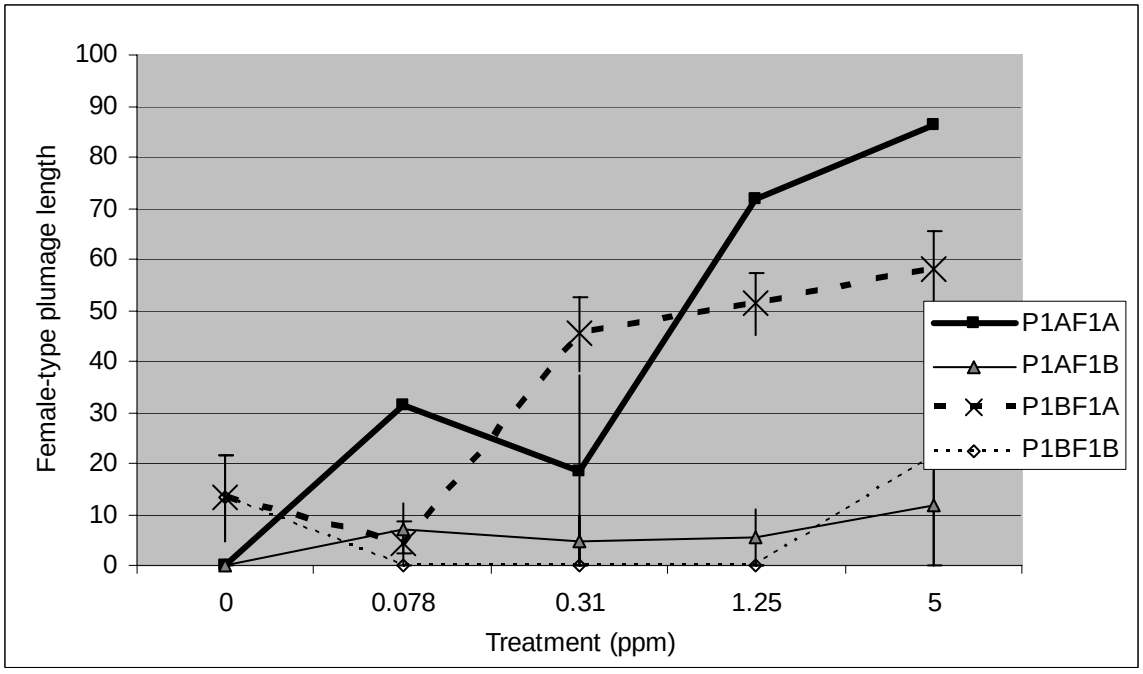

Figure 56. Female-type plumage length of males in F1 generation. A significant difference between control and treatment levels was observed in the P1AF1A and the P1BF1A groups. No significant differences were detected in the P1AF1B or P1BF1B exposure groups; thus the LOEC for those two exposure groups was greater than 5 ppm.

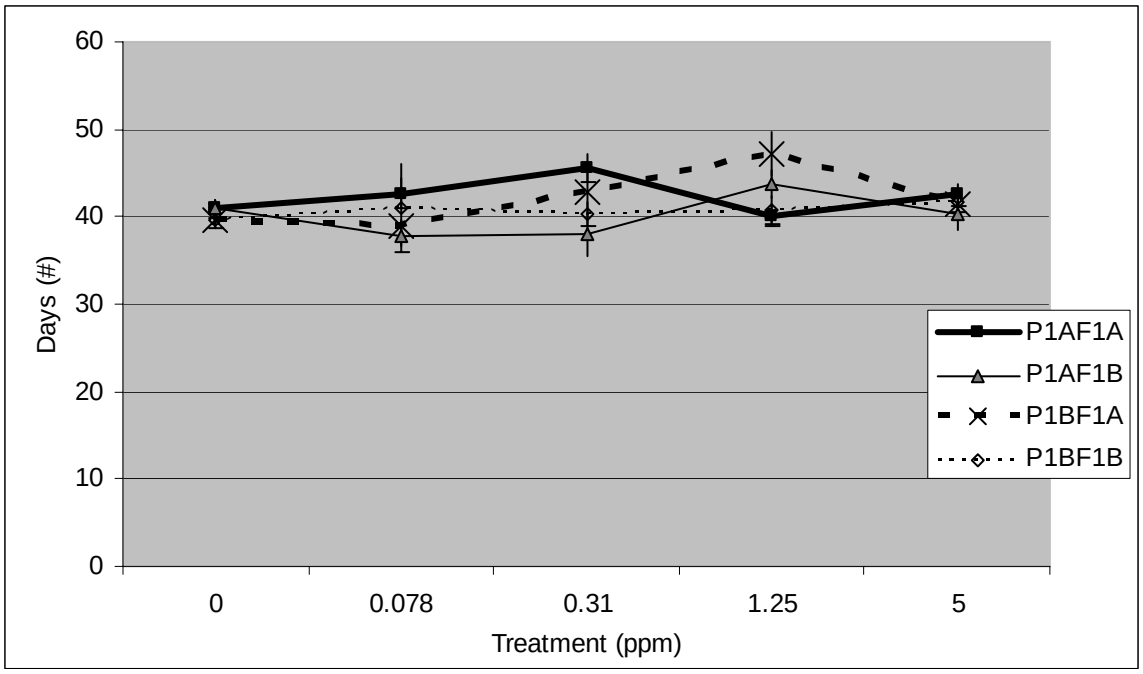

Figure 57. Onset of egg laying by F1 generation. A Kruskal-Wallis test detected a significant difference in the P1BF1A exposure group. No significant differences were observed for the other three exposure groups; thus the LOEC was greater than 5 ppm for those exposure groups.

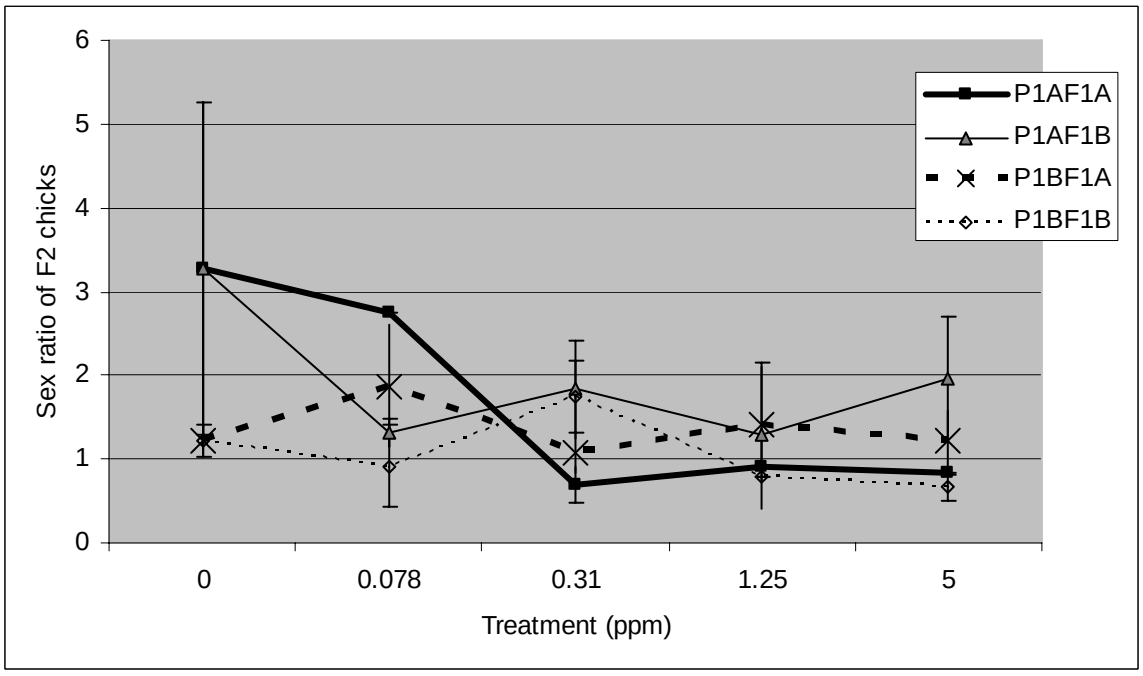

Figure 98. Sex ratio of F2 chicks. No significant differences were observed; thus the LOEC was greater than 5 ppm for all four exposure groups.