DRAFT FINAL REPORT

on

AVIAN DOSING STUDY

EPA CONTRACT NUMBER 68-W-01-023
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Prepared for

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1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA), in collaboration with the Organization for Economic Cooperation and Development (OECD), is developing a test guideline to assess the impact of chemicals on the reproduction and development of birds over two generations. The guideline will include both conventional and endocrine endpoints. Several methodological issues that could not be resolved from existing literature were discussed during an OECD Endocrine Disruptor Testing and Assessment Task Force consultation with member country experts (OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds). One of the key issues needing resolution prior to developing a test guideline is the selection of appropriate exposure scenario(s) during a two-generation test. Some experts argue that dietary treatment of the parental (P1) generation should begin after the onset of egg-laying to 1) allow the option of using pre-treatment measurements as covariates (internal controls) and 2) remove incompatible or infertile pairs before treatment to reduce non-treatment sources of variation and increase the power to statistically evaluate test parameters (i.e., increase the ability to detect treatment effects if they exist). Other experts believe that exposure should begin prior to sexual maturation to detect effects resulting from delayed or inhibited gonadal development and/or changes in the onset of laying of the P1 generation.

Debate over the exposure regimen also extends to the F1 generation, with some member country experts proposing that the F1 generation also receive dietary treatment of the test substance, while others argue that the F1 generation should not be exposed to the test chemical. Arguments in favor of exposing the F1 generation to the test substance during all critical life stages include the ability to account for endocrine–mediated effects that occur during growth and development of the F1 chicks and to represent a worst-case exposure scenario. Not treating the F1 generation focuses the test on the effects of *in ovo* exposure of the developing embryo (e.g., gonadal abnormalities, altered sex ratio) and the subsequent reproductive success of the F1 generation without the potentially confounding influence of direct toxicity of the test substance to the chicks and the sexually maturing juveniles. However, the response of the F2 generation may provide needed *in ovo* effects data if the F1 exposure regimen is used.

Therefore, a study to evaluate the appropriateness of the exposure scenarios of the P1 and F1 generations is essential to the development of a test guideline that assesses the impact of chemicals on the reproduction and development of birds over two generations.

This study describes the results of a study of 17ß–estradiol administered by dosed feed in Japanese quail (*Coturnix japonica*) under two P1 exposure scenarios (prior to and post maturation) and two F1 exposure strategies (from hatch through egg laying and no additional exposure above *in ovo*).

Section 2.0 presents the objectives and Section 3.0 presents the materials and methods used in this study. Section 4.0 presents the results from the parental (P1) generation, while Sections 5.0 and 6.0 present the results from the first-generation offspring (F1) quail and the second-generation offspring (F2), respectively. Subsections within Sections 4.0 through 6.0 describe various endpoints evaluated. Subsection 4.13 presents a discussion of feed analysis and phytoestrogen analysis results, which apply to both the P1 and F1 quail. Section 7.0 provides a discussion of the results, Section 8.0 provides conclusions, and Section 9.0 presents

recommendations. Section 10 is a list of references. Appendices in a separate volume provide supporting data.

In this report, a "generation" denotes hatchlings through adults. Eggs laid by a generation (P1 or F1) are discussed with their parental generation (e.g., P1 eggs are eggs laid by the P1 generation). Abbreviations for various generations, treatment groups, and compound concentrations, as defined in the methods section, are sometimes used to label graphs and tables.

2.0 OBJECTIVES

This study addresses the need for experimental data regarding the relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects over two generations[1] and whether the F1 generation should receive dietary treatment of the test substance.

The specific objectives of the study were to

1. Compare dose-response relationships of endocrine and fitness endpoints between the two P1 exposure scenarios to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Emphasis was placed on comparing the relationships on the basis of slope, relative sensitivity and relative variability of the endpoints, and determining endocrine-mediated effects that may not be observed by initiating treatment after the onset of egg laying.

2. Compare dose-response relationships of endocrine and fitness endpoints between the two exposure scenarios for the F1 generation of each P1 exposure scenario and between all F1 exposure groups to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Time series data were used to assess the daily/weekly/etc. within-class variation in response, time lag between exposure and response, and appropriateness of the exposure duration.

This report documents the results of the Endocrine Disruptor Screening Program (EDSP) Avian Dosing Study under work assignments (WAs) 2-17 and 5-7. The Avian Dosing Study is described in the Avian Dosing Study Plan (Battelle, 2003a) and associated Quality Assurance Project Plan (Battelle, 2003b).

[1] Please note that on page 2 of the Work Assignment Statement of Work the exposure options for the P1 generation are "initiated at sexual maturation or after proven egg-laying ability." Because sexual maturation is often determined by the onset of egg laying, it is assumed that "prior to or during maturation" was intended and conforms to the reference to "pre-breeding" dosing and the exposure scenarios discussed in the OECD documents.

3.0 MATERIALS AND METHODS

3.1 Study Design

The exposure options were evaluated in an experimental design that compared two P1 exposure scenarios (P1A receiving treated diet prior to sexual maturation and P1B receiving treatment after proven egg-laying ability has been established) and two F1 exposure options for each of the two P1 scenarios (Figure 3-1). F1 groups were comprised of two cohorts of chicks that survived *in ovo* exposure from the P1 parents. F1a chicks were exposed to treated diet from hatch through egg laying, while F1b birds received no dietary treatment.

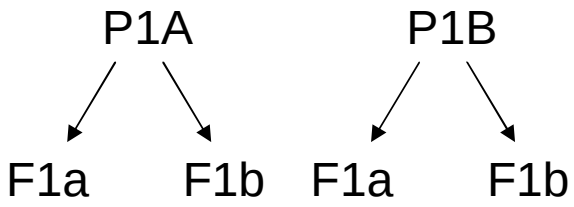

Figure 3-1. Exposure design. The F1 populations are obtained from the last week of eggs collected from the P1 birds.

The dietary route of administration was selected because it represents the most likely route of exposure to avian species in the environment.

In each P1 and F1 population, birds were assigned randomly to one of a geometrically spaced series of four dietary concentrations of the test substance. A concurrent control group was used for each of the two P1 test populations and each the two F1a populations. F1a control groups served as controls for both F1a and F1b populations. A shared control for these two populations was used to reduce the number of animals used and to defray cost. Offspring of the F1 birds (F2 chicks) did not receive dietary treatment.

Birds in the P1A population were separated, randomly assigned to treatment groups, and started on dietary treatment at approximately 3 weeks of age. The P1A birds continued on treatment through maturation and 10 weeks of egg laying. Males and females were housed separately until the fifth week of egg production, at which time they were paired together. P1B birds were also separated and randomly assigned to treatment groups at about 3 weeks of age, but without dietary treatment until they were paired. Only proven breeders (females laying at least 3 eggs per week by the fifth week post onset of egg production) were used in the P1B test groups. Treatment of the P1B birds lasted 5 weeks such that treatment ended at the same time as for the P1A birds. The F1 breeding populations were established from the last collection of P1 eggs. A subset of eggs (4 eggs per pen) from each of the treatment groups and the control group from the P1 test populations were collected from eggs in the last week of egg laying; however, due to a low number of eggs produced in some treatment groups, all eggs not designated for egg quality or steroid analysis were collected to form the F1 breeding populations. The eggs and hatchlings were marked to identify parental origin. At 3 weeks of age, chicks from each group were paired so that F1 breeding pairs were formed from non-siblings of their associated P1 parents. From

each P1 group, two F1 groups of eight pairs each were formed with equitable representation and assigned to one of the two F1 exposure regimens.

The experimental treatments are shown in Table 3-1.

Table 3-1. Treatment groups for the exposure comparison study. Test Concentrations were geometrically distributed from the high concentration using a factor of 0.25.

Onset of Exposure	Pens per P1 or F1 Group (1 cock and 1 hen per pen)	17̣-Estradiol Exposure Concentration (ppm)			
		adults	F1a	F1b	F2
P1A (pre-breeding; 3 wks old)	8	0[2]	0[2]	-----[3]	0
	8	1x	1x	0	0
	8	0.25x	0.25x	0	0
	8	0.063x	0.063x	0	0
	8	0.016x	0.016x	0	0
P1B (adult; proven layers)	8[1]	0	0	-----[3]	0
	8	1x	1x	0	0
	8	0.25x	0.25x	0	0
	8	0.063x	0.063x	0	0
	8	0.016x	0.016x	0	0

[1] 10 or more pairs were established in each group initially to provide for at least 8 breeding pairs during treatment.
[2] Control birds were from the same hatch as the test groups and were kept under the same experimental conditions as the test birds.
[3] No additional control group was used. The F1a control groups served as controls for both F1a and F1b populations.

3.2 Test Material and Exposure Regime

The following criteria for selecting a suitable test substance for comparing exposure regimens were applied to candidate compounds obtained from sources described in Appendix A:

1. The test substance should have the potential to affect the maturation of parents in such a way to determine what endocrine-mediated effects may be missed by starting treatment during the egg laying period (P1) or by not treating the F1 chicks.

2. The test substance should give rise to intergenerational effects so that the impact on reproductive/endocrine endpoints in the F1 generations of the two P1 exposure regimes can be compared. This also provides for a comparison of the reproductive performance of untreated F1 and treated F1 birds and the survivability of their offspring.

3. The test compound must clearly act on a hormone system (not simply alter a process that is under normal endocrine control).

4. There should be sufficient knowledge of the effects and/or mode of action of the test substance that appropriate, sensitive endpoints can be selected.

Candidate compounds reviewed for this study were evaluated relative to the above criteria and separated according to their potential to exert confounding, non endocrine-mediated effects in the F1 generation. In general, those compounds that have no or minimal maternal transfer to the egg were considered to be less useful for the study. Other compounds also were considered less useful if they appeared to affect pathways that could result in confusing results (e.g., TCDD is both anti-androgenic and anti-estrogenic in northern bobwhite quail, *Colinus virginianus*, depending upon dose and tissue; McMurry and Dickerson, 2001). Organochlorine compounds were not rated highly for selection because relatively great concentrations of these compounds are required to elicit effects (Feyk and Giesy, 1998). Too little work with anti-estrogenic, anti-androgenic, or thyroidogenic compounds in birds has been conducted to identify a useful test substance from these classes of endocrine disruptors.

The majority of the environmental chemicals identified as endocrine-active compounds are estrogenic, and existing data show that administration of endogenous estrogen in young birds, in adults, and *in ovo* causes clear changes in reproduction, sexual behavior, and sexual differentiation (Yoshimura et al., 2000). Furthermore, maternal transfer of estradiol to egg yolks in hens injected or implanted with the hormone has been demonstrated and resulted in changes in sexual differentiation of the offspring (Adkins-Regan et al., 1995). Estrogen has also been implicated in causing eggshell thinning and changes in reproductive behavior (Enstrom et al., 1997; Brewer et al. 2002a, b).

Accordingly, 17ß-estradiol (1,3,5[10]-estratriene-3,17 ß-diol; CAS Number: 50-28-2), was selected for use in the avian dosing study because it satisfied the general selection criteria (obvious action on a hormonal system, ability to affect maturation, documented transfer from hen to egg, and induction of intergenerational effects) for the dosing study and is applicable to evaluating the F1 exposure regimen in the absence of confounding toxicity. It was used specifically to evaluate the appropriateness of pre-breeding vs. proven breeder exposure regimens.

Because the available literature was insufficient to establish appropriate test concentrations, the dietary test concentrations were determined from a range-finding trial (Battelle, 2003c)**.** The range-finding trial was designed by EPA and consisted of three treatment levels with no controls. Each treatment level exposed three reproducing pairs of Japanese quail to dosed feed (six birds in three treatment levels or 18 birds total). The treatment levels were 1 ppm, 10 ppm, and 100 ppm 17ß-estradiol in feed. Adult birds were evaluated through 14 days of egg-laying for adult survival, body weight, egg production, and embryo viability at Day 8 of incubation. Eggs from the second week of egg laying were incubated until hatching and the hatchlings evaluated at 3 days of age for normality. Selection of the maximum concentration for this dosing study was based on the range finding study's criteria that the highest concentration be less than the lowest concentration manifesting effects (i.e., 10 ppm) and the next lower concentration (i.e. 1 ppm). The target concentrations determined from the range-finding test in consultation with representatives of EPA are summarized in Table 3-2.

Table 3-2. Target chemical concentrations.

Onset of Exposure	17β-Estradiol Exposure Concentration (ppm) [1]			
	adults	F1a	F1b	F2
P1A (pre-breeding; 3 wks old)	0	0	-----[2]	0
	0.078	0.078	0	0
	0.31	0.31	0	0
	1.25	1.25	0	0
	5	5	0	0
P1B (adult; proven layers)	0	0	-----[2]	0
	0.078	0.078	0	0
	0.31	0.31	0	0
	1.25	1.25	0	0
	5	5	0	0

[1] mg 17β-Estradiol per Kg feed
[2] No additional control group is used. The F1a control groups served as controls for both F1a and F1b populations.

3.3 Preparation and Sampling of Test Diets

The test substance, 17ß-estradiol (Sigma-Aldrich Corp. St. Louis, Missouri) was obtained from the Battelle EDSP chemical repository. Prior to conducting the dietary exposures, the purity of the test substance was determined and the stability of the compound in the commercial feed was evaluated under storage and animal room conditions. Stability of the test substance in feed was limited to 28 days refrigerated. However, the estradiol degraded more rapidly under the warmer conditions of the animal rooms. Test diet concentrations fell below test limits after 5 days in sealed feed bins and were stable for 4 days (using 20% loss) in the open feeders. Purity of the stock 17ß-estradiol was determined to be 96.17% by using High Performance Liquid Chromatography (HPLC). A summary of the purity and stability results is located in Appendix B.

Test diets were prepared by dissolving 17ß-estradiol in acetone (≥99.5%, Appendix C) and mixing the solution into the feed using a Gilson Model 59017 drum utility mixer (Figure 3-2) following procedures in MSL-T-061-00, *Test Diet Preparation*. Because of the flammability of acetone, the mixer was oriented so that the drum lid faced a fume hood. All ignition sources were removed from the food-preparation laboratory and the mixer was disconnected from the power supply. A weighed amount of feed was placed in the drum and the lid secured. Nitrogen gas (N_2) was introduced into the drum through one of three openings in the lid at a rate of 2 CFM for a period of 3 minutes to reduce the oxygen concentration within the drum to below 7% (operational safety limit). Once the drum was purged of oxygen, the nitrogen gas line was removed and the N_2 and purge openings were sealed to prevent gas loss. The estradiol-acetone solution was added through a third opening in the lid directly onto the feed using a glass funnel.

The feed was then mixed so that no dry feed was observed but minimal fines (powder from broken-down feed) were produced.

Each dietary concentration was mixed separately in a dedicated drum liner made of high density polyethylene (HDPE). Acetone was evaporated from the feed prior to use in the test on stainless steel evaporation trays lined with aluminum foil (Figure 3-2). Control diet was prepared in a similar manner, but without addition of the test substance. Acetone was added to the control diet in the highest concentration used for the test diets. To minimize cross-contamination of the test diets, the dietary treatments were mixed in increasing concentration from low to high concentration. A single diet concentration was evaporated at a time in a fume hood. Dedicated mixing vessels and evaporation trays were used for each dietary concentration. Foil liners were discarded and all mixing and evaporation equipment was cleaned between each dietary concentration. Uncontaminated feed was mixed and discarded, and a second batch made following cleaning of the equipment to assure no transfer of estrogen to the subsequent diet preparation.

Figure 3-2. Mixing and evaporation apparatus used in preparing the test diets.

Three composite samples of feed (~30 grams of feed from 5 different areas within the storage bin) were taken from each newly formulated batch of test diet on the day that it was prepared. Each composite sample was placed in a separate acetone-rinsed glass jar with aluminum foil lid liner and rotated at least 10 times to mix the contents. A second set of triplicate composite samples for each test diet were collected and stored as backup samples. To confirm that the dietary concentrations of the test diets were maintained in the feeders over the feeding periods, about 30 grams of feed from the center of three feeders in each test group were removed and placed in separate acetone-rinsed jars at the end of the first feeding period of the test (before the diet in the feeders was renewed) and again at the end of the last feeding period of the study for each feed type (Layena® and Startena®). Samples from three individual feeders, rather than a single composite sample, were collected to aid in detecting cross-contamination from neighboring pens. The samples were shipped on ice to the EDSP chemical repository

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(MSL-T-054-01, *Diet Sampling*). The chemicals were logged in for analysis following the procedures for sample receipt, handling, and storage (MSL-A-001, *Sample Log-In Procedure*, and MSL-A-002, *Sample Chain-of-Custody*). The samples were stored under appropriate conditions until analyzed. A copy of the chain-of-custody form accompanied all samples. A total of 19 batches of each test diet were mixed and sampled. The formulation dates and analysis schedule are reported in Appendix D.

3.4 Analysis and Recovery of 17ß-Estradiol in Feed Samples

Aliquots of the test diet samples were spiked with a surrogate internal standard (ethynyl-estradiol, EE2) and extracted with acetone and anhydrous sodium sulfate for 1 hour using a mechanical shaker. After centrifugation, an aliquot of the supernatant was filtered, concentrated to dryness with N_2 or He, spiked with a second surrogate internal standard (deuterated estradiol, DE2) and derivatized using N–methyl-N(trimethylsilyl) trifluoroacetamide:sublimed iodine (MSTFA/I_2; 1000:4 v/wt) at 60ºC $\pm$ 2ºC for 30 minutes. A recovery internal standard (phenanthrene-d_{10}) was then added and the sample analyzed by gas chromatography with mass detection (GC/MS). The calculated method detection limited (MDL) was 36.7 ng/g. Recoveries of 17ß-estradiol from newly formulated test diets, test diet concentrations collected from feeders at the end of feeding periods, and cross-contamination results are reported in Appendix D. Calibration data and specific analytic conditions can also be found in Appendix D and in MSL-O-018-00, *Estradiol Determination Using Gas Chromatography with Mass Detection*.

3.5 Sampling and Analysis of Natural Endocrine-Active Compounds in Basal Diet

Because of the potential influence of natural endocrine-active compounds in the basal diet on the response of the birds to the test substance, the level of endocrine-active phytoestrogens and mycotoxins in each lot of the basal diet was documented.

Samples for these contaminants were obtained from randomly selected 50 lb sacks of the commercial feed. Representative samples from each bag of a specific feed lot were collected using an open-handled slotted grain probe (Seedburo Equipment Co., Chicago, Illinois) (Figure 3-3). The probe was inserted into a bag diagonally from end to end and the slots opened only after the probe was fully inserted. The samples were composited in a large brown paper bag, gently mixed and then passed through a Boerner Divider (Seedburo Equipment Co., Chicago, Illinois). Two of the reduced samples (~500 g) were retained, one for contaminant analysis and one as an archived sample. Samples were stored at room temperature in brown paper bags in a metal cabinet and did not come in contact with plastics. Samples for analysis were sent in cardboard containers at ambient temperature with no plastics to Veterinary Medicine Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri for analysis. Each sample was analyzed for three primary phytoestrogens commonly found in feed products (genistein, daidzein, and glycitein) and the estrogenic mycotoxin zearalenone. (Because zearlenone was part of a standard mycotoxin screen, data on three other toxic

Figure 3-3. Sampling feed bag with grain probe. A Boerner Divider is in the foreground.

mycotoxins were also obtained: aflatoxin B_1, vomitoxin, and ochratoxin A.) The phytoestrogens were measured by HPLC. Mycotoxin content was determined by thin layer chromatography. A highly sensitive bioassay of total estrogenic activity by estrogen-dependent cell proliferation (Welshons et al. 1990) was also conducted on the first Layena® and Startena® lots. Values for the bioassay method are expressed in estradiol ng equivalents per gram of feed or zearalenone ppm equivalents. Confirmation of estrogenic mechanism of activity when present in the bioassay was made by suppression of activity by co-incubation of active sample with anti-estrogen ICI 182780 at 100 nM.

Details on the assay conditions, calibration, and data quality are in provided in Appendix E.

3.6 Test Animals and Husbandry

The species tested was the Japanese quail (*Coturnix japonica*). The P1 generation was raised from eggs obtained from a commercial *Coturnix* quail hatchery (Northwest Gamebirds, Kennewick, Washington) and delivered to the animal care facility at Battelle Northwest Laboratory. The exterior of the sealed paper carton in which the eggs were transported was disinfected with Clidox-S Base (Pharmacal Research Laboratories, Naugatuck, Connecticut) at the door of the barrier facility prior to crossing the barrier. Eggs were removed from the carton at the door of the animal room and transferred into clean egg trays in the room. F1 breeders were obtained from the eggs produced during the last batch of eggs laid by the P1 birds.

Eggs were incubated in a NatureForm model NMC-1620 incubator (NatureForm Hatchery Systems, Jacksonville, Florida) at 37.5ºC ± 0.3ºC with an average relative humidity of approximately 60%. The incubator was equipped with a fan that produces a mild breathing air movement that is designed to eliminate intra-cabinet temperature and humidity variation during incubation. The eggs were automatically rotated from 50º off of vertical in one direction to 50º off of vertical in the opposite direction (total arc of rotation of 100º) every 2 hours. On the 15th day of incubation, the eggs were transferred to a Natureform Model NMC-1620 hatcher in which they were housed in pedigree baskets to assure identification of parentage and incubated without rotation at 37ºC ± 0.3ºC and 70% relative humidity until hatched.

Hatchlings were housed in galvanized gamebird brooding pens (Georgia Quail Farm, Savannah, Georgia) by treatment group. Thermostats in the brooding compartment of each pen were set to maintain a temperature of approximately 38°C. Brooder temperature was lowered daily over 3 weeks until room temperature (23ºC) was reached. Brooding behavior of the hatchlings was monitored in each pen to assure that appropriate temperature adjustments were being made daily. The brooders provided a graded temperature across the length of the brooder so that the chicks could move to warmer or cooler areas of the pen as needed. Feeders and waterers were placed at the cool end of the brooder to encourage exercise and toys were placed on the floor to promote exploratory behavior and minimize aggressive pecking.

At 3 weeks of age the birds were placed in individual adult pens custom constructed of stainless steel with sloping floors, individual pen feeders (Apollo Sheet Metal, Kennewick, Washington), and automatic quail cup valve waterers (Edstrom Industries, Inc., Waterford, Wisconsin). Feeders were fitted with a see-through guard plate of Plexiglas® to retard food loss and cross contamination from "billing-out" activity of the birds. Red autoclaveable tags (Secure-Pull®) typically used for surgical instrument tracking were attached to several areas within each pen to provide enrichment and aid in displacement for aggressive behavior. Birds were also provided with dust baths (sterilized sand) 3 times per week to maintain their feather quality and provide them feather grooming activity. Because the 3-week-old birds initially had difficulty discovering the automatic waterers, the actuating lever of each waterer was colored red with a Sharpie pen to attract pecking.

The photoperiod in the rooms housing both the adults and hatchlings was maintained by time clocks. The photoperiod for adults, chicks, and juveniles (>3 weeks of age) was 16 hours of light per day throughout the test. Birds received about 6 lux of illumination at the level of the bird. Light was provided by fluorescent lights that emit a spectrum simulating that of daylight. Quail can become stressed with sudden-start light periods; therefore, lighting in the room was gradually increased to full power over a 1 to 1.5 h period and gradually dimmed over a 1 to 1.5 h period after 14 to 15 h of continuous light.

Control and test birds were kept under the same environmental conditions. The quail were acclimated to the test facilities and an untreated diet until test initiation. Acclimation typically occurred in brooding pens. Birds were weighed and randomly assigned to treatment and control pens. At test initiation, all birds were in good health and free of abnormalities or injuries that might affect test results. However, at pairing of the P1 populations (pairing was initiated

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5 weeks after onset of egg laying), about 30% of the females were so aggressive that the males had to be removed to separate cages. Some males were injured or killed by the female. Where possible, the male was replaced. The EPA Project Officer and Work Assignment Manager were consulted, and all the pairs were separated so that there was equivalent handling throughout all the treatment groups. Males were placed back into the female pens until copulation was verified (about 30 minutes) 3 times per week. Males were removed sooner if aggression by the female was significant. Daily observations and health records were maintained from hatch until test termination.

To avoid pairing siblings within control and treatment groups of the F1 breeder populations, birds were randomly assigned to pens by pairs with males from different parental pens than that of the female. The sex of the birds was determined by a visual examination of the plumage. If birds in a pen were incompatible, they were replaced or rearranged within a control or treatment group.

All birds were identified by individual wing bands. Each pen was identified with a unique number. Groups of pens were identified by exposure type (e.g., established breeder, P1B, or during maturation, P1A) and concentration. Eggs were marked according to the pen from which they were collected, and stored at an average temperature of 10 to 16°C and an overall relative humidity of 40 to 95%. Eggs selected for steroid analysis were stored separately at -20ºC $\pm$ 4ºC. Weekly batches of eggs were assigned a unique batch number.

All birds and their offspring were given feed and water *ad libitum* during acclimation and testing. Basal diet used to prepare the treated and control diets of both adults and offspring was obtained from Purina Mills, Spokane, WA. Through Animal Specialties (Hubbard, Oregon). The hatchlings were raised on Purina® Game Bird Startena® with a minimum crude protein and fat content of 30.0% and 2.5%, respectively. Crude fiber was less than 6.5% and calcium was supplemented to between 1.0 and 1.5% of the diet by weight. The adult diet was Purina® Game Bird Layena® and contained at least 20.0% crude protein, 2.5% fat, 2.5% to 3.5% calcium, and no more than 7.0% crude fiber.

All birds received filtered tap water from the municipal water system. Neither the adults nor offspring received any form of medication in their feed or water prior to or during the test.

3.7 Duration of Test

The duration of the in-life portion of the test was 38 weeks, from initiation on 09/25/03 with the incubation of the eggs from which the P1 populations would be obtained to its conclusion on 06/17/04 with the sacrifice of the last batch of F2 chicks. Dietary exposure of the P1A birds began on 11/05/03; P1B exposures began on 12/29/03. The primary phases of the study and their durations are shown in Table 3-3.

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Table 3-3. Duration of in-life phases of the avian dosing study.

Generation	Phase of In-life	Duration	Dates
P1	Incubation of Eggs	~ 17 days	09/25/03 – 10/12/03 [a]
	Growth of chicks to adult type plumage	~ 25 days	10/11/03 – 11/05/03
P1A	Pre-egg laying exposure	~ 11 to 33 days	11/05/03 to (11/16/03 – 12/08/03[b])
	Egg-laying exposure	~ 50-72 days	(11/16/03 – 12/08/03) to 01/27/04
	Total in-life exposure	91 days	11/05/03 - 02/04/04
P1B	Pre-egg laying exposure	0	
	Egg-laying exposure	28 days	12/29/03 – 01/26/04
	Total in-life exposure	38 days	12/29/03 – 02/05/04
F1	Incubation of Eggs	~ 18 days	01/27/04 – 02/14/04 [a]
F1a	Total Exposure	95 days	02/13/04 - 05/18/04
F1b	Total Exposure	0	
F2	Incubation of 8 batches of eggs	69 days	03/25/04 – 06/02/04
	Growth of chicks to 2 weeks of age	65 days	04/13/04 – 6/17/04
Total	P1 Egg set to last F2 sacrifice	266 days[c]	09/25/03 – 06/17/04

[a] Mean day of hatch
[b] Dates of when first and last female began laying eggs
[c] The in-life phase of the Range Finding Study was initiated on April 25, 2003 and concluded on June 12, 2003. Total in-life days for the Range Finding Study and the Avian Dosing Study was 314.

3.8 Endpoints

3.8.1 Selection of Study Endpoints

Selection of the endpoints for this prevalidation study were based on information provided in four documents:

1) "Discussion Document of Pre-Validation of an Avian Two-Generation Toxicity Test with the Japanese Quail," R. Bennett, K. Brugger, A. Fairbrother, A. Leopold, N. Mastrota, and M.A. Ottinger, OECD Draft Document, March 2001.

2) Draft protocol developed by Dr. Mary Ann Ottinger (University of Maryland) entitled "(Test Substance): A Two-Generation Reproduction Study with the Japanese quail *(Coturnix coturnix japonica)*".

3) Proposal for a New Test Guideline, "Avian Two-generation Toxicity Test in the Japanese quail *(Coturnix coturnix japonica),*" OECD Guideline for Testing of Chemicals, First Draft, December 1999.

4) Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) Final Report. Office of Prevention, Pesticides, and Toxic Substances, U.S. EPA 1998.

Battelle also consulted with individuals that have served on the OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds for input on selection of endpoint measures. The expert group members contacted were Drs. Rick Bennet (EPA-ORD), Nick Mastrota (EPA-OPP), Anne Fairbrother (formerly of Parametrix, Inc. and currently with EPA-ORD), and Mary Ann Ottinger. Dr. Ottinger also provided information on refinements to the above-mentioned endpoints and protocols based on preliminary results from two-generation endocrine studies conducted at the University of Maryland.

The general consensus of the expert group was to include in the pre-validation most of the "fitness" endpoints (Table 3-4) described in the above documents and to apply a subset of "physiological or "endocrine" endpoints that identify endocrine-mediated effects during sexual maturation and egg production. Because the test substance is 17ß-estradiol, the selected endpoints emphasize measures with underlying estrogenic mechanisms and measures for feminization of males (Table 3-5). A detailed account of endpoint selection is summarized in the Avian Dosing Study Plan (Battelle, 2003a).

3.8.2 Summary of Fitness Endpoint Measures

Eggs Laid per Pair/Number of Cracked Eggs at Set: Daily record of egg production was kept for each breeding pair of birds. Eggs were collected daily and marked with a soft lead pencil or permanent ink according to the pen from which they were collected and the date of collection. The labeled eggs were stored as weekly batches at 10 to 16ºC. Each batch was labeled with a unique batch number. At the end of the weekly interval, all eggs were removed from cold storage and selected eggs were taken for eggshell quality measurements (one egg per pen per week). The remaining eggs were candled with a Lyon High Intensity egg-candling lamp (Lyon Electric Company, Chula Vista, California) to detect eggshell cracks or abnormal eggs. Cracked or abnormal eggs were recorded and discarded.

Eggshell Strength Measurements: Shell strength was measured on one egg per pen per week with a Chatillon® LF Plus Series Universal Material Tester (Ametek, Inc., Largo, Florida). The egg was placed on its side on the test stand so that the compression head contacted the egg at the equator between two parallel stainless steel surfaces advancing at a constant rate of 0.4 mm/min with a 50 Newton (N) maximum load range until the egg cracked. The load (±1%) to rupture (breaking force or strength) and maximum load prior to rupture were recorded in Newtons. Simultaneously, shell stiffness (the slope of the force-deformation curve during the compression tests) was recorded.

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Table 3-4. Fitness endpoints for exposure comparison study indicating toxicity (T) and endocrine activity (E) measured

Endpoint	Endocrine Activity	
	Estrogenic	Thyroidogenic
For Breeding birds (P1 and F1)		
Body weight at start and end of treatment		E
Food consumption during treatment		E
Survival	T	
Clinical Signs	T	
Number of eggs laid per pair	E	
Number of fertile eggs per eggs laid	E	
Number of cracked eggs (at set)	Potential E	Potential E
Number of eggs hatched per eggs set[1]	E	
Eggshell strength and thickness	Potential E	
Early and late viability per eggs set[2]		
For F1 and F2 Chicks		
Sex ratio of chicks[3]	E	
Number of chicks surviving 14 days per eggs set and per eggs hatched[4]		
Growth rate of chicks (weight at days 1, 14)[4]	E	E

[1] Only F1 eggs from the last week of egg-laying (week 8) were hatched. F2 eggs from all collection periods were hatched.
[2] Late viability was determined on all F2 eggs and the F1 eggs from last week of egg-laying.
[3] Last batch (batch 8) of F2 chicks only.
[4] F2 chicks and those F1 chicks hatched from the last week of egg collection.

Eggshell Thickness Measurements: Following the shell strength test, the same egg was prepared for shell thickness measurements. Each egg was cut open at the equator, the contents removed, and the empty shell rinsed with tap water. The shell was then allowed to air dry with the membrane intact for at least 48 hours at room temperature. The mean thickness of the dried shell, including membranes, was determined by measuring five points around the waist of the egg with a micrometer. Measurements were made to the nearest 0.002 mm.

Table 3-5. Endocrine or physiological endpoints for exposure comparison study indicating endocrine activity (E).

Endpoints	Estrogenic	Thyroidogenic	Androgenic
For Breeding Birds (P1 and F1)			
Gross morphology & histology			
Weight of testes, ovaries, thyroid, adrenals, oviduct, cloacal gland, liver	E	E	E
Histology of thyroid, adrenals, gonads, brain	E	E	E
Testicular spermatid counts and morphology	E (feminization)	E	
Gross anomalies of the genital tract	E		E
Developmental Landmarks			
Feather dimorphism	E	E	
Cloacal gland size, 1st appearance of foam			E
1st egg laid	E		
Sexual behavior[1]	E (feminization)		
Fecal/urate hormones			
Steroid hormones (estradiol, testosterone)[2]	E		E
Egg hormone content			
Steroid hormones (estradiol, testosterone)	E		E
For F2 Chicks			
Gross morphology & histology			
Size and dimorphism of gonads	E		E
Histology of gonads (relative amount of cortex and oocytes), thyroid, oviduct	E		E
Presence, weight and differentiation of oviduct	E		E
Thyroid, cloacal gland, liver, brain, pancreas	E	E	E
Wing or bone length		E	

[1] Insufficient number of F1males were produced to form cohort for behavioral tests; therefore the behavioral tests were not conducted.
[2] Samples were collected for steroid analysis from both P1 and F1 populations, only the P1 samples were analyzed for steroids.

Early and Late Embryo Viability: Eggs were candled on Day 8 of incubation to determine early embryonation (embryo viability, fertility). Eggs produced by P1 birds (with the exception of those used to form the F1 breeding populations) were discarded after the Day 8 candling. Eggs set for the F2 chicks and the F1a and F1b breeding populations were also candled on Day 15 (late viability) and the number viable and not viable were recorded per pair.

Hatching Success (number of eggs hatched per eggs set): On Day 15, viable eggs were placed in pedigree baskets and transferred to a hatching incubator where they were allowed to hatch. Eggs that did not hatch within about 24 hours of the majority of chicks were considered unhatched. The number of hatched and unhatched eggs were recorded per pair.

Survivability of Hatchlings: F1 offspring (F2) were observed over a 14-day period beginning when birds were first removed from the incubator. The number surviving to 14 days were recorded per pen. Survivability of chicks hatched for F1a and F1b populations were also recorded. Insufficient offspring were available to form a cohort for behavioral tests.

Body Weight of Chicks (growth rate of chicks): The mean weight of all surviving offspring was determined both at hatch and at 14 days of age. Mean weights were determined from individual body weight measurements and was determined for all offspring originating from a given parental pen during a specific week of egg laying.

Genetic Sex Ratio: Uniquely labeled sample collection and transport cards ("PermaCode," Avian Biotech, Tallahassee, Florida) or Whatman filters (Whatman Inc., Florham Park, NJ) were used to collect blood samples from 14 day old chicks from the last batch of eggs laid by F1 parents. Blood samples were obtained from the chicks by claw clip. The collection cards or filters were air dried and sent to Avian Biotech International Laboratories (Tallahassee, Florida) for analysis. A chain of custody accompanied all samples. When samples arrived at the testing laboratory, each sample was inspected and given an additional unique tracking number. The genetic sex data were compared to gender data acquired from necropsy.

Deoxyribonucleic acid (DNA) was prepared for polymerase chain reaction (PCR) analysis using Chelex 100 chelating resin developed for extracting DNA from forensic-type samples for use with the PCR (Walsh et al. 1991, FBI manual). PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems, Inc., Foster City, California). Specific control samples were used to confirm the results for each PCR run using pre-run verified male and female samples of Japanese quail. Blood samples of known male and female Japanese quail from Battelle were sent blind to the testing laboratory prior to submission of the chick samples to verify the laboratory's ability to identify the sex of this species. Final results including controls were compared to identify intensity and variations in band patterns. Abnormal samples were excluded from the results and were reprocessed. The laboratory used three different ABI models. A Kodak Gel Logic 200 (Kodak, Rochester, N.Y) was used for gel documentation. Sample Data Entry, DNA preparation, PCR preparation, PCR, electrophoresis, and gel documentation were conducted in separate rooms to limit contamination.

Clinical Observations: All adults and offspring were observed daily throughout the test for overt signs of toxicity or abnormal clinical observations. A record of all mortalities and observations was kept.

Adult Body Weight: Individual body weights of the adults were measured at start and end of treatment and weekly prior to egg laying. Body weights were not measured after egg laying was established due to possible adverse effects that handling may have on egg production.

Feed Consumption: Feed consumption for each pen was measured at least every 4 days and at test termination. Feed consumption was determined by weighing the freshly filled feeder on Day 0, recording the amount of any additional diet added during the feeding period, and weighing the feeder and remaining feed at the end of the 4-day feeding period. Estimates of feed consumption were conservative due to unavoidable wastage of feed by birds that could not be accounted for.

3.8.3 Summary of Endocrine Endpoint Measures

Gross Necropsy and Organ Weights: All adult test birds that died during the course of the test and all adults remaining at the termination of the adult portion of the test were subjected to a gross necropsy. The necropsy included an examination of the overall condition of the birds, as well as any external or internal observations. The examination included, but was not limited to, gross observations of the liver, gonads, and general condition of the organs. Gonads, oviduct, thyroid, adrenal glands, liver, brain, and cloacal gland were excised and their weight recorded. All lesions were recorded.

Eight batches of F2 chicks were raised. Because of the reduced number of F2 chicks in treatment groups of 0.31 ppm estradiol or greater and in consultation with a representative from EPA, necropsies were conducted on all chicks in all groups for Batches 1 through 6. Chicks from batch 7 were weighed but not necropsied. Because of their large number, necropsies were conducted on a subset of the F2 chicks from the control and 0.078 ppm estradiol groups and all chicks from the remaining treatment groups. The gonads, oviduct, cloacal gland, and thyroid of necropsied birds from Week 8 eggs were examined histologically. A leg was removed from each chick and the tibiotarsus removed, weighed and the length and width measured in F2 chicks.

Organ weights were normalized by body weight (organ weight/body weight) and by brain weight (organ weight/brain weight). The testis weight asymmetry (left testis weight/right testis weight) was also calculated.

Histology: Tissues were excised at Battelle and preserved in fixative. Ovaries were preserved in paraformaldehyde and glutarldehyde fixative and testes of the F1 and F2 males were immersed in Bouin's solution. All other tissues were placed in 10% buffered formalin and shipped to Experimental Pathology Laboratories, Inc. (EPL®). The samples were sent in accordance with Battelle chain-of-custody procedures. At EPL, tissues were trimmed, processed, embedded in paraffin, microtomed, placed on glass microscope slides, and stained with hematoxylin and eosin. Testicular injury was evaluated in a stage-aware manner (Creasy 1997) using characterized stages of seminiferous tubules for the *coturnix* described by Lin et al. (1990) and

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Lin and Jones (1993). Routine histological procedures were used to assess the condition of the remaining tissues. The following tissues were evaluated histologically:

1) The thyroid, adrenal, liver, brain, pineal gland, and pituitary of adult male and female quail from the control and 5 ppm groups in the P1 and F1 generations

2) Cloacal gland, epididymis, and testes of all male birds in the P1, F1 and F2 generations

3) The ovary and oviduct of all female birds in the P1, F1 and F2 generations

4) Thyroid of both male and female chicks from the control and 5 ppm groups in the F2 generation.

Microscopic findings for each animal were graded on a scale of 1 to 5 depending on severity. For ovarian tissue, an increase in follicles was defined as an increase of small follicles over baseline numbers when the mature follicles adjacent to the ovary were also enlarged. An increase over baseline in the numbers of follicles undergoing degeneration was used to define a condition of degenerating follicles.

Sexual Maturation: Sexual maturation of males was determined by the protrusion and secretion of foam from the cloacal gland. Cloacal gland measurements were taken weekly. Female maturation was recorded as the day the first egg was laid. The number of follicles in rapid development (>4 mm in diameter and yellow in color) was determined at necropsy.

Feather Color and Pattern: Appearance of feather dimorphism (color, appearance of spots) and length of spotted plumage (female-type) was recorded at necropsy. Gender was confirmed at necropsy and recorded.

Steroid Content of Fecal-Urate Samples: Fecal-urate matter (0.2 to 2 g) was collected from the drop pans under each of the breeder cages at termination of the P1 birds. Samples were collected to avoid contamination by feed and adjoining cage occupants. Samples were stored at approximately -20 ºC until shipped on ice to the EDSP chemical respository. The samples were logged in for analysis following the procedures for sample receipt, handling, and storage (MSL-A-001, *Sample Log-In Procedure*; and MSL-A-002, *Sample Chain-of-Custody*). The samples were freeze dried and stored at approximately -20 ºC until processed. A copy of the chain-of-custody form accompanied all samples. The samples were prepared for analysis of estrogen conjugates as described by Kahn et al. (2002). The fecal-urate samples were dried, ground into a powder, sifted through a # 40 sieve, and the steroids extracted from the sample in a solvent containing 90% methanol and 10% water. The samples were mixed for 30 minutes using a vortex mixer to dissolve the steroids. Steroids were separated from the solid matrix by centrifugation. The supernatant solution was assayed for estrogen and testosterone by competitive-binding enzyme immunoassays (EIA) (Munro et al. 1991) using production-quality assay kits purchased from Cayman Chemical Company (Ann Arbor, Michigan). The assays were carried out on a Bio-Tek Synergy HT multi-detection microplate reader interfaced to a Dell computer, employing the Bio-Tek KC4 assay analysis software.

Steroid Content of Eggs: A subset (1 egg per pen per week) of eggs was collected during the 7th and 10th weeks of egg laying of the P1 birds. One egg per pen per week was collected during F1 egg laying and those collected during week 5 of egg laying were sent for steroid analyses. Eggs from each group were combined into composites for the analyses. The compositing scheme is reported in Appendix F. Yolks were separated from albumin by differential thawing and sections of the yolks were homogenized in H_2O at a ratio of yolk:H_2O of 1:2. Free steroids were extracted from the homogenates and purified using a three-step method. The steroids were first extracted using a solvent of 30% petroleum ether 70% diethyl ether. The ether was evaporated and the primary extract was dissolved/suspended in a solution of 90% ethanol:10% H_2O. This ethanol solution precipitated the majority of the lipids and proteins while leaving the steroids in solution. The ethanol solvent of this secondary extract was evaporated and the steroids dissolved in a solvent of 10% ethyl acetate in isooctane. In the final step, the steroids were purified using a Celite (diatomaceous earth) chromatography column. Extraction methods are described in detail by Schwabl (1993) and Lipar et al. (1999). The extracts were analyzed for steroid content using Enzyme Immunological Assay kits for testosterone and estradiol as described in the fecal-urate section.

Male Sexual Behavior: Because of the reduced hatch due to aggression and treatment effects, there were insufficient numbers of males in the groups to form a separate cohort for the behavioral test.

3.9 Statistical Analyses

The overall objective of the statistical analysis was to determine which exposure scenario for the P1 generation birds (during maturation or after proven breeding ability is established) and F1 birds (exposure from hatch or no additional exposure above *in ovo* exposure) were more biologically sensitive to chemically induced reproductive/endocrine disrupting impacts to species fitness. The study design produced a time series of reproductive parameters for P1 adults under both exposure scenarios for each concentration; a dose-response curve for each generation; plus the pen mean responses for each concentration, exposure scenario, and generation. Thus, three statistical approaches were used:

- A regression against time for a given concentration, exposure scenario, and generation
- A regression against chemical concentration for a given exposure scenario and generation
- An analysis of variance (ANOVA) approach based on the mean pen responses between concentrations for a given exposure scenario and generation.

A generalized linear model was used to compare the main effects of exposure scenarios, treatment doses, and their interaction.

The time series produced by the P1 and F1 generation birds for a given concentration and exposure scenario allowed the evaluation of 1) a possible delay in response time; 2) the form of the time series response (i.e., linear, curvilinear, spline); and 3) the potential carry-over effect of the reproductive response to the F1 generation. The dose response for each generation and exposure scenario allowed the estimation of the slope and/or EC_{50} of the response. The

difference in the slopes between P1-F1 combined exposure scenarios allowed for the evaluation of a potential carry-over (*in ovo*) effect.

Appropriate data transformations were applied to maintain homogeneity of the within-class variances (i.e., data expressed as a ratio were arcsine-square root transformed, counts were square root transformed, and continuous data were transformed to the natural logarithm). Nonparametric statistics were used when the data transformation was not successful in controlling heterogeneity.

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles (Q1, median, and Q3), were used to characterize each endpoint measured in the study. All summary data reported in the results section tables were based on more significant figures than are shown in the summary tables. Hand calculations of means and coefficients of variance may not yield the exact results shown. Statistical significance for each endpoint was evaluated based on the difference in the mean characteristics between the treated and control groups using analysis of variance, Tukey's multiple comparisons test, and the nonparametric Kruskal-Wallis test (Snedecor and Cochran 1980). Box plots were used to visually characterize the effect of each treatment (Minitab 2000). A boxplot portrays the data as a rectangle bounded by Q1 and Q3 and divided at the median value. A red dot was used to portray the mean value for a given group. Whiskers or lines above and below the rectangle indicate a nonparametric distributional upper and lower limit based on the quartiles. Extreme values are represented as an asterisk and are values outside the whisker limits defined as Q1 – 1.5 (Q3-Q1) and Q3 + 1.5 (Q3-Q1). Biological and statistical outliers would have biological reasons for exclusion from data analysis and were defined as values outside Q1 – 3 (Q3-Q1) and Q3 + 3 (Q3-Q1). The symbol "o" was used to portray these outliers if they should exist.

For purposes of statistical testing, an alpha less than 0.05 was considered statistically significant. An alpha less than 0.15 was also reported because the sample sizes may not have been adequate to detect a difference at the 0.05 level. Statistical power is the probability of rejecting the null hypothesis of equal means when the alternative is true—that is, detecting a difference when there is a difference. Statistical power is a function of the variability among replicate experimental units within a treatment, the number of replicate experimental units, the size of the Type I error, and the percentage difference one wishes to detect. One can control the latter three components; however, the variability in response is inherent in the test organism. Thus, the relevant endpoints to measure should include a comparison of inherent variability or coefficients of variation (CVs), defined as standard deviation/mean x 100%. High CVs have low power for detecting small-scale differences.

The power assuming a Type I error rate of α[alpha] = 0.05 was calculated to allow comparison of the sensitivity of selected endpoints. Power is the probability that a significant response will be detected at $\alpha = 0.05$ when a true difference of δ[delta]% exists. The achieved power, given the observed maximum difference between treatment means and the control, was calculated. Further, the achieved power, given a 10%, 20%, and 50% difference from the control mean, was calculated to evaluate the sensitivity of the endpoint. When a significant difference was not achieved between potentially biologically important differences in means, and the power was low (<50%), then there should be a concern that the null hypothesis was falsely concluded. In

contrast, when the power was high (>80%) and a significant difference was not achieved, then it can be more certain that the null hypothesis was not falsely concluded.

Statistical analysis was performed on each of the following parameters for the P1, F1, and/or F2 generations identified in Tables 3-4 and 3-5:

1. Adult Body Weight. Individual body weight was measured during testing and at adult termination. Statistical comparisons were made by sex between the control group and each treatment group to evaluate growth and termination weight using dose response and Tukey's multiple comparisons. All birds that were randomized into the design, treated, and had a minimum of three observations were used to calculate individual growth rates within linear segments of growth.

2. Adult Feed Consumption. Feed consumption expressed as grams of feed per bird per day was examined by pen at weekly intervals during the test. Statistical comparisons were made between the control group and each treatment group to evaluate the slope, average, and coefficient of variation of food consumption across time using dose response and Tukey's multiple comparisons. All birds that were randomized into the design, treated, and had a minimum of three observations for estimation of rate of consumption were included in the analysis.

3. Eggs Laid of Maximum Laid (%). This variable was defined as the number of eggs laid per hen divided by the largest number of eggs laid by any one hen. This transformation was used to convert the number of eggs laid to a ratio value less than or equal to 1. The value was correlated with the total eggs laid per pen. Statistical analysis of egg production included evaluating the slope of egg production across time and the ratio of the total to maximum laid using dose response and ANOVA comparing the overall mean pen responses. All eggs from birds that were randomized into the design, treated, and measured were analyzed.

4. Eggs Cracked of Eggs Laid (%). This variable was defined as the number of cracked eggs (determined by candling) divided by the number of eggs laid per pen. Statistical analysis of the percentage of eggs cracked included the evaluation of the slope of the proportion cracked across time and the total number of eggs cracked divided by the number laid using dose response and ANOVA comparing the treatment mean responses. All eggs from birds that were randomized into the design, treated, and measured were analyzed.

5. Viable Embryos of Eggs Incubated (%). This variable was defined as the number of viable embryos as determined by candling on Days 8 and 15 divided by the number of eggs set per pen. Statistical analysis of the percentage of viable embryos included evaluation of the slope across bird age and the total number of viable embryos divided by the number set using dose response and ANOVA comparing the treatment mean responses. Only eggs from mated pairs that were randomized into the design, treated, and measured were analyzed.

6. Hatchlings of Viable Embryos (%). This variable was defined as the number of hatchlings removed from the hatcher divided by the number of viable embryos per pen. Statistical analysis of the percentage hatching was conducted using dose response and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

7. Hatchlings of Fertile Eggs (%). This variable was defined as the number of live hatchlings divided by the number of fertile eggs per pen. Statistical analysis of the percentage hatching was conducted using dose response and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

8. 14 Day Old Survivors of Normal Hatchlings (%). This variable was defined as the number of hatchlings divided by the number of eggs set per week by pen. Statistical analysis of the percentage normal was conducted using dose response and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

9. Normal Hatchlings as a Percentage of the Maximum Number of Eggs Incubated. This variable was defined as the number of hatchlings per hen divided by the largest number of eggs set from any one hen. This transformation was used to convert the number of hatchlings to a ratio value equal to or less than 1. Statistical analysis of the percentage normal was conducted using dose response and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

10. 14 Day Old Survivors of Eggs Set (%). This variable was defined as the number of 14 day old survivors divided by the number of eggs set per week by pen. Statistical analysis of the percentage surviving was conducted using dose response and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

11. 14 Day Old Survivors of Maximum Set (%). This variable was defined as the number of 14 day old survivors per pen divided by the largest number of eggs set. Statistical analysis of the percentage surviving was conducted using dose response, and ANOVA comparing the treatment mean responses. Only hatchlings from mated pairs that were randomized into the design, treated, and measured were analyzed.

12. Hatchling Body Weight. The group body weights of surviving hatchlings and 14-day old survivors was measured by parental pen group and was analyzed by dose response and ANOVA. All hatchlings from birds that were randomized into the design, treated, and measured were analyzed.

13. 14 Day Old Survivor Body Weight. The group body weights of surviving hatchlings and 14 day old survivors was measured by parental pen group and was analyzed by dose response and ANOVA. All hatchlings from birds that were randomized into the design, treated, and measured were analyzed.

14. Eggshell Thickness and Eggshell Strength. The average eggshell thickness and strength of indiscriminately selected eggs per pen were measured during testing. The average, coefficient of variation on the natural logarithm scale, and slope across bird age also on the natural logarithm scale were analyzed by dose response and ANOVA. All eggs from birds that were randomized into the design, treated, and measured were analyzed.

15. Hormone level in egg contents and fecal/urate matter. Concentrations of hormones averaged per groups and pen respectively were analyzed by dose response and ANOVA. All fecal matter and eggs from birds that were randomized into the design, treated, and measured were analyzed.

16. Sexual Maturation. The time to sexual maturation averaged per pen was analyzed by dose response and ANOVA. Onset of lay was recorded as the number of days to first egg laid by the hen. All birds that were randomized into the design, treated, and measured were analyzed.

17. Genetic Sex Ratio. The ratio of the number of males to females in the last batch of F2 chicks by blood analysis was analyzed by dose response and ANOVA and compared to gender determined at necropsy. All birds that were randomized into the design, treated, and measured were analyzed.

18. Incidence of Abnormal Reproductive Structures. The number of abnormal reproductive structures found in the 14 day old chicks and adults was analyzed by regression analysis. All birds that were randomized into the design, treated, and measured were analyzed.

19. Organ Weights. The absolute value, the somatic index of organ weight to body weight, and the organ weight to brain weight of 14 day old chicks and adults were analyzed by the time series analysis by estimating a slope across bird age when appropriate, dose response, and ANOVA. All birds that were randomized into the design, treated, and measured were analyzed.

20. Oocyte Development. The number of oocytes in rapid development (>4 mm in diameter and yellow in color) per adult female were analyzed by dose response and ANOVA. All birds that were randomized into the design, treated, and measured were analyzed.

21. Cloacal gland size. Cloacal gland size was calculated as the area of the cloacal gland using the following formula:

$$\text{Area} = ab$$

where a is the length of the long axis, and b is the length of the short axis. The cloacal area was analyzed by dose response and ANOVA. All birds that were randomized into the design, treated, and measured were analyzed.

22. Organ Lesions. Histological scores of tissue abnormalities were analyzed as proportions of observed abnormalities by dose response and ANOVA. All birds that were randomized into the design, treated, and measured were analyzed.

3.10 Quality Assurance

3.10.1 Technical Systems Audits

The Battelle Sequim Quality Assurance (QA) Unit performed assessments on activities and operations affecting data quality, the raw data, and final report. Any findings were reported to the Work Assignment Principal Investigator and management to ensure that the requirements in relevant Standard Operating Procedures (SOPs), WA protocol, Quality Assurance Project Plans (QAPP), and the Quality Management Plan (QMP) were met. The QAPP, Protocol, and Study Plan are presented in Appendix K. The assessments for this study included technical systems audits (TSAs) and audits of data quality (ADQs) that included reviews of project notebooks, data base entry verifications from raw data sheets, and reviews of statistical analyses performed.

TSAs were performed at the start of the study, and for critical elements during the study such as the following:

- Personnel training files for documentation that EDSP SOPs, work plan and WA QAPP read and understood by WA personnel before startup
- Calibration status of project instrumentation
- Food consumption, egg collection, candling, body weights, and clinical observations
- Chemical analysis of test chemicals
- Termination of each experiment.

During TSA activities, the QA Unit recorded observations to be used later in preparing the audit report. The QA Unit observed completion of permitting requirements, implementation of procedures, data recording and record keeping, and equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the work plan, and the QAPP, applicable SOPs, and the QMP. Any findings were communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., misdosing an animal). If necessary, the EDSP QA team members immediately notified the WA leader/Principal Investigator by telephone and/or e-mail of any adverse findings that could affect the conduct of the WA. This direct communication was also documented in the audit report.

3.10.2 Audits of Data Quality

Audits of data quality (ADQs) focused on the accuracy of data collection, recording, traceability, and calculations to ensure that the report accurately describes the materials and methods used in the WA. The assessment criteria for ADQs were that data collection, analysis, and reporting met the requirements of the applicable facility and program SOPs, the work plan and QAPP, and the EDSP QMP, and that deviations be documented according to the requirements of the procedure. Deviation reports relative to the work assignment were submitted to the WA leader and were included in the project records.

Direct and frequent communication between the project manager, laboratory staff, and the QA Unit manager was designed to provide for sufficient time to perform an ADQ so that the submission date of the audited final report met those specified in the work plan.

TSAs and ADQs were conducted throughout the duration of this WA. Neither of these activities resulted in any major findings nor any stop work associated with the conduct of the experiments.

Raw and transcribed data have gone through quality control and quality assurance checks. Preliminary QC and QA have been performed on synthesized data and the initial report. However, final QC and QA checks will need to be performed upon receipt of EPA comments on the draft report, and upon completion of the final report. Results are subject to change until the final report is submitted.

3.10.3 Storage of Records and Data Management

The data for this study were collected on preprinted data collection forms. The data forms included, as appropriate, the following items: study code, protocol number, cage or container number, treatment code, and others. The forms had preprinted dates for collection of data when possible. Otherwise, the dates for data collection were hand printed on the forms as needed prior to or on the day of collection of the data. Data forms were initialed and dated by the person collecting the data, and all forms received documented technical review and signature approval. Corrections to data entries were made by drawing a single line through the error and recording the correct entry, initials, date, and error code that explained the reason for the correction.

The data were entered into a Microsoft Excel database. Data entry included transferring information on the written form to the database form. These database forms and tables associated with the forms had data integrity such that deletions were not allowed by the data entry personnel. Also, there was a quality control (QC) process during data entry to identify and correct any obvious discrepancies in the data.

The original raw data collected on the data forms will remain in the project file until there is a signed final report, at which time they will be inventoried and archived on compact disks with read-only memory (CD-ROM) for at least 2 years (longer if required by study protocol or government regulations), unless the sponsor requests that the data be transferred to an alternative archive location other than at Battelle.

All specimens and records remain the responsibility of Battelle Pacific Northwest Division (PNWD) and are retained for the length of time stipulated in the contract, which is typically 5 years. The archive is located at Battelle's facility in Richland, Washington, and is maintained according to a policy of limited access. The Battelle sample custodian is responsible for archiving and retrieving work assignment materials. An archive inventory is maintained, and storage capability is provided for the expedient retrieval of materials. Specimens and samples are disposed only after an assessment is made that they no longer afford evaluation.

4.0 PARENTAL GENERATION (P1) RESULTS

4.1 Adult Body Weight, Growth, and Tibiotarsus and Tarsometatarsus Measurements (P1)

4.1.1 Body Weight

Body weights of adult females and males at termination of the P1 phase of the study are shown in Table 4.1-1 and Table 4.1-2, respectively. The mean body weight of the 16-week-old hens was not significantly affected (p>0.66) by dietary treatment or exposure scenario (p=0.45). Male body weight at 16 weeks was also unaffected (p>0.44) by dietary treatment or exposure strategy (p=0.53) (Table 4.1-2).

Table 4.1-1. Body weight (g) of adult P1 female quail at 16 weeks of age.[a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	295	296	30.0	248	335	272	327	10%
P1A-0.078 ppm	8	279	273	32.0	239	321	247	313	12%
P1A-0.31 ppm	8	270	277	45.0	175	310	251	307	17%
P1A-1.25 ppm	8	288	292	22.0	258	311	262	309	8%
P1A-5 ppm	7	291	287	34.0	241	348	272	314	12%
P1B-0 ppm	7	291	301	19.0	261	318	274	301	7%
P1B-0.078 ppm	8	286	281	24.0	249	320	271	312	8%
P1B-0.31 ppm	8	286	266	43.0	251	360	257	330	15%
P1B-1.25 ppm	9	281	288	47.0	174	347	270	305	17%
P1B-5 ppm	8	294	286	40.0	244	363	264	328	14%

[a] No significant differences between dietary treatments (p>0.66) or exposure scenario (p=0.45)were found (General Linear Model analysis).

Table 4.1-2. Body weight (g) of adult P1 male quail at 16 weeks of age. [a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	214	219	29.0	151	251	210	227	13%
P1A-0.078 ppm	8	232	231	10.0	221	253	223	237	4%
P1A-0.31 ppm	8	229	233	34.0	157	266	213	256	15%
P1A-1.25 ppm	8	227	227	21.0	190	257	217	245	9%
P1A-5 ppm	8	237	241	20.0	204	259	220	255	8%
P1B-0 ppm	7	226	229	15.0	206	251	211	232	7%
P1B-0.078 ppm	8	240	240	18.0	211	271	229	250	7%
P1B-0.31 ppm	9	225	225	15.0	204	246	209	238	7%
P1B-1.25 ppm	9	241	237	32.0	211	318	220	251	13%
P1B-5 ppm	9	228	234	43.0	131	271	209	264	19%

[a] No significant differences between dietary treatments (p>0.44) or exposure scenario (p=0.53) were found (General Linear Model analysis).

4.1.2 Growth Rates

Growth rate of the birds from 3 weeks of age to termination at 16 weeks of age was determined using the terminal body weight, body weight at pairing (Week 11), and weekly body weights obtained prior to onset of egg laying (Weeks 4, 5, 6, and 7). Rate of growth of the birds was analyzed in two segments, one encompassing the pre-maturation measurements from 22 to 45 days of age, and the other representing the post-maturation period between 44 and 116 days of age (Figure 4.1-1). Body weight was regressed against age for each growth phase and the slopes (growth rate) of each phase compared between dietary treatments and between the P1A and P1B exposure scenarios by General Linear Model analysis.

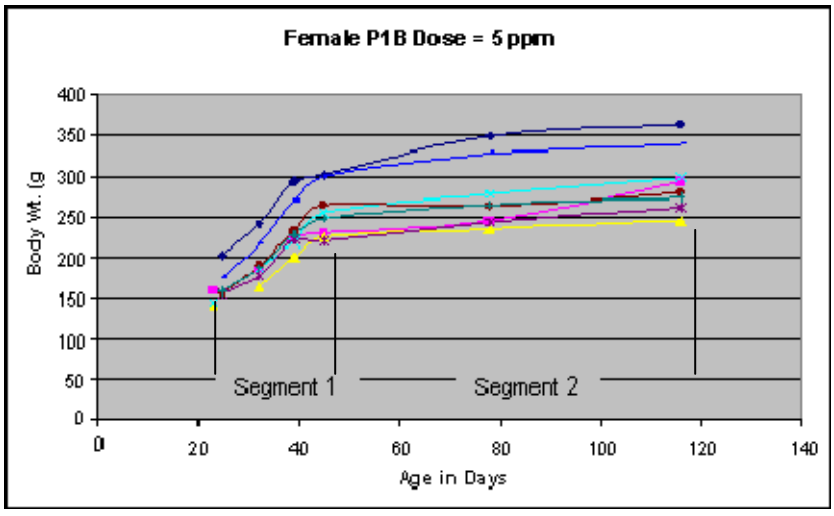


Figure 4.1-1. Two segments of the body weight time series used to compare growth rates of individual quail (grams versus days). Segment 1 encompasses the pre-maturation period from 22 to 45 days of age; segment 2 encompasses the post-maturation period between 44 and 116 days of age.

No significant differences in growth rates ($p \geq 0.30$) between dietary treatments or exposure strategies were found for either growth segment of female quail (Tables 4.1-3 and 4.1-4). In males, pre-maturation exposures (P1A) to E2 tended to result in a faster growth rate ($p=0.101$) within the pre-maturation phase was observed (Figure 4.1-2). However, extreme values in some groups (Figure 4.1-3) appear to be exaggerating the effect, and the biological importance of the pre-puberty exposure on this period of growth is likely minimal. Growth rates post-maturation were not significantly different between the two exposure scenarios ($p>0.98$).

Table 4.1-3. Growth rate (g/d) of P1 female quail between 22 and 45 days of age. [a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	4.32	4.47	0.74	3.33	5.59	3.58	4.70	17%
P1A-0.078 ppm	8	3.91	3.58	0.94	2.88	5.60	3.14	4.66	24%
P1A-0.31 ppm	8	3.96	4.11	0.54	3.01	4.64	3.46	4.32	14%
P1A-1.25 ppm	8	4.09	4.00	0.41	3.54	4.60	3.70	4.54	10%
P1A-5 ppm	7	3.90	4.03	0.41	3.37	4.46	3.47	4.18	11%
P1B-0 ppm	7	3.61	3.42	0.56	2.82	4.52	3.34	4.11	15%
P1B-0.078 ppm	8	3.62	3.67	0.45	2.92	4.20	3.22	4.01	13%
P1B-0.31 ppm	8	4.32	3.75	1.67	2.60	7.80	3.20	5.32	39%
P1B-1.25 ppm	9	3.82	3.97	0.67	2.89	4.89	3.12	4.28	18%
P1B-5 ppm	8	3.85	3.89	0.75	2.83	5.09	3.16	4.36	19%

[a] No significant differences between dietary treatments ($p=0.77$) or exposure scenario ($p=0.30$) were found (General Linear Model analysis).

Table 4.1-4. Growth rate (g/d) of P1 female quail between 44 and 116 days of age. [a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	0.64	0.59	0.22	0.44	1.01	0.44	0.82	34%
P1A-0.078 ppm	8	0.55	0.57	0.38	-0.23	1.02	0.38	0.86	69%
P1A-0.31 ppm	8	0.34	0.43	0.38	-0.49	0.70	0.20	0.62	112%
P1A-1.25 ppm	7	0.54	0.53	0.19	0.32	0.85	0.40	0.70	34%
P1A-5 ppm	7	0.67	0.68	0.33	0.12	1.06	0.43	1.00	49%
P1B-0 ppm	7	0.51	0.55	0.12	0.35	0.64	0.42	0.62	22%
P1B-0.078 ppm	8	0.48	0.47	0.20	0.14	0.86	0.41	0.52	42%
P1B-0.31 ppm	7	0.59	0.53	0.29	0.29	1.12	0.42	0.83	49%
P1B-1.25 ppm	8	0.56	0.60	0.15	0.31	0.74	0.44	0.71	27%
P1B-5 ppm	8	0.54	0.57	0.25	0.22	0.87	0.29	0.80	46%

[a] No significant differences between dietary treatments ($p=0.64$) or exposure scenario ($p=0.87$) were found (General Linear Model analysis).

No significant difference ($p \geq 0.71$) in growth rate as a function of dietary treatment was observed in the P1A males or P1B males (Tables 4.1-5 and 4.1-6).

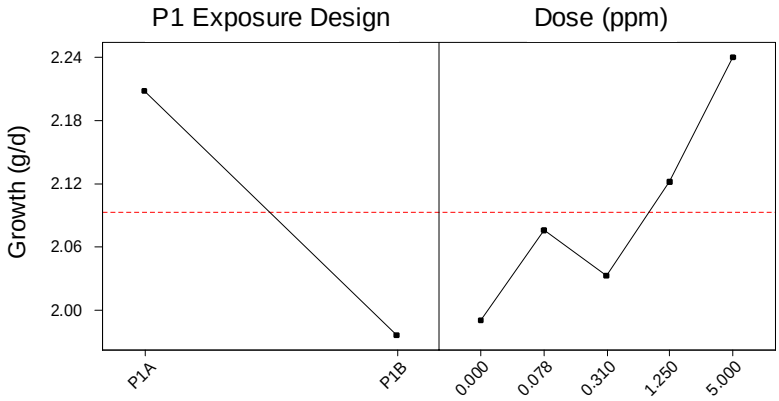


Figure 4.1-2. General Linear Model analysis of the slopes of the pre-maturation growth segment in male quail from the P1 generation (g/d). A nearly significant difference (p=0.101) increase in rate of growth was observed for males exposed to estradiol (E2) from 3 weeks of age (P1A).

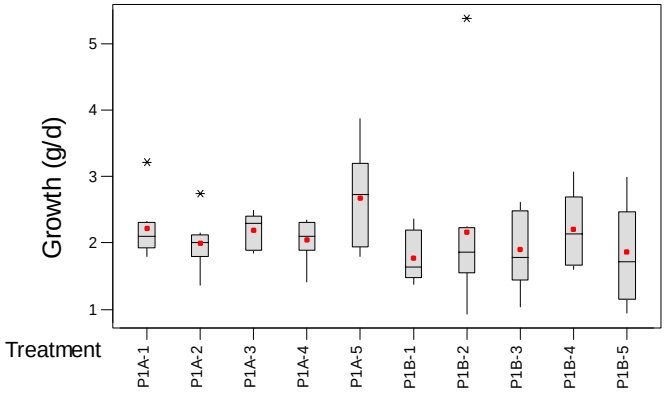


Figure 4.1-3. Box plots of the growth rate (g/d) between 22 and 45 days for male P1 birds showing extreme samples that may be influencing the significance of the growth rate. Means are indicated by solid circles. Asterisks indicate extreme values.

Table 4.1-5. Growth rate (g/d) of P1 male quail between 22 and 45 days of age. [a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	2.22	2.10	0.44	1.80	3.21	1.93	2.31	20%
P1A-0.078 ppm	8	1.99	2.01	0.39	1.36	2.75	1.80	2.13	20%
P1A-0.31 ppm	8	2.19	2.30	0.26	1.85	2.49	1.89	2.39	12%
P1A-1.25 ppm	8	2.05	2.10	0.31	1.41	2.35	1.89	2.31	15%
P1A-5 ppm	8	2.67	2.73	0.71	1.80	3.88	1.95	3.20	27%
P1B-0 ppm	9	1.77	1.64	0.38	1.38	2.36	1.48	2.19	21%
P1B-0.078 ppm	9	2.16	1.87	1.28	0.93	5.38	1.55	2.23	59%
P1B-0.31 ppm	10	1.90	1.78	0.57	1.03	2.61	1.44	2.48	30%
P1B-1.25 ppm	9	2.20	2.14	0.54	1.60	3.07	1.67	2.69	25%
P1B-5 ppm	9	1.86	1.71	0.73	0.93	2.99	1.16	2.46	39%

[a] No significant differences between dietary treatments (p=0.82) or exposure scenario (p=0.10) were found (General Linear Model analysis).

Table 4.1-6. Growth rate (g/d) of P1 male quail between 44 and 116 days of age. [a]

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	0.25	0.35	0.32	-0.49	0.50	0.19	0.45	127%
P1A-0.078 ppm	8	0.51	0.45	0.14	0.33	0.75	0.41	0.64	28%
P1A-0.31 ppm	8	0.38	0.45	0.42	-0.58	0.72	0.33	0.66	111%
P1A-1.25 ppm	8	0.45	0.46	0.14	0.26	0.67	0.31	0.56	31%
P1A-5 ppm	8	0.37	0.39	0.21	-0.02	0.60	0.20	0.55	58%
P1B-0 ppm	9	0.42	0.37	0.09	0.32	0.60	0.34	0.49	23%
P1B-0.078 ppm	9	0.28	0.50	0.56	-1.17	0.63	0.26	0.54	199%
P1B-0.31 ppm	10	0.32	0.36	0.28	-0.38	0.65	0.25	0.48	87%
P1B-1.25 ppm	9	0.48	0.59	0.30	0.10	1.05	0.22	0.62	62%
P1B-5 ppm	9	0.36	0.46	0.46	-0.75	0.87	0.27	0.60	128%

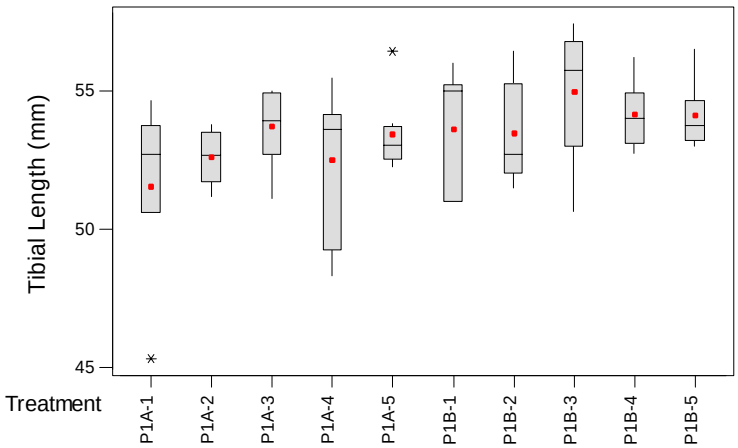
[a] No significant differences between dietary treatments (p=0.71) or exposure scenario (p=0.98) were found (General Linear Model analysis).

4.1.3 Measurements of the Tibiotarsus and Tarsometarsus of Adult Quail at Necropsy (P1)

Note that the term "tibia" is used in some graphic labels as the equivalent of "tibiotarsus". Likewise, the term "tarsus" is the equivalent of the term "tarsometatarsus".

Males

Significant differences in tibiotarsus and tarsometatarsus length (p=0.004 and p< 0.001, respectively) between exposure scenarios were found for male quail. Overall, the bone length was less in males exposed to E2 prior to puberty (Figures 4.1-4 and 4.1-5). No significant differences between dietary treatments of E2 were found for these two measures, though there was a nearly significant (p=0.123) increase in tibiotarsal length as a function of dietary concentration of E2, but the response was not linear (Figure 4.1-4). The weight and diameter of the tibiotarsus were unaffected by exposure scenario or dietary treatment (Figures 4.1-6 and 4.1-7).



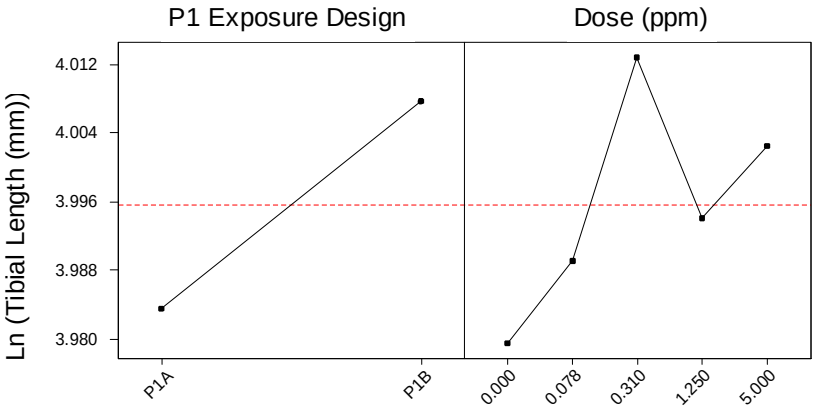


Figure 4.1-4. Box plots of tibiotarsus length (mm) by dietary treatment for male quail exposed from 3 weeks of age (P1A) and after the onset of puberty (P1B) to E2 (above). Main effects of the General Linear Model analysis of the natural log-transformed lengths of the tibiotarsus in male quail exposed to E2 (below). Difference in exposure scenarios was significant (p=0.004); tibiotarsal length affected by dietary concentrations was nearly significant (p=0.123).

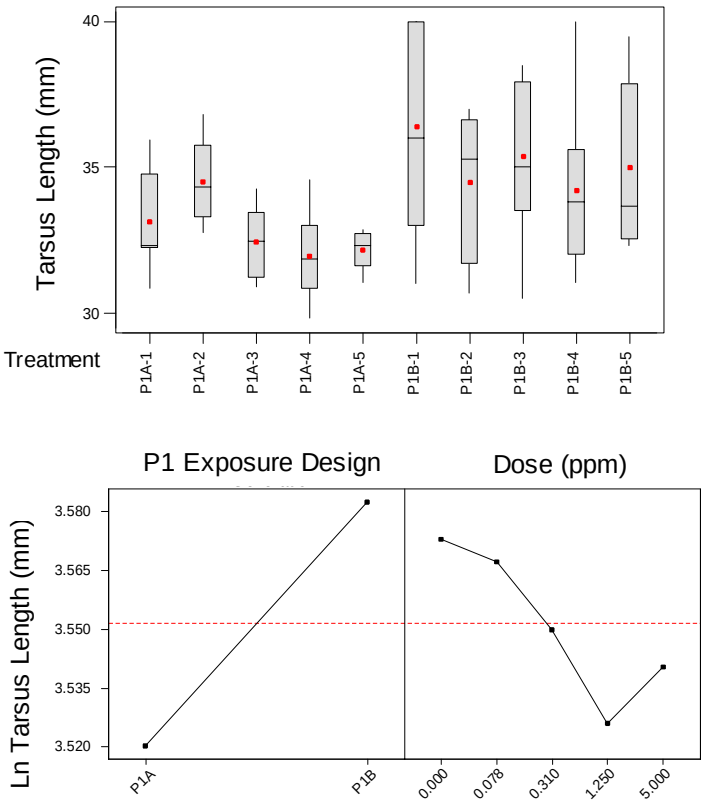


Figure 4.1-5. Box plots (above) of tarsometatarsus length (mm) by dietary treatment for male quail exposed from 3 weeks of age (P1A) and after the onset of puberty (P1B) to E2. Apparent increase in tarsometatarsus length was found to be significant ($p<0.001$) by General Linear Model analysis of the natural log-transformed lengths (below) of the bone.

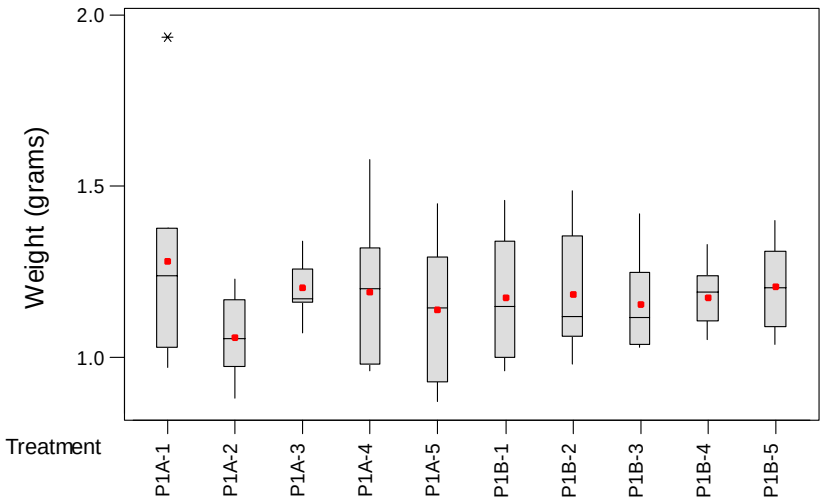


Figure 4.1-6. Box plots of tibiotarsus weight (g) in male quail by dietary treatment within two exposure scenarios, dietary exposure to E2 from 3 weeks of age (P1A) and dietary exposure after the onset of puberty (P1B).

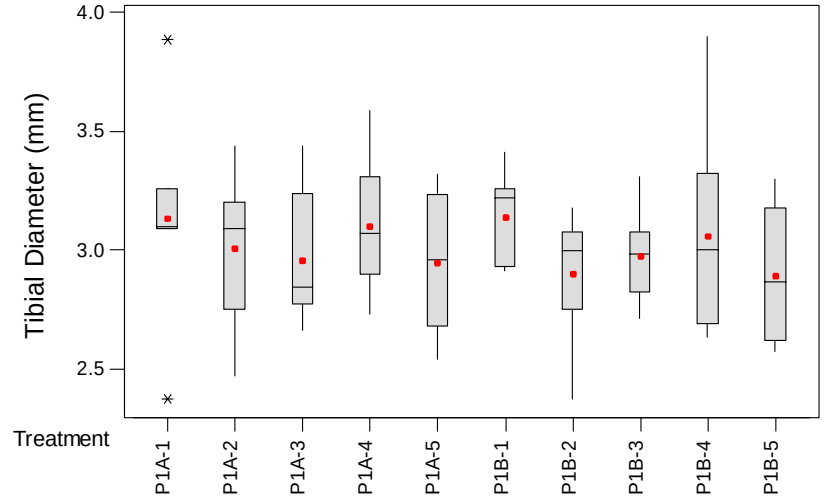


Figure 4.1-7. Box plots of tibiotarsus diameter (mm) of male quail by dietary treatment within two exposure scenarios, dietary exposure to E2 from 3 weeks of age (P1A) and dietary exposure after onset of puberty (P1B).

Females

The only significant effect of E2 exposure on female bone measurements was an increase in tarsometatarsus length under the P1B exposure scenario ($p<0.016$). However, as seen in Figure 4.1-8, the difference between the median P1A and P1B controls suggest that the observed difference in bone length were not in response to E2 treatment. No difference between E2 dietary treatments was observed (Figure 4.1-8). The distribution of tibiotarsus lengths and diameters in female quail by dietary concentration within the two P1 exposure scenarios are shown in Figure 4.1-9).

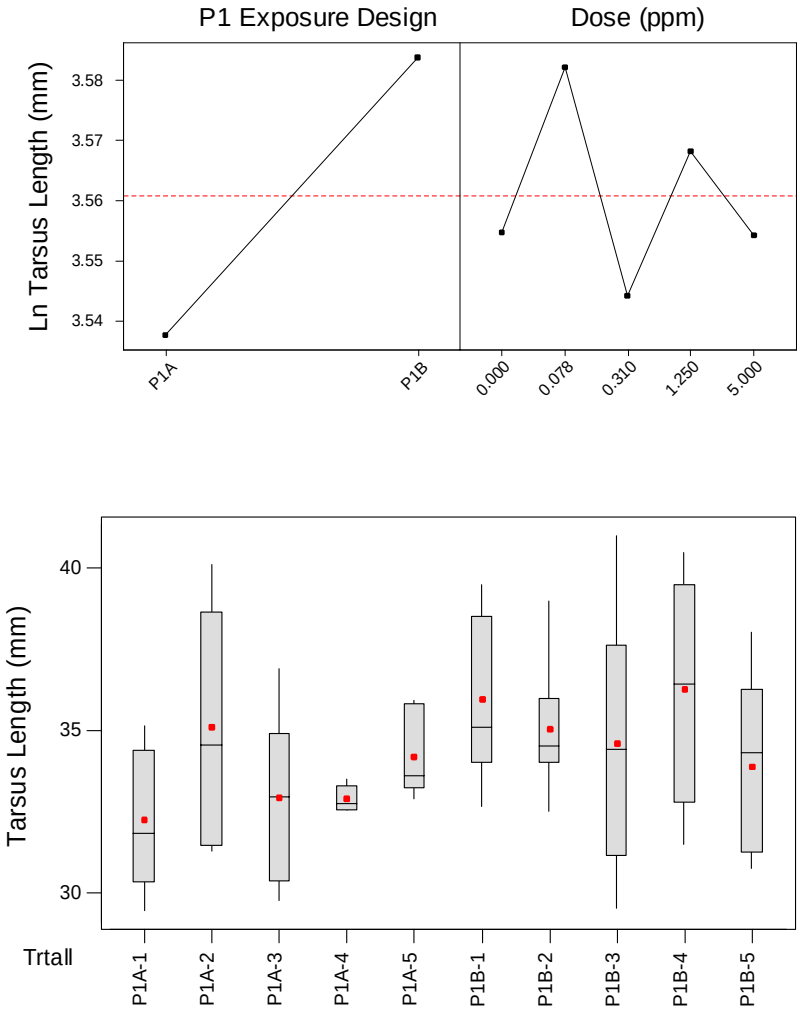


Figure 4.1-8. Main effects of the General Linear Model analysis of the natural log-transformed lengths of the tarsometatarsus (mm) in female quail exposed to E2 (above), and boxplots of the tarsometatarsus length (mm) in the female quail. Difference in exposure scenarios was significant ($p<0.016$); no difference ($p=0.706$) between dietary treatments was found.

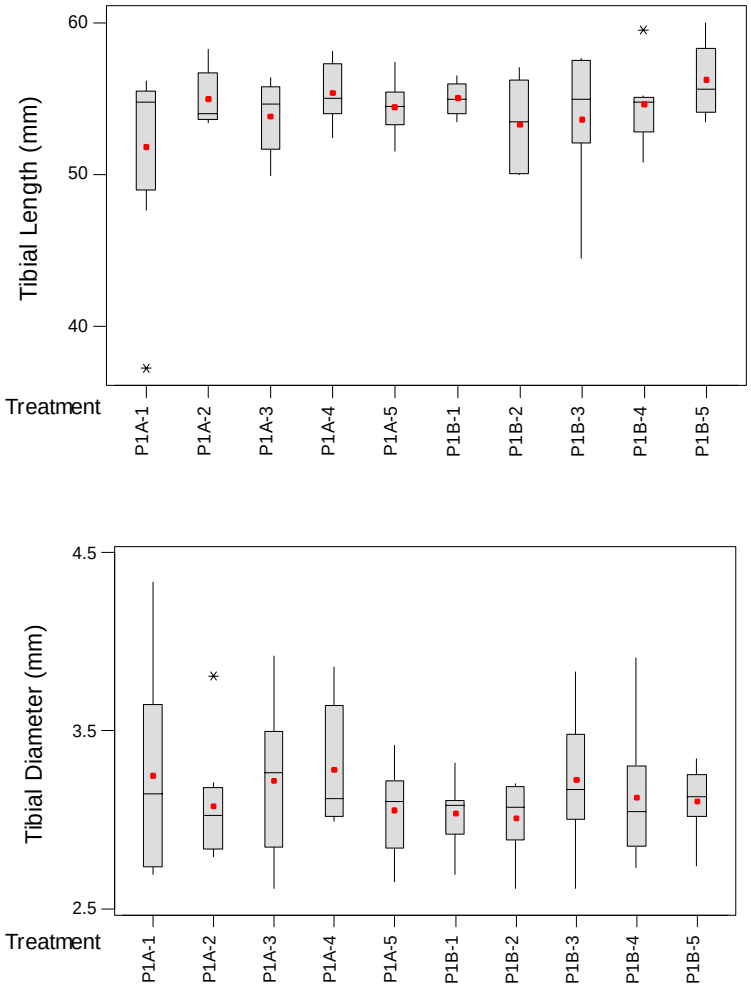


Figure 4.1-9. Box plots showing the distribution of tibiotarsus lengths (above) and diameters (below) in female quail by dietary concentration within two exposure scenarios. Dietary exposure to E2 was from 3 weeks of age (P1A) and dietary exposure after the onset of puberty (P1B). Means are indicated by solid circles.

4.2 Food Consumption (P1)

Females

Average food consumption during the post-pairing period for the P1A and P1B female quail is shown in Figure 4.2-1. Average post-pairing food consumption rates of the hens did not differ significantly (p= 0.88) between the two exposure scenarios and was about 36 grams of food per hen per day overall (Figure 4.2-2). Food consumption tended to be affected by treatment concentration (p=0.10); however, the response was non-concentration linear (Figure 4.2-2).

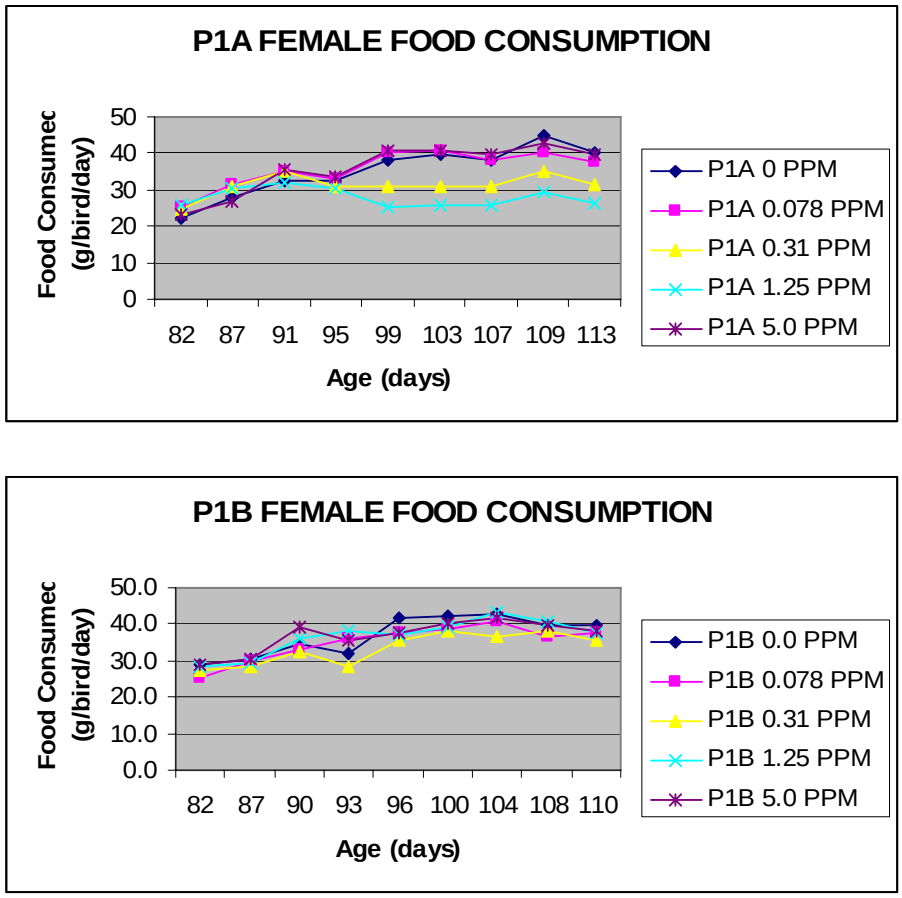


Figure 4.2-1. Average food consumption per hen per day post pairing. All pairs were separated during week 13. P1A birds were fed E2 from 3 weeks of age; P1B birds were fed E2 from 11 weeks of age.

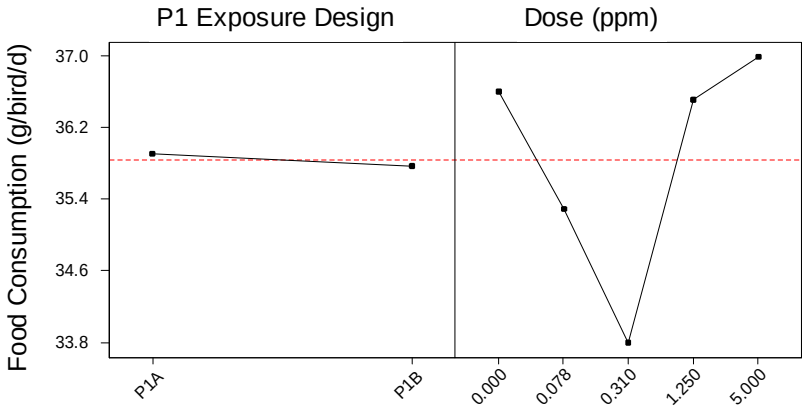


Figure 4.2-2. Main effects of the General Linear Model analysis of food consumption rate (g/bird/d) in female quail fed E2 treated diets for 13 weeks from pre-maturation through egg laying (P1A exposure scenario) or 5 weeks during egg laying (P1B exposure). No significant difference in food consumption was observed between exposure scenarios (p=0.88); a nearly significant (p=0.10) dietary treatment effect (non-concentration linear) was observed.

Food consumption was regressed against age for the post-pairing period (Weeks 11 through 16 of age) when birds from both exposure scenarios were consuming treated feed and the slopes were compared between dietary treatments and between the P1A and P1B exposure scenarios by General Linear Model analysis. For female quail, a significant difference in food consumption rate over time (age) between the two exposure scenarios (p=0.03) was observed. The mean rate of food consumption was greater over time in females in the P1A exposure scenario compared to their P1B counterparts. There was also a significant dietary treatment effect on food consumption over time (p=0.03), but the dose response was not linear (Figure 4.2-3).

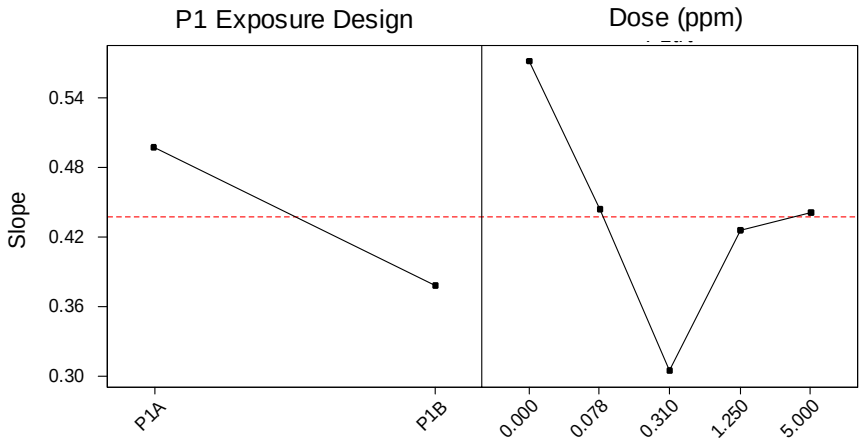
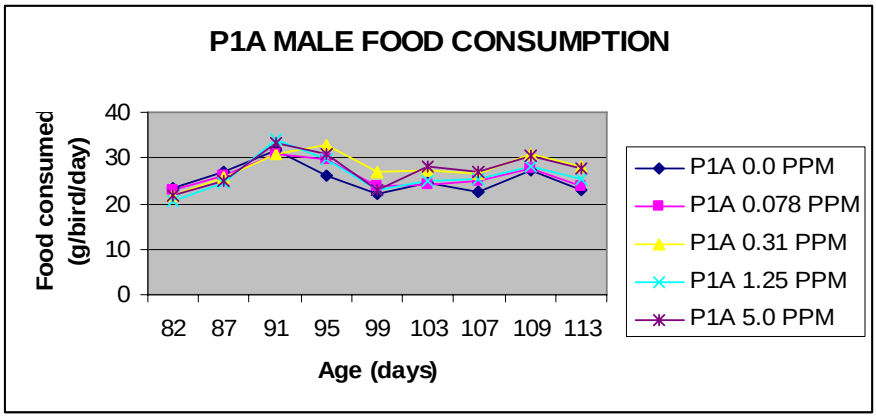


Figure 4.2-3. General Linear Model analysis of the effect of exposure scenario and dietary concentration on the slopes of food consumption regressed against time post-pairing (g/bird/d) in female quail fed E2 treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from pre-maturation; in the P1B design, breeding quail were exposed to E2 for 5 weeks. Significant difference in food consumption rate over time was observed between exposure scenarios (p=0.03); a significant (p=0.03) dietary treatment effect (non-dose linear) was observed.

Males

Average food consumption during the post-pairing period for the P1A and P1B male quail is shown in Figure 4.2-4. Average post-pairing food consumption rate for males was about 25 to 28 grams per bird per day (Figure 4.2-5) and was not significantly different between exposure scenarios or dietary treatments (p=0.317 and p=0.893, respectively). Dietary treatment had no effect (p=0.52) on the slopes of the food consumption curves of either the P1A or P1B male Japanese quail; however, there was a nearly significant though small negative slope in the food consumption rate of P1B males (Figure 4.2-6).



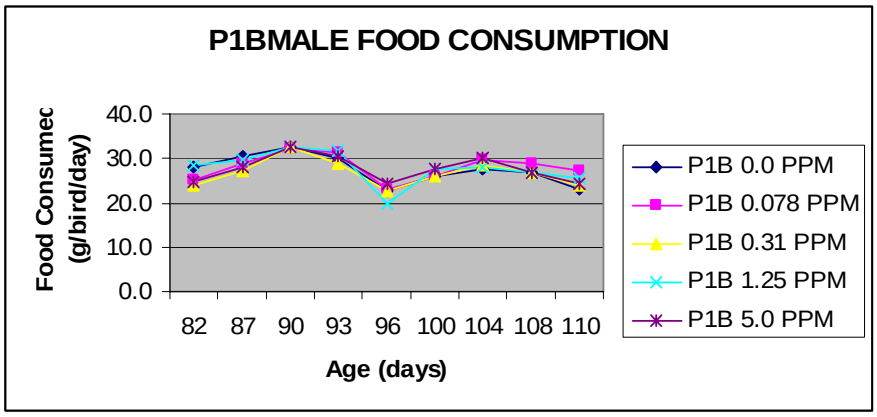


Figure 4.2-4. Average food consumption per male Japanese quail per day post pairing. Pairs were separated on day 93. P1A birds were fed E2 from 3 weeks of age; P1B birds were fed E2 from 11 weeks of age.

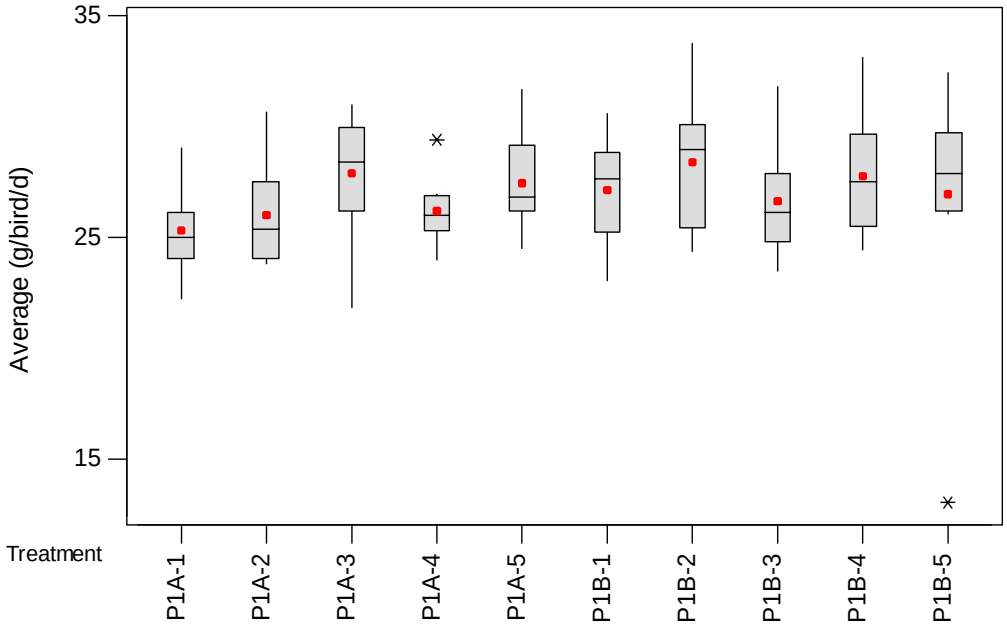


Figure 4.2-5. Box plots of average food consumption by males fed E2 treated feed (g/bird/d) under two exposure scenarios, from 3 weeks of age (P1A) and from 11 weeks of age (P1B). Means are indicated by solid circles.

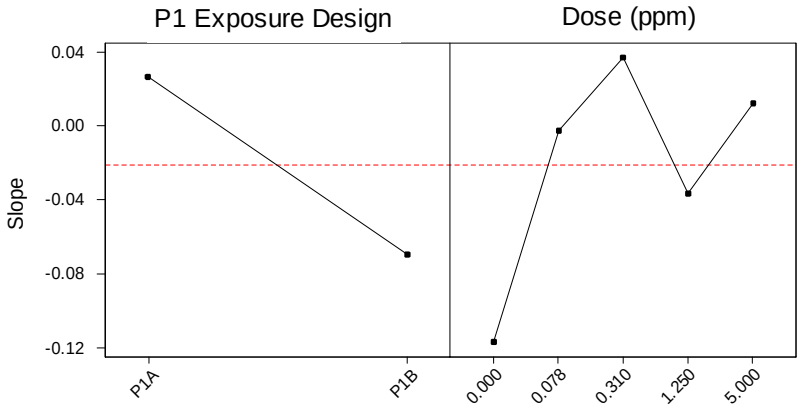


Figure 4.2-6. General Linear Model analysis of the effect of exposure scenario and dietary concentration on the slopes of food consumption regressed against time post-pairing (g/bird/d) in male quail fed E2 treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from pre-maturation; in the P1B design, breeding age quail were exposed to E2 for 5 weeks. A negative food consumption rate was observed in P1B males (p=0.11); no significant dietary treatment effect (p=0.52) was observed.

4.3 Steroid Content of Fecal-Urate Samples from P1 Birds

4.3.1 17ß-Estradiol

Females

In both the P1A and P1B hens, fecal-urate E2 increased significantly ($p<0.001$) with increasing dietary concentration (Table 4.3-1). Regardless of the length of exposure, E2 excretion was similar in the two parental exposure populations at the 5th week of egg laying. Figure 4.3-1 shows the average slope of the linear fit of the natural log-transformed E2 fecal-urate concentrations for the P1A and P1B populations to be nearly identical (0.566 and 0.563 ng/g/ppm diet, respectively).

Table 4.3-1. Estradiol levels (ng/g) in fecal-urate samples of female quail at the fifth week of egg laying under two exposure scenarios: Dietary exposure from 3 weeks of age (P1A) or from onset of egg laying (P1B).

Design	Dose (ppm)	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A	0	8	141	116	56.1	109	274	112	153	40%
P1A	0.078	8	159	128	76.3	97.1	313	111	217	48%
P1A	0.31	7	363	359	55.2	294	453	320	413	15%
P1A	1.25	7	1062	1099	170	845	1312	861	1168	16%
P1A	5	7	2882	2820	470	2325	3464	2404	3352	16%
P1B	0	8	120	122	19.2	89.0	154	105	127	16%
P1B	0.078	8	173	180	41.0	98.3	213	149	207	24%
P1B	0.31	7	376	359	67.3	285	463	320	459	18%
P1B	1.25	8	944	946	126	783	1119	819	1057	13%
P1B	5	8	2924	2992	334	2414	3452	2611	3123	11%

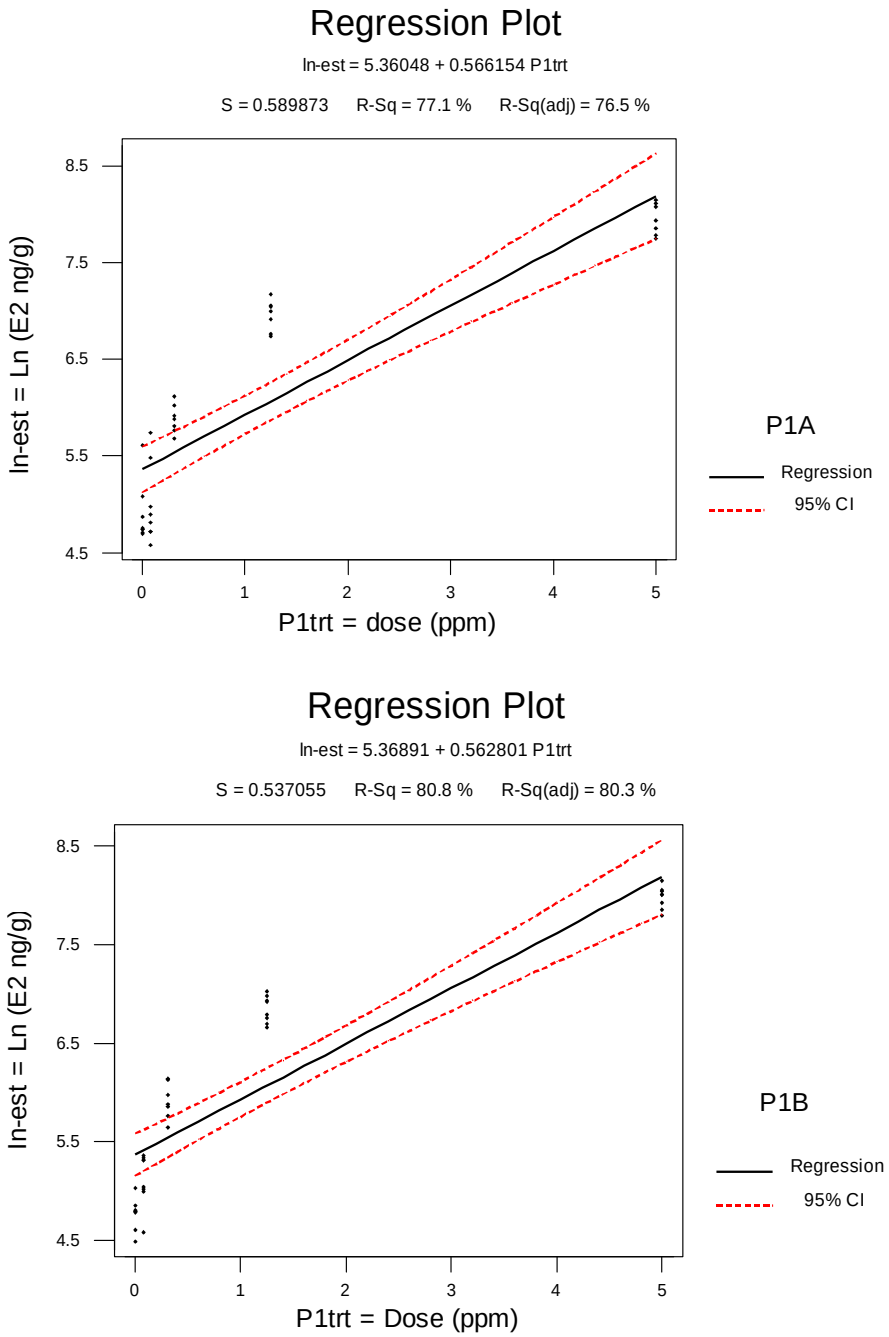


Figure 4.3-1. Linear fit of the natural log-transformed E2 fecal-urate concentrations (ng/g) by dose (ppm) at the fifth week of egg laying for the P1A and P1B exposure scenarios.

Males

Excretion of estradiol in fecal-urate was similar to that observed for hens. P1A and P1B fecal-urate E2 levels were similar over exposure range and increased significantly ($p<0.001$) with increasing dietary concentration (Table 4.3-2). The slopes of the linear fit of the natural log-transformed fecal-urate E2 concentrations by P1A and P1B males were similar (0.650 and 0.613 ng/g/ppm diet, respectively).

Table 4.3-2. Estradiol levels (ng/g) in fecal-urate samples of male *Corturnix* at the fifth week of egg laying under two exposure scenarios: Dietary exposure from 3 weeks of age (P1A) or from onset of egg laying (P1B).

Design	Dose (ppm)	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A	0	7	47.2	47.7	11.5	32.9	69.2	39.4	49.8	24%
P1A	0.078	8	81.4	72.7	28.5	55.4	139	60.1	101	35%
P1A	0.31	7	435	417	104	261	574	388	524	24%
P1A	1.25	8	1170	1164	254	804	1560	956	1394	22%
P1A	5	8	2660	2745	495	1878	3412	2227	2973	19%
P1B	0	8	51.7	52.2	12.3	34.5	69.4	39.3	62.2	24%
P1B	0.078	8	155	123	94.4	70.0	336	76.0	229	61%
P1B	0.31	8	344	341	128	129	512	256	470	37%
P1B	1.25	9	1164	1091	172	1033	1559	1050	1257	15%
P1B	5	8	2480	2430	292	2027	2953	2279	2714	12%

4.3.2 Testosterone

Females

Exposure scenario had a marginally significant effect on the testosterone levels excreted in fecal-urate matter by hens ($p=0.145$), as shown in Table 4.3-3. However, the Kruskal-Wallis non-parametric test found no significant difference ($p=0.812$) in median testosterone concentrations between dietary treatments for P1A or P1B females. Two P1A female birds had elevated testosterone concentrations in the 5 ppm test diet, which greatly influenced the mean and produced a significant regression for the P1A females ($p=0.02$); however, these two results may be spurious.

Table 4.3-3 Testosterone levels (ng/g) in fecal-urate samples of female quail at the fifth week of egg laying under two exposure scenarios: Dietary exposure from 3 weeks of age (P1A) or from onset of egg laying (P1B).

Design	Dose (ppm)	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A	0	8	103	101	21.0	70.0	134	88.0	122	21%
P1A	0.078	8	106	110	18.0	82.0	129	88.0	120	17%
P1A	0.31	7	93.0	93.0	26.0	66.0	139	69.0	108	28%
P1A	1.25	7	103	92.0	45.0	50.0	188	67.0	127	44%
P1A	5	8	160	112	122	70.0	366	76.0	289	76%
P1B	0	8	96.0	93.0	40.0	44.0	151	62.0	137	41%
P1B	0.078	8	96.0	95.0	24.0	56.0	129	78.0	120	25%
P1B	0.31	7	107	101	22.0	84.0	144	95.0	131	20%
P1B	1.25	8	97.0	96.0	24.0	64.0	128	71.0	119	25%
P1B	5	8	81.0	80.0	14.0	62.0	106	70.0	90.0	17%

Males

No difference (p=0.455) in fecal-urate concentrations of testosterone were observed between the P1A and P1B populations of males (Figure 4.3-2). A significant reduction (p = 0.009) in median fecal-urate testosterone concentration with increasing dietary exposure to E2 was observed for P1B males, but the Kruskal-Wallis nonparametric test found no such response in fecal-urate testosterone levels of males from the P1A exposure population (Table 4.3-4). However, when averaged over the exposure scenarios (i.e., P1A and P1B populations), a trend (p=0.084) toward decreased mean testosterone levels with increasing dietary exposure was observed (Figure 4.3-2).

Table 4.3-4 Testosterone levels (ng/g) in fecal-urate samples of male quail at the fifth week of egg laying under two exposure scenarios: Dietary exposure from 3 weeks of age (P1A) or from onset of egg laying (P1B)

Design	Dose (ppm)	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A	0	7	290	299	83.8	168	428	223	330	29%
P1A	0.078	8	315	271	95.0	203	478	249	400	30%
P1A	0.31	7	293	290	49.8	225	364	257	351	17%
P1A	1.25	8	267	268	58.3	192	365	210	309	22%
P1A	5	8	256	222	116	150	513	177	298	45%
P1B	0	8	317	321	38.6	265	372	278	347	12%
P1B	0.078	8	299	292	56.1	225	410	259	324	19%
P1B	0.31	8	300	271	106	195	533	231	335	35%
P1B	1.25	9	296	299	41.7	212	356	276	328	14%
P1B	5	8	244	238	37.7	200	314	213	274	15%

No significant difference in fecal-urate concentration of testosterone between dietary concentrations was found (p=0.529, Kruskal-Wallis nonparametric test) within the P1A exposure scenario.
A significant reduction in fecal-urate testosterone content with increasing dietary exposure to E2 (p=0.009, Kruskal-Wallis nonparametric test) was found within the P1B exposure scenario.

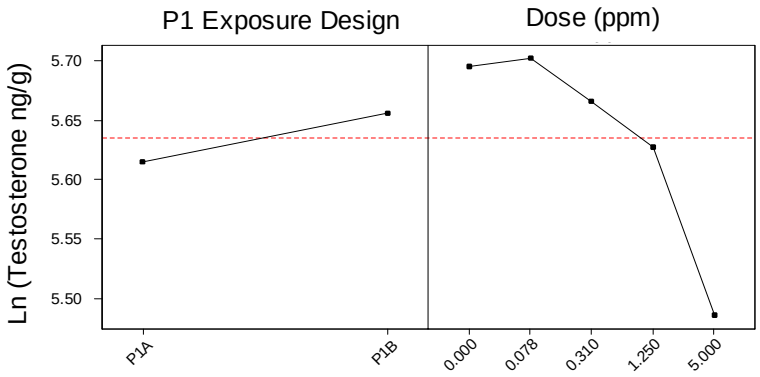


Figure 4.3-2. Main effects of the General Linear Model analysis of the natural log-transformed concentrations of testosterone (ng/g) in fecal-urate samples at the fifth week of egg laying; trend (p=0.084) was toward decreased testosterone excretion with increasing dietary treatment (ppm).

4.4 Clinical Observations, Aggression, Early Deaths, and Abnormalities (P1)

4.4.1 Clinical Observations

Most clinical signs were of injury, aggressive behavior, pododermatitis (bumble foot) or egg binding. Feather damage and pecking or other injuries were tabulated as a measure of aggression post-pairing. Feather loss on the head and back of neck region of females was associated with male mounting attempts. Also during mating, some females received gashes or cuts from the claws of the males. Females were also aggressive, causing numerous sometimes fatal pecking injuries to males and several deaths (Section 4.4.2, Incidence of Aggression). Because of the number of pairs that had to be separated because of aggression, all pairs were separated to equalize mating opportunity and stress across pairs. Feather loss and damage to the breast were observed in both females and males and appeared to be the result of rubbing the feathers over the food trough in attempts to reach birds in adjacent pens. Feather loss on the head and back of neck of males was from female pecking. Egg binding was observed in a few females. When clinical signs of egg binding became evident, an attempt was made to rupture the egg *in utero* and remove the egg. Most birds, however, succumbed to egg binding between observation periods or were found moribund and were euthanized.

4.4.2 Incidence of Aggression

Both P1A and P1B pairs were raised from 3 weeks of age in separate cages. At 11 weeks of age, well after the onset of egg laying, the birds were paired by introduction of males into the female cages. Seven P1 males were killed by females (Table 4.4-1 and Section 4.4.3, Incidence of Unscheduled Deaths) during the first day after introduction of the male. An additional 15 males were injured by their pen mate to the point of having to be separated to another pen. Of the total number of male injuries from female aggression (22), over two thirds were from the P1B population.

The incidence of male aggression resulting in injury to females was less than half that observed for female injury of males (Table 4.4-1).

Table 4.4-1. Incidence of aggression[a] injury cases.

Treatment	Injured Females	Injured Males	Males Killed by Females	Total
P1A-0 ppm	3	0	1	4
P1A-0.078 ppm	0	2	0	2
P1A-0.31 ppm	1	1	1	3
P1A-1.25 ppm	0	1	0	1
P1A-5 ppm	0	1	0	1
P1B-0 ppm	1	3	0	4
P1B-0.078 ppm	2	3	1	6
P1B-0.31 ppm	0	1	1	2
P1B-1.25 ppm	1	0	0	1
P1B-5 ppm	2	3	3	8

[a] Aggression is defined here as behavior that causes death or open wound and/or trauma sufficient to require separation of the pair.

Because of the number of affected pairs and the continued aggressive behavior of the females, all pairs were subsequently separated and the males were introduced into the female cages three times per week. The males were removed after at least one successful mount but before aggression by the female inflicted injury to the male.

4.4.3 Incidence of Unscheduled Deaths

A total of 16 unscheduled deaths occurred in the P1 generation (Table 4.4-2). Five deaths occurred after initiation of E2 exposure of the P1A birds. Of the P1A deaths, 3 were females and 2 were males. Two of these females, one in the 0.31 ppm dietary treatment group and one in the 1.25 ppm group, died from egg binding. The third female died as a result of a skull fracture. Both males died from rapid, severe aggression by females at pairing (males were paired with females after initiation of egg laying).

The incidence of male death due to female aggression was greater in the P1B treatments, with a total of 5 males dying as result of pairing with an aggressive female. P1B birds had not received treated food prior to initial pairing. Only two P1B females died during the exposure period, one from egg binding and one from pecking injuries by a male. Additionally, prior to the exposure/pairing period, two P1B females died of egg binding and another from unknown cause. One male also died of unknown cause prior to exposure/pairing.

Table 4.4-2. Incidence of unscheduled deaths of P1 birds.

Treatment	Female			Male
	Egg Binding	Aggression (pecked by male)	Accident (skull facture)[b]	Aggression (pecked by female)[c]
P1A-0 ppm	0	0	0	1
P1A-0.078 ppm	0	0	0	0
P1A-0.31 ppm	1	0	0	1
P1A-1.25 ppm	1	0	0	0
P1A-5 ppm	0	0	1	0
P1B-0 ppm	0[a]	0	0	0
P1B-0.078 ppm	0	0	0	1
P1B-0.31 ppm	1	0	0	1
P1B-1.25 ppm	0	1	0	0
P1B-5 ppm	0	0	0	3

Values in the table are deaths of birds that had been exposed to E2.
[a] Two additional birds died from egg binding in the P1B exposure scenario prior to exposure to E2.
[b] One additional female died of unknown causes prior to exposure to E2.
[c] One additional male died of unknown causes prior to exposure to E2.

4.4.4 Incidence of Abnormalities Observed at Necropsy (P1)

Females

There was a significant difference ($p < 0.05$) in the incidence of right oviducts between the two exposure scenarios. P1B females, exposed after the onset of egg production, had greater numbers of right oviducts than females exposed prior to maturation through egg laying (P1A) predominately in the lower concentrations (Figure 4.4-1, Table 4.4-3). P1A females, however, tended to have more injuries than P1B females ($p = 0.08$), and significantly greater ($p = 0.005$) incidence of abnormalities (Figure 4.4-2 and Figure 4.4-3). The incidence of injury appeared to be unrelated to dietary exposure to E2. However, the incidence of abnormalities decreased significantly ($p=0.012$) as a function of dietary E2 (Figure 4.4-3 and Table 4.4-3).

Table 4.4.3. Incidence of abnormalities and injuries of P1 female quail by dietary treatment and exposure scenario.

Generation and Treatment Dose	N	Presence of Right Ovary	Presence of Right Oviduct	Presence of Injuries	Indication of Pecking	Presence of Abnor-malities[a]	Chest feathers rubbed off	Head-neck feathers lost
P1A-0 ppm	8	0	1	2	1	5	0	5
P1A-0.078 ppm	8	1	1	4	2	3	1	6
P1A-0.31 ppm	7	0	0	0	0	2[b]	2	3
P1A-1.25 ppm	7	0	1	1	1	2[b]	0	5
P1A-5 ppm	7	0	1	3	3	3	1	5
P1B-0 ppm	7	0	2	0	0	3	0	6
P1B-0.078 ppm	8	0	5	2	2	1	1	6
P1B-0.31 ppm	7	0	5	0	0	1[b]	1	4
P1B-1.25 ppm	8	0	2	1	0	1	1	3
P1B-5 ppm	8	0	3	0	0	2	2	5

[a] Abnormalities include organ lesions.
[b] One incident of egg binding.

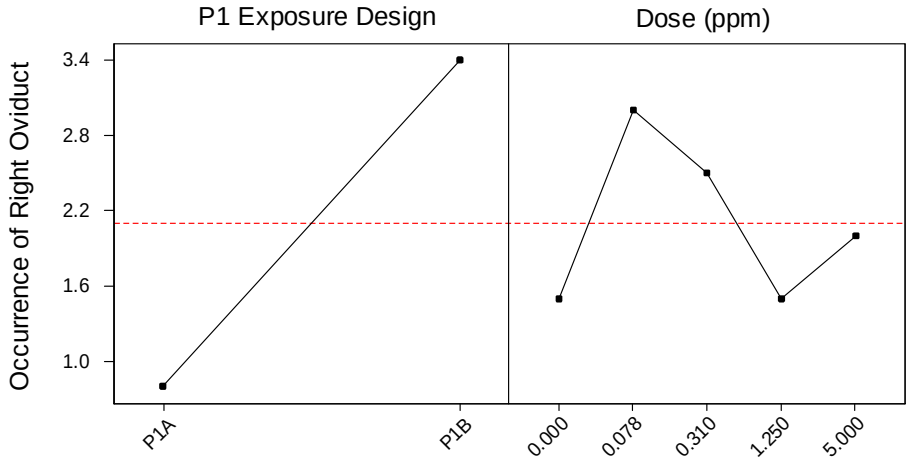


Figure 4.4-1. Main effects of the General Linear Model analysis of the incidence (mean number of occurrences per dose group) of right oviducts in female quail exposed to E2. Difference in exposure scenarios was significant ($p<0.05$); no difference ($p=0.732$) between dietary treatments was found.

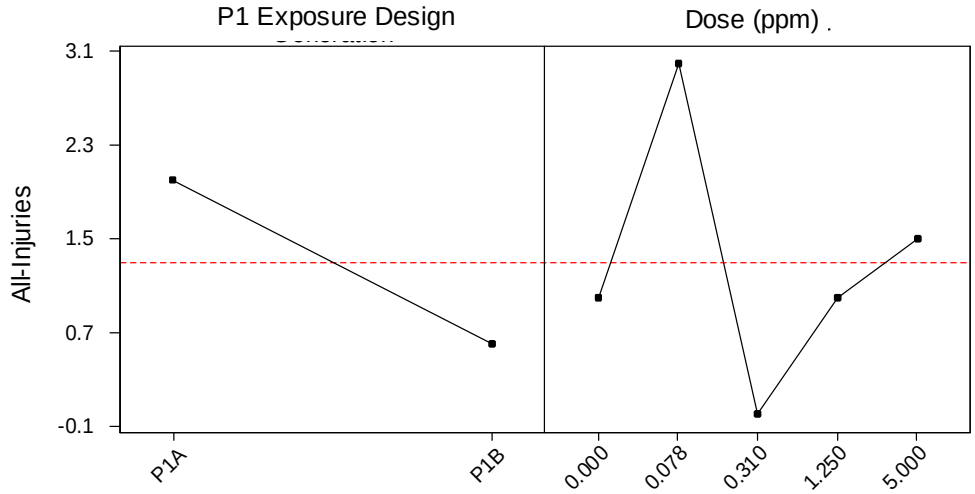


Figure 4.4-2. Main effects of the General Linear Model analysis of the incidence of all injuries (mean per dose group) in female quail exposed to E2. A nearly significant difference between exposure scenarios ($p=0.08$) was observed; no difference ($p=0.183$) between dietary treatments was found.

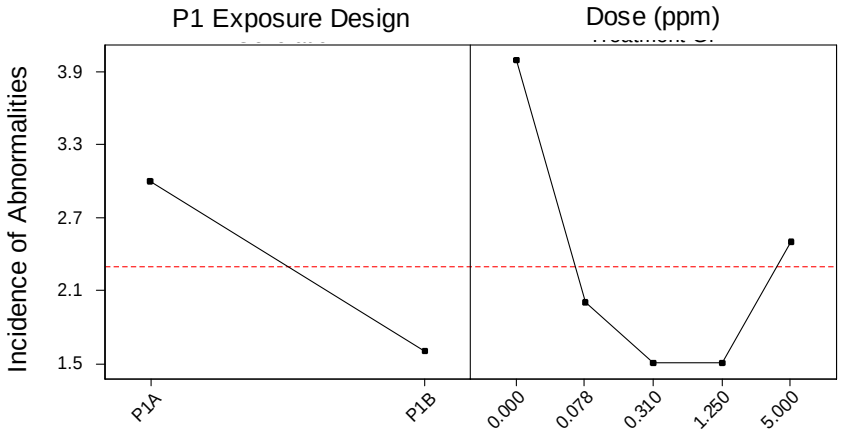


Figure 4.4-3. Main effects of the General Linear Model analysis of the incidence of abnormalities (organ lesions, egg-binding, mean per dose group) in female quail exposed to E2. Difference between exposure scenarios was significant (p=0.005); a significant increase (p=0.012) with decreasing dietary treatments was found.

Males

The incidence of abnormalities and injuries are shown in Table 4.4-4. Although there were few abnormalities in male quail, there was a significant difference in the presence of organ lesions ($p < 0.033$) between the P1 dosing exposures. P1A males showed greater numbers of abnormalities. Figure 4.4-4 shows results of analysis of abnormalities in males.

Figure 4.4-4. **Incidence of abnormalities and injuries of P1 male quail by dietary treatment and exposure scenario.**

Generation and Treatment Dose	N	Presence of Injuries	Indication of Pecking	Presence of Abnormalities [a]	Chest feathers rubbed off	Head-neck feathers lost
P1A-0 ppm	7	1	0	1	2	0
P1A-0.078 ppm	8	0	0	2	3	2
P1A-0.31 ppm	7	3	2	2	4	1
P1A-1.25 ppm	8	1	0	1	1	3
P1A-5 ppm	8	3	2	0	2	3
P1B-0 ppm	6	2	1	0	2	4
P1B-0.078 ppm	8	2	2	0	4	0
P1B-0.31 ppm	8	2	2	0	2	3
P1B-1.25 ppm	9	1	1	0	2	2
P1B-5 ppm	8	1	0	0	3	0

[a] Abnormalities denote various organ lesions

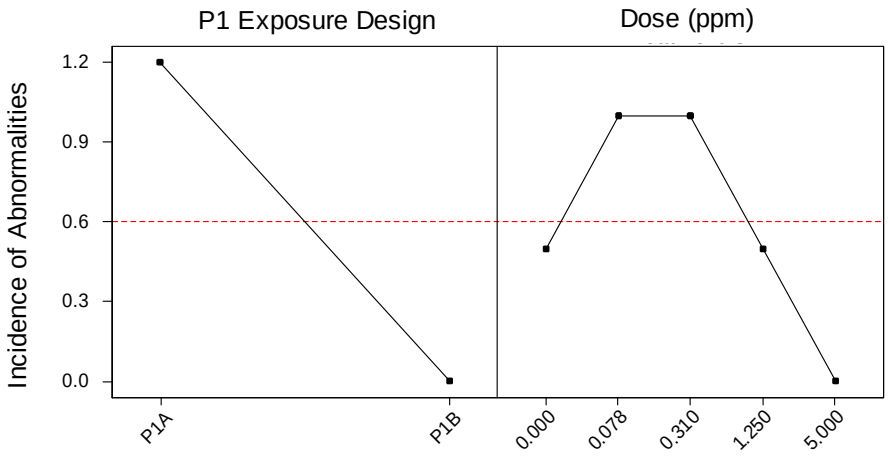


Figure 4.4-4. Main effects of the General Linear Model analysis of the incidence of abnormalities (organ lesions, mean per dose group) in male quail exposed to E2. Difference between exposure scenarios was significant ($p<0.033$); no significant differences between dietary treatments were found ($p=0.500$).

4.5 Organ Weights of Adult Quail at Necropsy (P1)

Females

Absolute and relative weights (organ weight to body weight and organ weight to brain weight ratios) of the reproductive organs of female quail were unaffected by exposure scenario (Table 4.5-1) or dietary treatment with E2. No significant difference in the number of oocytes in active growth (i.e., oocytes >4 mm in diameter and yellow in color) in the ovary per hen between dietary treatments (p=0.266) or exposure scenarios (p=0.417) was found (Figure 4.5-1).

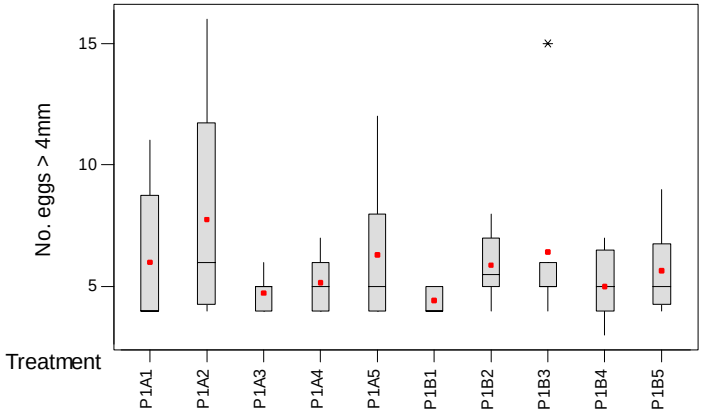


Figure 4.5-1. Box plots of the number of eggs in rapid growth (>4 mm in diameter and yellow in color) by dietary treatment for female quail exposed from 3 weeks of age through egg laying (P1A) and female quail exposed from the onset of egg laying (P1B). Means are indicated by solid circles.

Two organs in female quail were affected by E2. Absolute weights of the thyroid and adrenal glands differed significantly between the two exposure scenarios (p<0.05) (Figures 4.5-2 through 4.5-5) with greater gland weight occurring in birds exposed under the P1B scenario (Figures 4.5-2 through 4.5-5). Relative weights (i.e., weights normalized to body weight or to brain weight) of these glands were also significantly greater in the P1B exposed hens (p<0.04). However, for the thyroid gland, elevated thyroid weights were also seen in the P1B controls. Indeed, the P1B control mean was greater than all other group means. The overall mean effect of dietary treatments was not significant for either gland (Figure 4.5-3 and 4.5-5) or all remaining organs.

Table 4.5-1. Necropsy body weight (g) and organ weights (g) of female quail by exposure scenario of the P1 generation. Birds were exposed to E2.

Variable	Generati	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Body Weight	P1A	37	288	288	27.9	239	348	268	309	10%
	P1B	38	291	284	30.6	244	363	271	305	11%
Thyroid [a]	P1A	37	0.037	0.036	0.012	0.021	0.073	0.028	0.043	33%
	P1B	38	0.045	0.043	0.017	0.022	0.101	0.033	0.056	38%
Adrenal Gland [a]	P1A	36	0.031	0.031	0.014	0.005	0.066	0.021	0.042	45%
	P1B	38	0.040	0.037	0.020	0.012	0.091	0.023	0.052	49%
Liver	P1A	37	10.1	9.52	1.72	7.46	13.9	8.64	11.2	17%
	P1B	38	9.71	9.55	1.61	7.06	14.2	8.46	10.7	17%
Brain	P1A	37	0.818	0.822	0.063	0.642	0.941	0.782	0.864	8%
	P1B	38	0.813	0.809	0.067	0.623	0.938	0.775	0.863	8%
Left Ovary	P1A	37	8.32	7.89	2.81	4.19	19.5	6.65	9.34	34%
	P1B	38	8.68	8.28	2.15	3.7	13.3	7.26	10.5	25%
No. eggs > 4mm	P1A	37	6.03	5.0	2.89	4.0	16.0	4.0	7.0	48%
	P1B	38	5.47	5.0	2.04	3.0	15.0	4.0	6.0	37%
Left Oviduct	P1A	37	12.0	11.0	3.63	6.47	23.0	9.71	14.0	30%
	P1B	38	11.4	11.3	1.53	8.07	15.5	10.4	12.4	13%
Ratio of Thyroid to BW [a]	P1A	37	0.00013	0.00013	0.00004	0.00006	0.00022	0.0001	0.00015	31%
	P1B	38	0.00016	0.00016	0.00005	0.00007	0.00028	0.00011	0.00019	31%
Ratio of Adrenal to BW [a]	P1A	36	0.00011	0.00011	0.00005	0.00002	0.00021	0.00007	0.00014	45%
	P1B	38	0.00014	0.00012	0.00007	0.00004	0.00033	0.00008	0.00019	50%
Ratio of Liver to BW	P1A	37	0.03508	0.03490	0.00507	0.02735	0.04821	0.03115	0.03788	14%
	P1B	38	0.03343	0.03214	0.00480	0.02693	0.05089	0.03032	0.0357	14%
Ratio of Brain to BW	P1A	37	0.00287	0.00280	0.00037	0.00214	0.00351	0.00261	0.0032	13%
	P1B	38	0.00282	0.00285	0.00035	0.00173	0.00359	0.00267	0.00303	12%
Ratio of Left Ovary to BW	P1A	37	0.02877	0.02717	0.00859	0.01602	0.06224	0.02345	0.03333	30%
	P1B	38	0.0298	0.03021	0.00645	0.01274	0.04164	0.02536	0.0332	22%
Ratio of Left Oviduct to BW	P1A	37	0.04189	0.03895	0.0125	0.02254	0.0718	0.03256	0.04883	30%
	P1B	38	0.03933	0.03866	0.0053	0.02621	0.04833	0.03627	0.04411	13%
Ratio of Thyroid to Brain [a]	P1A	37	0.046	0.045	0.016	0.024	0.094	0.034	0.053	35%
	P1B	38	0.057	0.054	0.025	0.026	0.163	0.040	0.070	44%
Ratio of Adrenal to Brain [a]	P1A	36	0.038	0.036	0.018	0.006	0.075	0.026	0.047	46%
	P1B	38	0.049	0.045	0.023	0.015	0.108	0.032	0.064	47%
Ratio of Liver to Brain	P1A	37	12.4	11.6	2.21	8.94	17.7	10.8	13.9	18%
	P1B	38	12.0	11.4	2.26	9.10	20.0	10.4	12.8	19%
Ratio of Left Ovary to Brain	P1A	37	10.2	9.64	3.65	4.94	26.3	7.90	11.6	36%
	P1B	38	10.8	10.3	3.01	4.67	19.9	8.60	12.0	28%
Ratio of Left Oviduct to Brain	P1A	37	14.7	13.3	4.52	8.43	27.7	11.7	16.9	31%
	P1B	38	14.1	14.2	1.90	9.81	17.5	12.7	15.2	14%

[a] Significant ($p<0.05$) difference between P1A and P1B exposure scenarios (General Linear Model analysis)

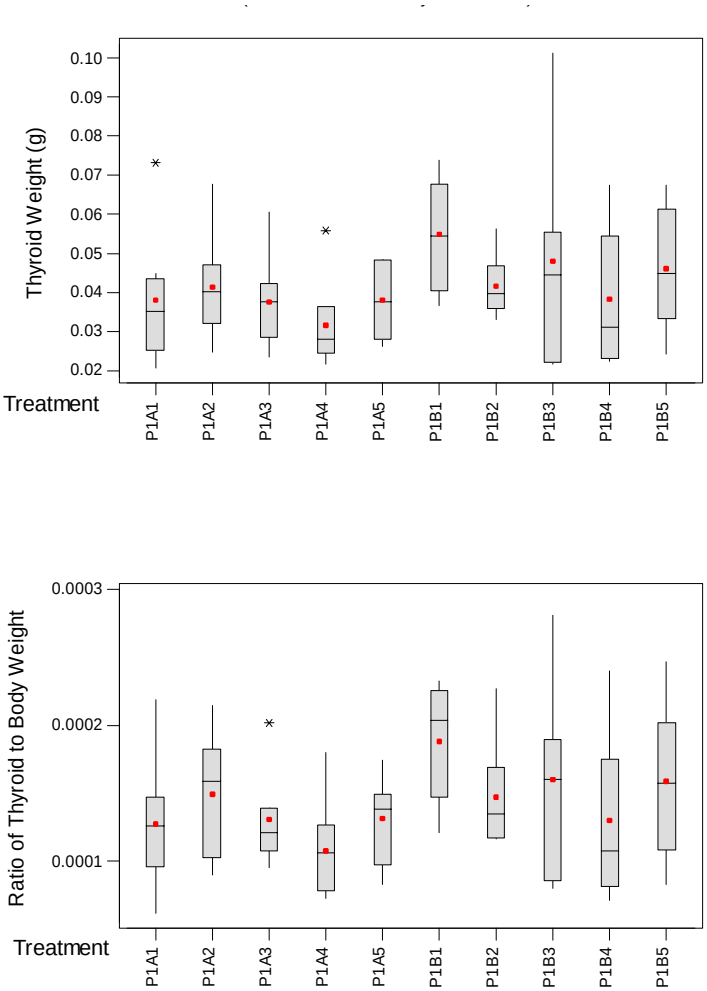


Figure 4.5-2. Box plots of thyroid gland weight (g) by dietary treatment for female quail exposed from 3 weeks of age through egg laying (P1A) and female quail exposed from the onset of egg laying (P1B). Absolute and relative (thyroid to body weight ratio) weights are shown. Means are indicated by solid circles. An * indicates an extreme value.

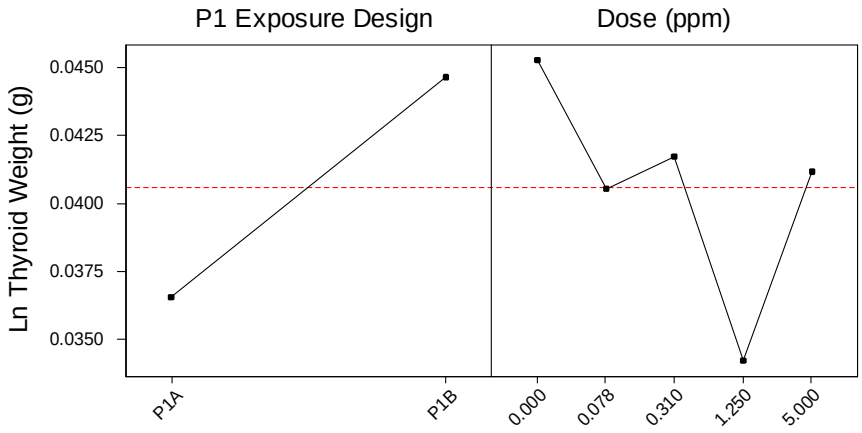


Figure 4.5-3. Main effects of the General Linear Model analysis of the natural log-transformed weights (g) of the thyroid gland. Difference in exposure scenario is significant ($p<0.02$); no significant difference in dietary treatment was found.

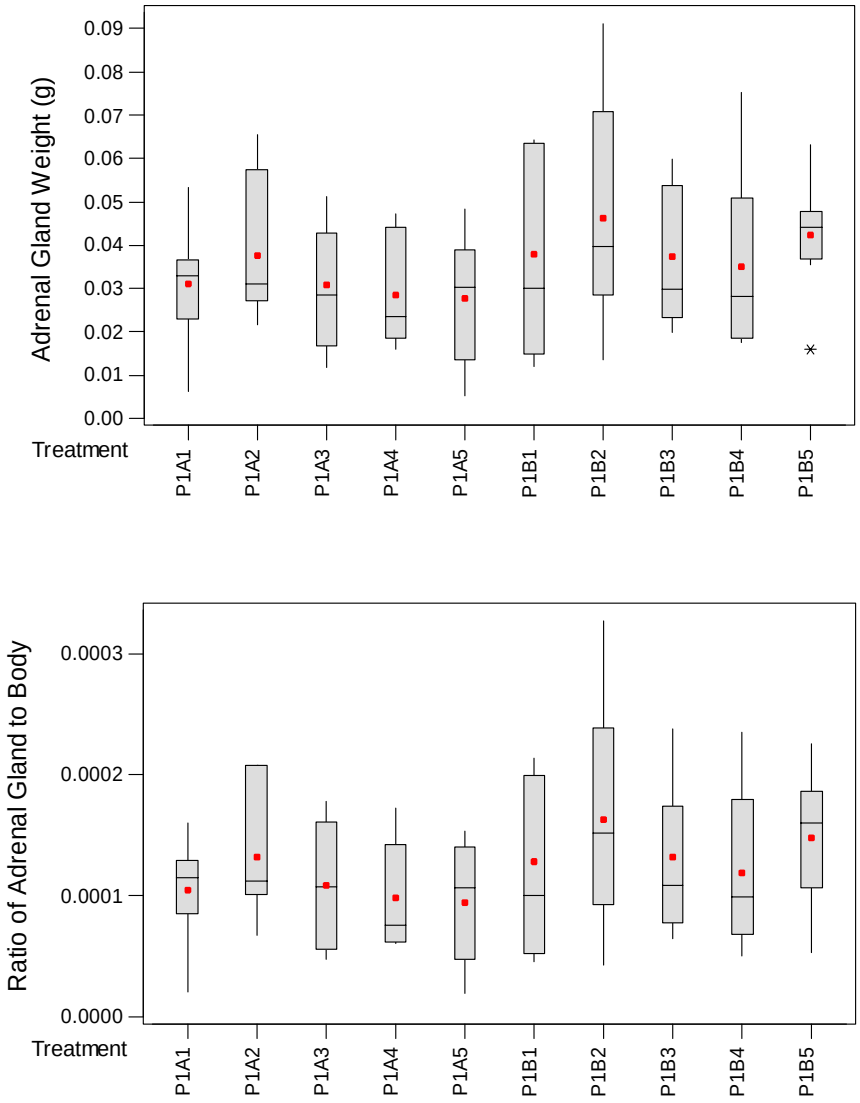


Figure 4.5-4. Box plots of adrenal gland weight (g) by dietary treatment for female quail exposed from 3 weeks of age through egg laying (P1A) and female quail exposed from the onset of egg laying (P1B). Absolute and relative (adrenal to body weight ratio) weights are shown. Means indicated by solid circles.

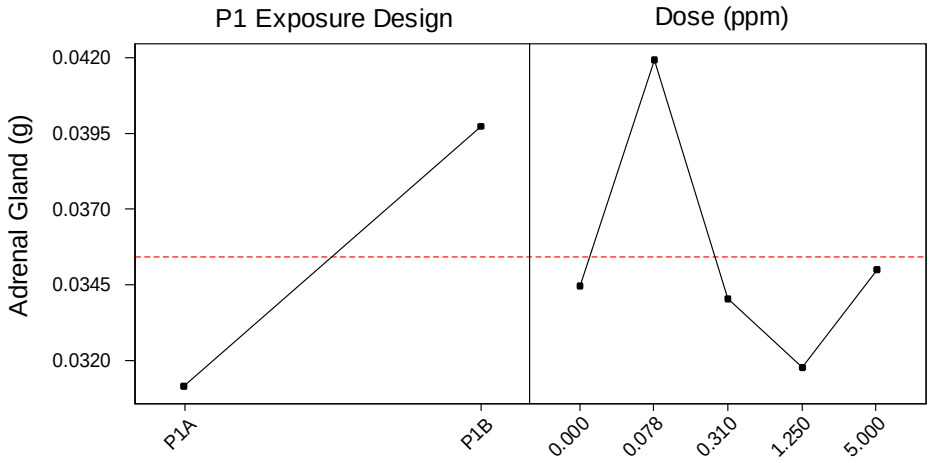


Figure 4.5-5. Main effects of the General Linear Model analysis of the absolute weights (g) of the adrenal gland in female *Corturnix* exposed to E2. Difference in exposure scenarios was significant ($p<0.05$); no significant difference in dietary treatment was found.

Males

In males, no absolute or relative weights of reproductive organs were significantly affected by dietary exposure to E2. Testis asymmetry (ratio of left to right testis) was also unaltered by E2 exposure. Of all organs, only the adrenal gland showed a significant ($p=0.049$) change in weight in response to exposure scenario (Table 4.5-2). As in females, the adrenal weight was increased in birds exposed to E2 after the onset of egg laying (P1B), and no significant dietary exposure response was observed ($p>0.47$) (Figure 4.5-6). While the difference between absolute gland weights was significant between exposure scenarios, the relative weights were nearly significantly ($p< 0.091$) enlarged in the P1B population. However, the enlargement appears to be attributable, not to greater effect of E2 post purberty, but to the greater mean gross or relative weights of the controls and 0.078 ppm groups. Indeed, there is a significant negative regression ($p=0.046$) of the organ weight against dietary concentration in the P1B population, although the mean weights are not different between groups.

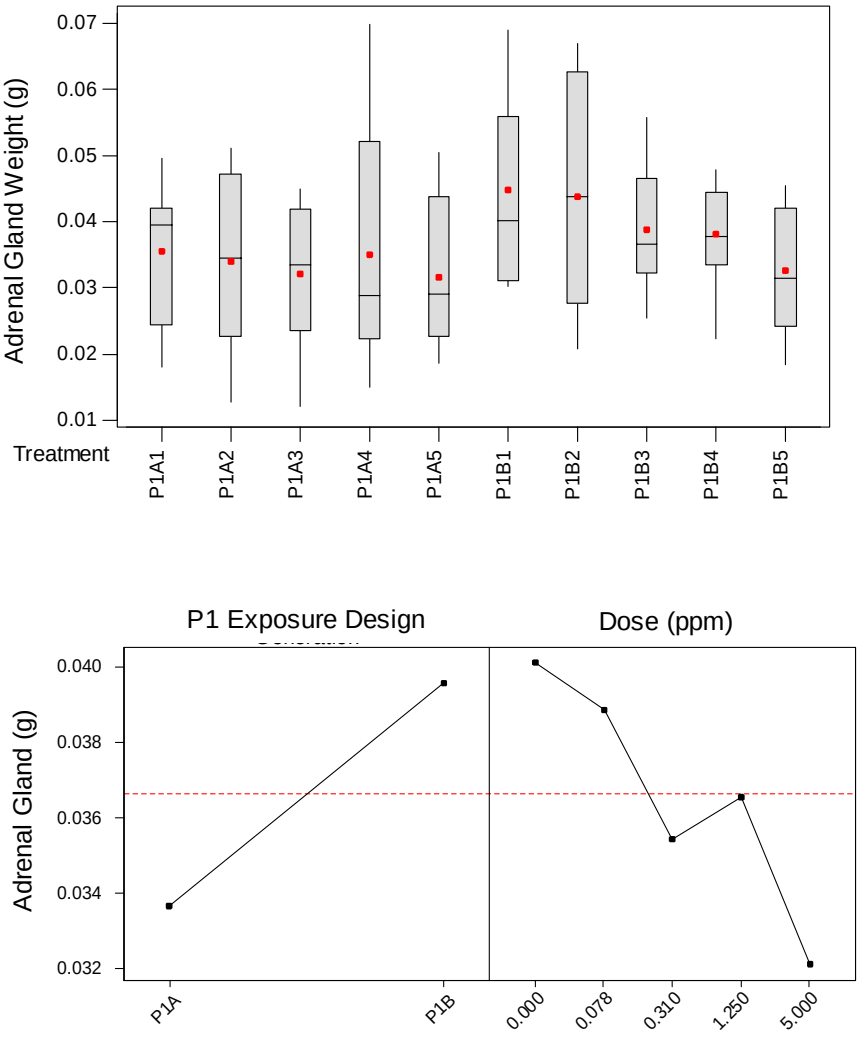


Figure 4.5-6. Box plots (above) of adrenal gland weight (g) by dietary treatment for male quail exposed from 3 weeks of age through egg laying (P1A) and male quail exposed from the onset of egg laying (P1B). Main effects of the General Linear Model analysis (below) of the absolute weights of the adrenal gland of male quail exposure to E2. Difference in exposure scenario is significant ($p<0.05$); no significant difference in dietary treatment was found. Means are indicated by solid circles.

Table 4.5-2. Necropsy body weight (g) and organ weights (g) of male quail by exposure scenario of the P1 generation. Birds were exposed to E2.

Variable	Scenario	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Body Weight	P1A	38	232	230	17.5	190	266	220	249	8%
	P1B	40	235	233	22.4	198	318	222	246	10%
Cloacal Gland	P1A	38	1.61	1.73	1.01	0.139	3.23	0.37	2.43	62%
	P1B	40	1.82	2.05	1.25	0.168	4.37	0.431	2.81	69%
Thyroid	P1A	38	0.0278	0.0259	0.013	0.011	0.0713	0.0167	0.0360	47%
	P1B	40	0.0316	0.0306	0.0099	0.0167	0.0563	0.0221	0.0389	32%
Adrenal[a]	P1A	38	0.0337	0.0330	0.013	0.0121	0.0698	0.02335	0.04193	39%
	P1B	40	0.0394	0.0382	0.012	0.0184	0.069	0.0308	0.04593	31%
Liver	P1A	38	4.92	4.84	0.64	3.84	6.52	4.41	5.36	13%
	P1B	40	4.87	4.65	0.81	3.61	7.35	4.44	5.11	17%
Left Testis	P1A	38	3.34	3.40	0.54	2.26	5.07	2.89	3.60	16%
	P1B	40	3.27	3.29	0.53	2.05	4.26	3.00	3.71	16%
Right Testis	P1A	38	3.27	3.37	0.49	1.96	4.18	2.91	3.67	15%
	P1B	40	3.29	3.24	0.52	2.44	4.68	2.89	3.76	16%
Brain Weight	P1A	38	0.813	0.829	0.10	0.365	1.006	0.788	0.845	13%
	P1B	40	0.829	0.832	0.067	0.659	0.972	0.787	0.869	8%
Ratio of Thyroid to BW	P1A	38	0.00012	0.00011	0.00005	0.00005	0.00028	0.00007	0.00016	42%
	P1B	40	0.00014	0.00013	0.00004	0.00007	0.00024	0.0001	0.00016	29%
Ratio of Adrenal to BW	P1A	38	0.00015	0.00014	0.00006	0.00005	0.00032	0.0001	0.00018	40%
	P1B	40	0.00017	0.00017	0.00005	0.00007	0.00032	0.00013	0.00019	29%
Ratio of Liver to BW	P1A	38	0.0213	0.0213	0.0021	0.0182	0.0264	0.0197	0.0225	10%
	P1B	40	0.0207	0.0204	0.0024	0.0167	0.0268	0.0188	0.0224	12%
Ratio of Left Testis to BW	P1A	38	0.0145	0.0144	0.0022	0.0090	0.0196	0.0131	0.0164	15%
	P1B	40	0.0139	0.0142	0.0020	0.0090	0.0182	0.0128	0.0151	14%
Ratio of Right Testis to BW	P1A	38	0.0142	0.0143	0.0022	0.0089	0.0199	0.0127	0.0160	16%
	P1B	40	0.0140	0.0141	0.0019	0.0101	0.0178	0.0126	0.0151	13%
Ratio of Cloacal Gland to BW	P1A	38	0.0070	0.0077	0.0044	0.00058	0.0146	0.0017	0.0107	63%
	P1B	40	0.0078	0.0086	0.0053	0.00073	0.0196	0.0020	0.0112	69%
Ratio of Brain to BW	P1A	38	0.0035	0.0036	0.00050	0.0016	0.0045	0.0033	0.0038	14%
	P1B	40	0.0036	0.0036	0.00042	0.0023	0.0044	0.0033	0.0038	12%
Ratio of Thyroid to Brain	P1A	38	0.0343	0.0321	0.016	0.0139	0.0848	0.0211	0.0457	45%
	P1B	40	0.0381	0.0361	0.012	0.0187	0.0656	0.0286	0.0447	31%
Ratio of Adrenal to Brain	P1A	38	0.0417	0.0406	0.016	0.0153	0.0827	0.0285	0.0508	39%
	P1B	40	0.0477	0.0463	0.015	0.0230	0.0919	0.0386	0.0545	32%
Ratio of Liver to Brain	P1A	38	6.18	6.01	1.30	4.47	11.6	5.29	6.69	21%
	P1B	40	5.91	5.61	1.08	4.34	10.0	5.30	6.61	18%
Ratio of Left Testis to Brain	P1A	38	4.18	3.95	0.94	2.89	7.92	3.58	4.53	23%
	P1B	40	3.97	4.07	0.71	2.47	5.70	3.57	4.42	18%

Table 4.5-2. Necropsy body weight (g) and organ weights (g) of male quail by exposure scenario of the P1 generation. Birds were exposed to E2 (continued).

Variable	Scenario	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Ratio of Right Testis to Brain	P1A	38	4.10	4.04	0.90	2.43	7.79	3.48	4.43	22%
	P1B	40	3.99	3.89	0.75	2.95	6.38	3.36	4.50	19%
Ratio of Cloacal Gland to Brain	P1A	38	2.12	2.08	1.61	0.143	8.07	0.451	2.85	76%
	P1B	40	2.25	2.44	1.63	0.195	6.64	0.544	3.40	72%
Ratio of Left to Right Testis	P1A	38	1.04	0.996	0.21	0.791	1.96	0.930	1.087	21%
	P1B	40	1.00	1.01	0.13	0.785	1.30	0.900	1.072	13%

[a] Significant ($p<0.05$) difference between P1A and P1B exposure scenarios (General Linear Model analysis)

4.6 Histology Results (P1)

4.6.1 Females

Ovary

Although a normal part of the reproductive cycle of the avian ovary, the incidence of increased follicles[2] in E2-treated hens was elevated over that of the controls in the P1A exposure scenario. This change was found in 88% (7/8) to 100% of (7/7 or 8/8) of the birds in each of the dietary treatment groups compared to 0% (0/7) in the control group (Table 4.6-1). An increased occurrence of degenerating follicles[3] in treated birds (13% to 43%) of the P1A population over that found in controls (0%) for this exposure scenario was also observed, but the proportion of affected birds per group was not concentration-linear (Table 4.6-1). In contrast, no differences in the incidence of elevated follicle number or follicle degeneration were seen in birds exposed as proven breeders (Table 4.6-1). Unlike controls in the P1A (0% incidence) exposure design, however, the occurrence of both follicle changes was elevated (57% to 100% incidence) in the P1B controls. Overall, ovarian histological response to E2 appeared to be non-concentration linear (Table 4.6-1).

Table 4.6-1. Incidence of Histological Changes in Ovarian Tissue from P1A and P1B Female Japanese Quail Exposed to E2 from Prior to Puberty and Post-Puberty, Respectively

Treatment (ppm diet)	N	Decreased Follicles	Degenerating Follicles	Increased Follicles	Infiltration of Macrophages
P1A					
0	7	1	0	0	0
0.078	8	0	3	7	1
0.31	8	0	1	7	0
1.25	8	0	1	8	0
5	7	0	3	7	0
P1B					
0	7	0	4	7	0
0.078	7	0	5	5	0
0.31	8	0	5	6	0
1.25	9	0	1	8	1
5	8	0	2	8	0

The severity of the ovarian changes in each bird were graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change. Ovarian changes recorded for both the P1A and P1B hens ranged between 1 and 3 (minimal to moderate) and did not appear to increase with increasing dietary concentration (Table 4.6-2), but were somewhat more severe in the treated P1A birds.

[2] Increased follicles were diagnosed when the number of small follicles in the ovary was increased over baseline.
[3] Degenerating follicles were diagnosed when the number of follicles undergoing degeneration was increased over baseline.

Table 4.6-2. Average severity of histological changes in ovarian tissue from P1A and P1B female Japanese quail exposed to E2 from prior to puberty and post-puberty, respectively.

Treatment (ppm diet)	N	Decreased Follicles	Degenerating Follicles	Increased Follicles
P1A				
0	7	2.0 [a]	0	0
0.078	8	0	1.3	1.7
0.31	8	0	2.0	2.0
1.25	8	0	1.0	2.1
5	7	0	1.0	2.0
P1B				
0	7	0	1.3	2.0
0.078	7	0	1.0	1.4
0.31	8	0	1.0	1.3
1.25	9	0	1.0	1.5
5	8	0	1.0	1.8

[a] Average was calculated based on the number on hens with the lesion.

Oviduct

The incidence of cellular response in the oviduct was similar to that observed for ovarian histological changes in P1A and P1B hens: an increased incidence (63% to 100%) of change (hypertrophy/hyperplasia epithelial cells lining the oviduct) in the E2 dietary treatment groups compared to a lower incidence rate (43%) in the controls of the P1A exposure regime with no comparable increase in the lesion incidence in P1B hens because of a high incident rate (100%) in the their controls (Table 4.6-3). The treatment concentration response was nonlinear. Average severity of oviduct changes did not appear to be a result of dietary treatment, but was somewhat elevated in treated P1B birds compared to treated birds in the P1A exposure scenario (Table 4.6-4).

Table 4.6-3. Incidence of histological changes in oviducts from P1A and P1B female quail exposed to E2 from prior to puberty or from post-puberty, respectively.

Treatment (ppm diet)	N	Epithelial Hyperplasia/Hypertrophy	Glandular Atrophy
P1A			
0	7	3	0
0.078	8	5	1
0.31	8	8	3
1.25	8	7	0
5	6	5	0
P1B			
0	7	7	0
0.078	8	8	0
0.31	8	7	0
1.25	9	9	0
5	8	6	0

Table 4.6-4. Average severity of histological changes in oviducts from P1A and P1B female Japanese quail exposed to E2 for 12 weeks from prior to puberty or post-puberty, respectively.

Treatment (ppm diet)	N	Epithelial Hyperplasia/Hypertrophy	Glandular Atrophy
P1A			
0	7	2.3[a]	0
0.078	8	1.4	1.0
0.31	8	2.3	1.3
1.25	8	2.0	0
5	6	2.4	0
P1B			
0	7	1.9	0
0.078	8	2.5	0
0.31	8	3.0	0
1.25	9	2.2	0
5	8	2.8	0

[a] Average was calculated based on the number on hens with the lesion.

Adrenal Gland

Only adrenal glands from the controls and the 5 ppm E2 treated birds of the two exposure scenarios were examined histologically. All P1B hens (8/8) exposed to 5 ppm E2 had diffuse hypertrophy of cortical and medullary cells of the adrenals, whereas the lesion was present in less than half (2/5) of the females exposed prior to puberty (P1A) to the 5 ppm diet (Table 4.6-5). Mean severity of the lesion in all P1A groups with incidence of the lesion was minimal. In the 5 ppm group of the P1B exposure scenario, there were more hens (5/8) with greater lesion severity than the minimal observed in the P1A birds, but the injury was still only slight. The incidence and severity of the adrenal lesion are shown in Table 4.6-5.

Table 4.6-5. Incidence and average severity of diffuse hypertrophy in adrenal glands from P1A and P1B females exposed to E2 from prior to puberty or post puberty, respectively.

Treatment (ppm diet)	N	Diffuse Hypertrophy	Average [a] Severity
P1A			
0	6	0	0
5	5	2	1
P1B			
0	7	1	1
5	8	8	1.6

[a] Average was calculated based on the number on hens with the lesion.

Brain, Liver, Thyroid

The incidence and severity of cellular level changes in the brain, liver and thyroid of female quail did not appear to be affected by exposure scenario or dietary concentration of E2. Appendix J contains the histopathology incidence and severity of all tissues examined.

4.6.2 Males

Testes

Dietary E2 induced degenerative changes in the seminiferous tubules of male Japanese quail exposed to the steroid prior to puberty. The degeneration was not present in control birds. Degenerative changes included reduced numbers of primary and secondary spermatocytes and increased cytoplasm granularity of the affected cells. These changes occurred in at least 50% of the males in each E2 treated group of the P1A males; however, the incidence rate did not appear to be concentration dependent in the P1A birds. Post puberty exposure to E2 induced the degenerative lesions only in the 5 ppm treatment group (Table 4.6-6). The degeneration was more severe in testes of the P1A males, ranging from minimal to moderate. In the P1B birds, E2 induced only minimal to slight testicular changes (Table 4.6-6).

Table 4.6-6. Incidence and average severity of degenerative changes in testes from P1A and P1B male Japanese quail exposed to E2 from prior to puberty or post puberty, respectively.

Treatment (ppm diet)	N	Diffuse Degeneration of the Seminiferous Tubules	Average [a] Severity
P1A			
0	9	0	0
0.078	8	4	2.5
0.31	7	7	2.1
1.25	8	5	2.0
5	8	7	1.7
P1B			
0	7	1	2
0.078	8	0	0
0.31	9	0	0
1.25	10	0	0
5	8	4	1.3

[a] Average was calculated based on the number of quail with the lesion.

A single incidence of focal infiltration of mononuclear cells also occurred in the 0.078 ppm E2 group of the P1A exposure scenario.

Epididymis

A reduction in mature spermatids in the epididymal lumens (hypospermia) was found in about 50% to 71% of the males in the E2 treated groups that were exposed to the steroid prior to puberty (Table 4.6-7). Hypospermia was not found in the controls exposed under this scenario. About 10% to 33% of males in all test groups, including controls, that began treatment after puberty (P1B) had reduced numbers of spermatids in the epididymal lumens (Table 4.6-7). The magnitude of the spermatid reduction ranged between slight to severe in both the P1A and P1B populations.

Table 4.6-7. Incidence and average severity of hypospermia in epididymis from P1A and P1B male Japanese quail exposed to E2 for starting prior to puberty or starting post puberty, respectively

Treatment (ppm diet)	N	Hypospermia	Average Severity
P1A			
0	8	0	0
0.078	7	4	3.8
0.31	7	5	3.6
1.25	8	4	3.3
5	5	3	4.3
P1B			
0	6	2	5.0
0.078	8	2	4.5
0.31	8	2	2.0
1.25	10	1	4.0
5	7	1	4.0

Cloacal Gland

Early exposure (P1A) to E2 appeared to induce atrophy of the submucosal glands of the cloacal gland (Table 4.6-8). The lesion was absent in birds exposed after puberty (P1B). The overall incidence rate of the lesion among the P1A males was modest (14%), but was higher (38%) in the 5 ppm E2 treatment group. Severity of the atrophy was also increased in males consuming the 5 ppm E2. Of the three affected birds in the 5 ppm E2 diet, one had a cloacal gland with moderate atrophy of the submucosal glands and two birds had cloacal glands with severe atrophy. In males on lower dietary treatments, the lesion was graded as slight (Appendix J).

Table 4.6-8. Incidence of histological changes in cloacal glands from P1A and P1B male Japanese quail exposed to E2 from prior to puberty and post puberty, respectively

Treatment (ppm diet)	N	Atrophy of Submucosal Glands	Dilation of Submucosal Glands	Epithelial Hyperplasia	Diffuse Hypertrophy of Submucosal Glands
P1A					
0	7	0	0	0	0
0.078	8	1	0	0	0
0.31	6	0	0	0	1
1.25	6	1	0	1	0
5	8	3	0	0	0
P1B					
0	7	0	0	0	0
0.078	7	0	3	1	0
0.31	7	0	0	0	0
1.25	9	0	0	0	0
5	8	0	0	0	0

Adrenal Gland

Adrenal glands from the controls and the 5 ppm E2 treated birds of the two exposure scenarios were examined. Only adrenal glands from treated males in the P1A exposure scenario showed a diffuse hypertrophy of cortical and medullary cells. No incidence of the lesion was observed in the adrenals of the P1A controls or any of the examined P1B male birds (Table 4.6-9). Lesion severity was moderate in 2 of the 4 birds from the P1A 5 ppm group and slight for the remaining 2 birds.

Table 4.6-9. Incidence of histological changes in adrenal glands from P1A and P1B male Japanese quail exposed to E2 from prior to puberty and post puberty, respectively.

Treatment (ppm diet)	N	Diffuse Hypertrophy	Cortical Hypertrophy
P1A			
0	7	0	0
5	4	4	0
P1B			
0	7	0	0
5	8	0	1

Brain, Liver, Thyroid

The incidence and severity of cellular level changes in the brain, liver, and thyroid of male Japanese quail were not affected by exposure scenario or dietary concentration of E2 (Appendix J).

4.7 Sexual Maturation (P1)

4.7.1 Foam Production in Males

The age at which the foamy exudate of the cloacal gland first appears did not differ (p=0.512, Kruskal-Wallis) across dietary concentrations in the males in the P1A exposure scenario. No difference in days to first production of foam was observed in the P1B population (p=0.31, Kruskal-Wallis). (P1B males were not exposed to the test substance until after puberty.) Exposure of juvenile birds to E2 during maturation (P1A) resulted in a significantly (p= 0.001) earlier production of cloacal foam than in males that were not treated prior to puberty (P1B). However, the overall mean age of onset was shortened only slightly (~1.4 days) compared to that observed in the P1B males (Figure 4.7-1). The mean, median, and variability of the maturation data for each group in each exposure scenario are shown in Figure 4.7-2.

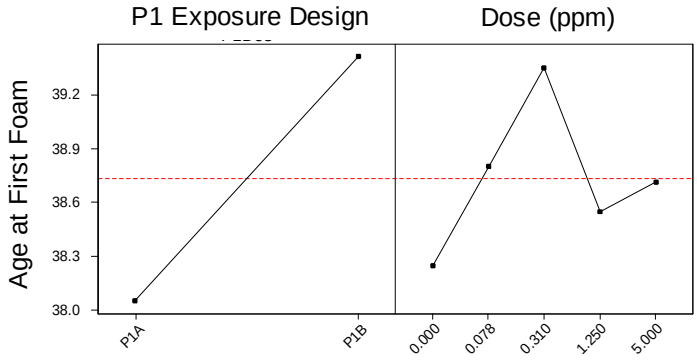


Figure 4.7-1. General Linear Model analysis of the effects of exposure scenario (pre-maturation or post maturation) and dietary treatment on the age of males (days) at first production of cloacal foam. Differences between exposure scenarios were significant (p=0.001); no significant difference in dietary treatment was found (p=0.512).

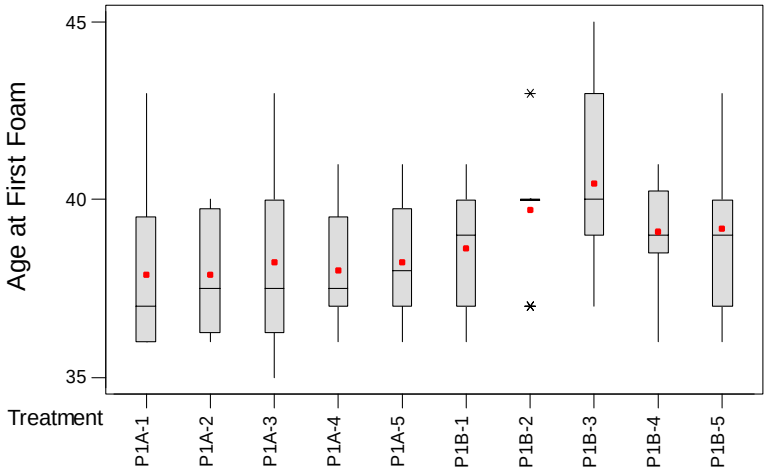


Figure 4.7-2. Box plots of age of males (days) at first production of cloacal foam by dietary treatment in each of the two exposure scenarios, exposure prior to maturation through reproduction (P1A) and exposure after the onset of reproductive maturity (P1B). Means are indicated by solid circles.

Age at first production of cloacal foam was also separately evaluated for male birds that developed phenotypic male plumage and males that developed either phenotypic female plumage or characteristics of both male and female type plumage (non-male phenotype). For birds with male phenotype plumage as adults, exposure to E2 during puberty (P1A) significantly shortened maturation age (p=0.022) compared to phenotypic adult males that were exposed to E2 after the first production of cloacal foam (P1B), but only by about 1 day (Figure 4.7-3). Adult P1A males with non-male plumage matured 2 days earlier (p= 0.042) than their P1B counterparts (Figure 4.7-4).

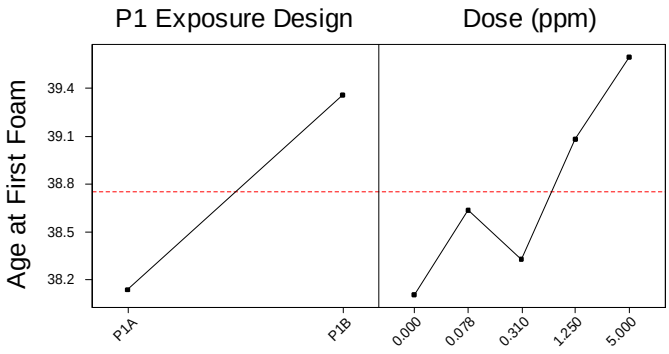


Figure 4.7-3. General Linear Model analysis of the effects of exposure scenario (pre-maturation or post maturation) and dietary treatment on the age at first production of cloacal foam in males (days) with adult phenotypic male plumage. Difference in exposure scenario was significant (p=0.022). Males in the P1B scenario are exposed after production of foam is attained.

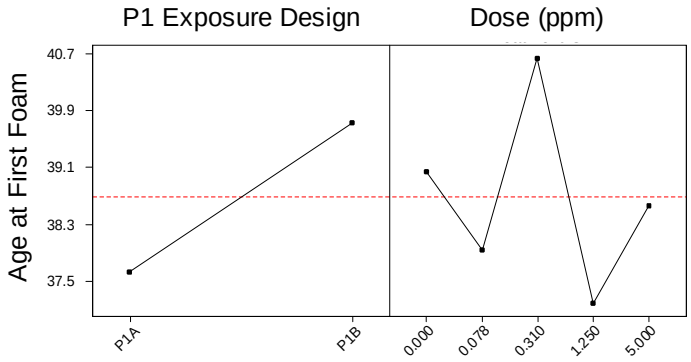


Figure 4.7-4. General Linear Model analysis of the effects of exposure scenario (pre-maturation or post maturation) and dietary treatment on the age at first production of cloacal foam in males (days) with adult non-phenotypic male plumage. Difference in exposure scenario was significant (p=0.042). Males in the P1B scenario are exposed after production of foam was attained.

4.7.2 Onset of Egg Laying in Females

The onset of sexual maturity (egg laying) in female quail exposed prior to puberty (P1A) was not affected by dietary concentration of E2 (p=0.71) (Table 4.7-1), nor did onset of egg laying of birds in the P1A exposure scenario differ significantly (p=0.29) from that of birds not treated with E2 prior to onset of egg laying (P1B). The age at which 33% of the hens in each test group were in egg production ranged between 38 and 41 and is shown in Table 4.7-2.

Table 4.7-1. Effect of estradiol (E2) on the onset of egg laying.[a] Values are in days to first egg production.

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	8	44	43	6	37	57	39	46	14%
P1A-0.078 ppm	8	43	43	5	35	51	40	48	12%
P1A-0.31 ppm	8	41	41	4	36	45	37	44	9%
P1A-1.25 ppm	8	42	42	3	37	45	38	44	8%
P1A-5 ppm	7	41	41	3	37	45	38	42	7%
P1B-0 ppm	11	41	39	5	36	52	37	43	12%
P1B-0.078 ppm	9	40	41	3	38	45	38	43	6%
P1B-0.31 ppm	9	41	40	4	39	50	39	43	8%
P1B-1.25 ppm	11	41	40	3	38	47	39	42	6%
P1B-5 ppm	9	42	42	4	37	51	39	44	10%

[a] No significant differences in the mean days to first egg production were found between dietary treatments in the P1A exposure scenario (p=0.71, Kruskal-Wallis), nor between P1A and P1B (P1B had not received treatment prior to onset of egg laying, p=0.29, General Linear Model Analysis).

Table 4.7-2. The age at which 33% of the hens in each group were in egg production.

Dose (ppm)	P1A Age	P1B Age
0	40	39
0.078	41	38
0.31	39	39
1.25	39	40
5	40	39

4.8 Plumage Dimorphism (P1)

Males

Dietary treatment with E2 resulted in increased incidence of males with phenotypic female or mixed-gender plumage (plumage with both male and female characteristics). The incidence of male-type and mixed-gender plumage among the P1A and P1B males is shown in Tables 4.8-1 and 4.8-2. Under the P1A exposure scenario, the proportion of males with mixed plumage increased significantly ($p<0.04$) in those groups fed E2 in concentrations greater than 0.078 ppm. This polynomial regression of the proportion of males with non-male plumage against the natural log-transformed dose ($R^2=0.90$) is shown in Figure 4.8-1. A similar trend ($p=0.06$) was seen in the P1B population (Figure 4.8-2).

Table 4.8-1. Incidence of male phenotypic plumage and non-male plumage in quail fed estradiol from prior to puberty through 16 weeks of age (P1A).

Dose (ppm)	Female-type Plumage		Male-type Plumage		Mixed Plumage		All Plumage Types	
	Females	Males	Females	Males	Females	Males	Females	Males
0	8	0	0	6	0	1	8	7
0.078	8	0	0	8	0	0	8	8
0.31	7	0	0	6	0	1	7	7
1.25	7	0	0	6	0	2	7	8
5	7	0	0	2	0	6	7	8
All Doses	37	0	0	28	0	10	37	38

Table 4.8-2. Incidence of male phenotypic plumage and non-male plumage in quail fed estradiol from post-maturation through 16 weeks of age (P1B).

Dose (ppm)	Female-type Plumage		Male-type Plumage		Mixed Plumage		All Plumage Types	
	Females	Males	Females	Males	Females	Males	Females	Males
0	7	0	0	7	0	0	7	7
0.078	8	0	0	5	0	3	8	8
0.31	7	0	0	6	0	2	7	8
1.25	8	0	0	6	0	3	8	9
5	8	0	0	5	0	3	8	8
All Doses	38	0	0	29	0	11	38	40

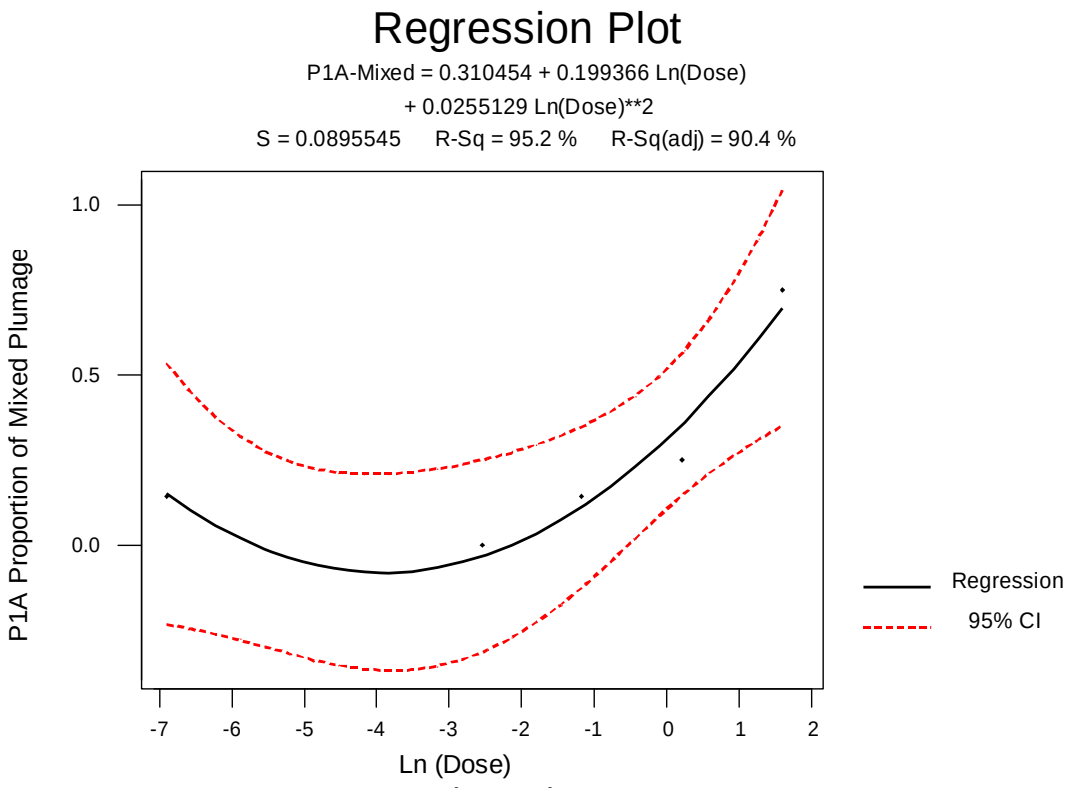


Figure 4.8-1. Proportion of mixed plumage for male P1A birds regressed against the natural logarithm transformed dietary concentration (ppm) (p<0.04).

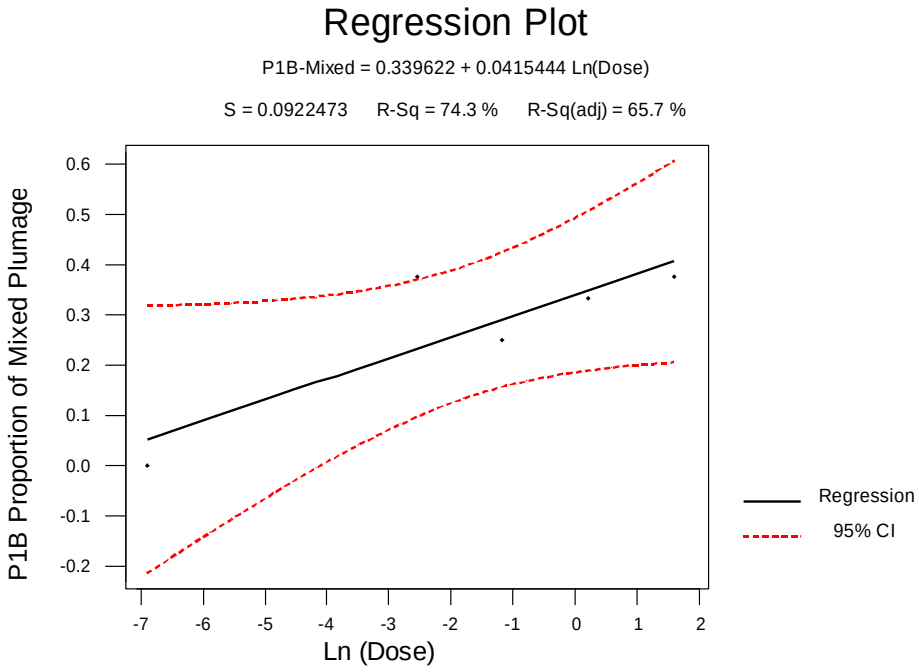


Figure 4.8-2. Proportion of mixed plumage for male P1B birds regressed against the natural logarithm transformed dietary concentrations (ppm) (p=0.06).

Length of the spotted region of non-male plumage (mm) in male Japanese quail was measured to quantify the degree of male feminization and is reported by treatment group within the P1A and P1B exposure scenarios in Table 4.8-3. Although no statistically significant change in the length of the spotted region of the breast feathers was detected in P1A or P1B males with feminized feathers ($p \geq 0.43$), there appeared to be an increase in the number of non-phenotypic males with elongated spotted breast feathers in the 5 ppm E2 treatment group of the P1A exposure scenario (Figure 4.8-3). When combined with P1B birds, there was no significant treatment effect ($p=0.45$) and no significant difference in response to exposure scenario ($p=0.60$) was found (Figure 4.8-4).

Table 4.8-3. Length of P1 non-male plumage (mm) in male Japanese quail by dietary treatment under two exposure scenarios, from pre-puberty (P1A) or from post-puberty (P1B).

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
P1A-0 ppm	1	24.2	24.2	NC	24.2	24.2	NC	NC	NC
P1A-0.078 ppm	0	NA	NA	NA	NA	NA	NA	NA	NA
P1A-0.31 ppm	0 [a]	NA	NA	NA	NA	NA	NA	NA	NA
P1A-1.25 ppm	2	26.5	26.5	5.9	22.4	30.7	NC	NC	22%
P1A-5 ppm	6	33.7	33.6	9.1	20.2	44.0	26.2	43.1	27%
P1B-0 ppm	0	NA	NA	NA	NA	NA	NA	NA	NA
P1B-0.078 ppm	2 [b]	27.5	27.5	4.9	24.0	30.9	NC	NC	18%
P1B-0.31 ppm	2	32.7	32.7	14.3	22.5	42.8	NC	NC	44%
P1B-1.25 ppm	3	24.7	22.0	6.1	20.4	31.7	20.4	31.7	25%
P1B-5 ppm	3	31.2	30.6	1.7	29.8	33.2	29.8	33.2	6%

NC Not calculable because n is too small.
NA Not applicable, n=0.
[a] One male with female-type plumage was not measured.
[b] No plumage length measurement for 1 of the 3 males with mixed plumage at this dose.

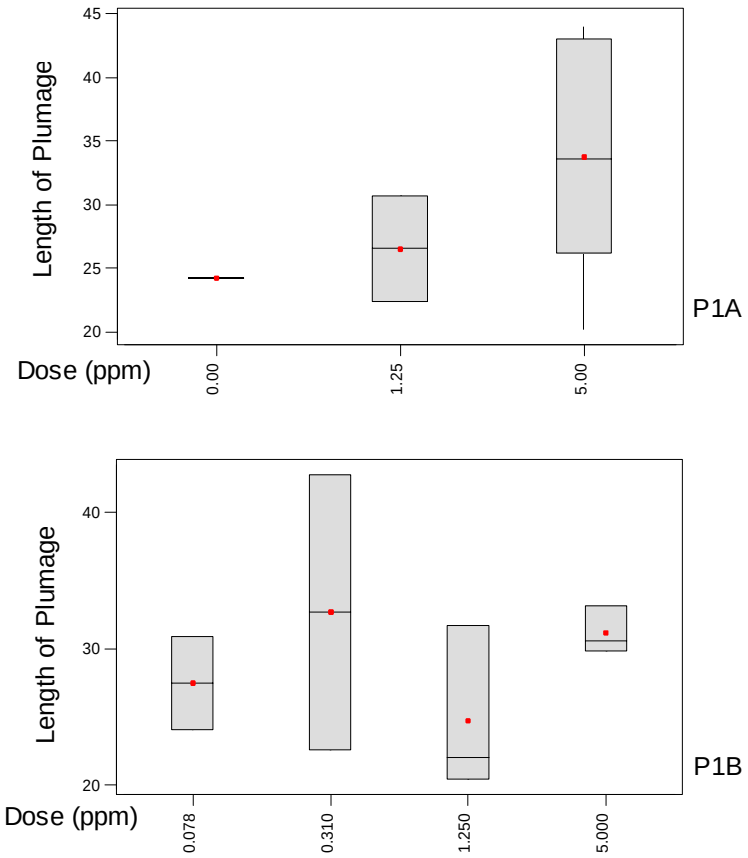


Figure 4.8-3. Length (mm) of feminized plumage in males exposed prior to puberty (P1A top) and post-maturation (P1B bottom). An increase in variability (the number of males with elongated female-type plumage) was found in the 5 ppm E2-treated group from the P1A exposure scenario. Means are indicated by solid circles.

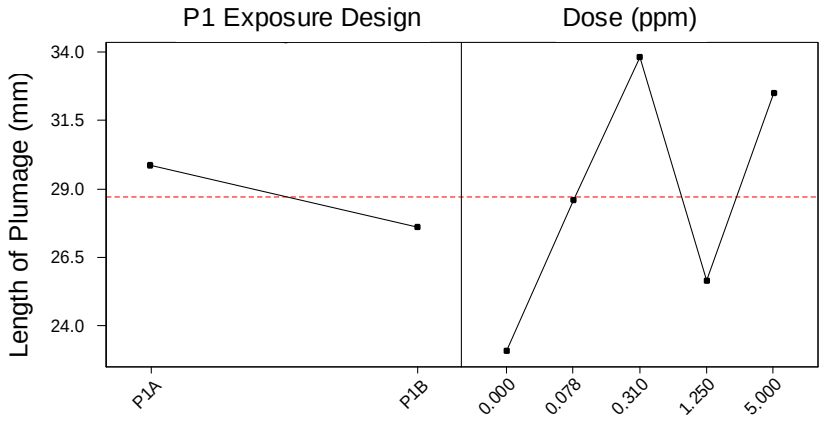


Figure 4.8-4. General Linear Model analysis of dietary concentration of E2 and the pre-maturation (P1A) or post maturation (P1B) exposure scenarios on the length (mm) of feminized feathers in male Japanese quail. No significant effect of exposure design (p=0.60) or dietary concentration (p=0.45).

Females

Although no alteration in female-type plumage coloration was observed in any hen, a nearly significant interaction between the P1 exposure scenario and E2 dietary concentration affecting the length of the spotted area covering the breast was detected (p=0.11). Hens exposed prior to maturation (P1A) that consumed the 5 ppm diet had greater lengths of the spotted area (p=0.053, Kruskall-Wallis test) than all other exposure design-dietary treatment combinations (Figures 4.8-5 and 4.8-6). When analyzed separately, the area of the spotted breast feathers was significantly (p=0.016) elongated in the 5 ppm group under the P1A exposure scenario, but was unaffected by E2 dietary treatment under the P1B design (p=0.545).

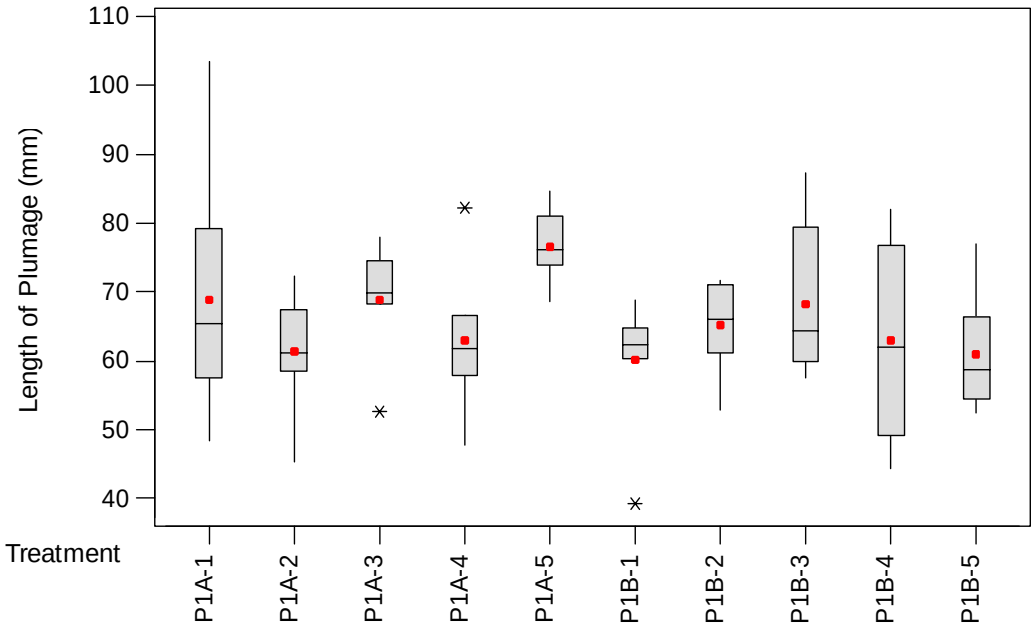


Figure 4.8-5. Box plots of the length (mm) of female phenotypic breast feathers in Japanese quail hens by dietary treatment under two exposure scenarios. Exposure beginning prior to puberty (P1A) and exposure beginning after the onset of egg laying (P1B). Means are indicated by solid circles.

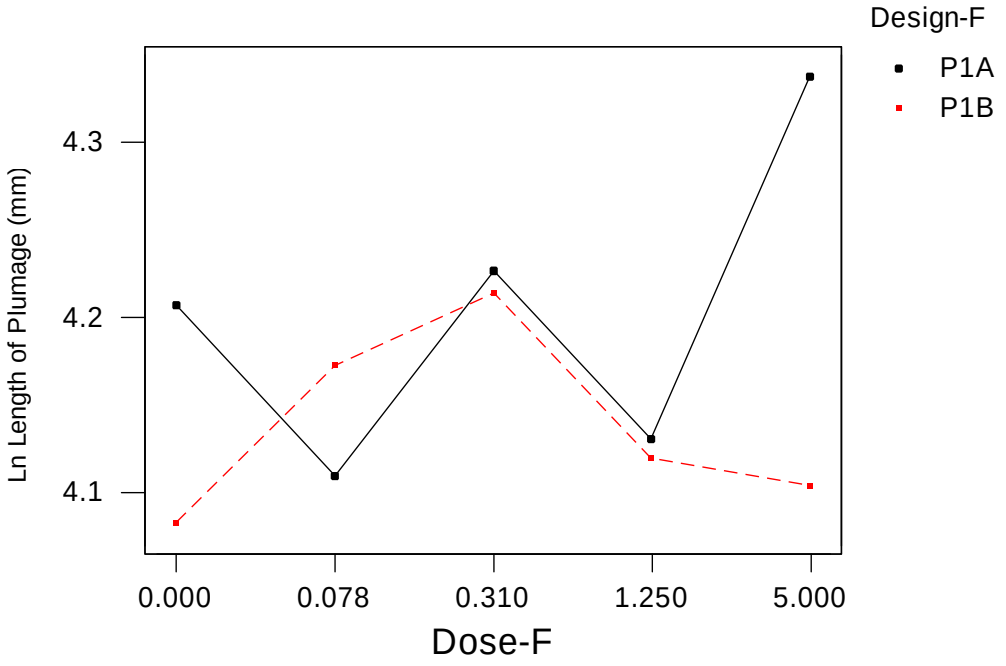


Figure 4.8-6. Interaction (p=0.11) between dietary concentration of E2 (ppm) and the pre-maturation (P1A) or post maturation (P1B) exposure scenarios on the natural log-transformed length (mm) of female phenotypic feathers of Japanese quail hens.

4.9 Cloacal Gland Size (P1)

At necropsy (Day 116 of age), the cloacal gland surface area, a measure of the androgen status of male Japanese quail, was not significantly affected ($p > 0.178$) by dietary concentrations or the pre-puberty or post-puberty exposure scenarios (Figure 4.9-1).

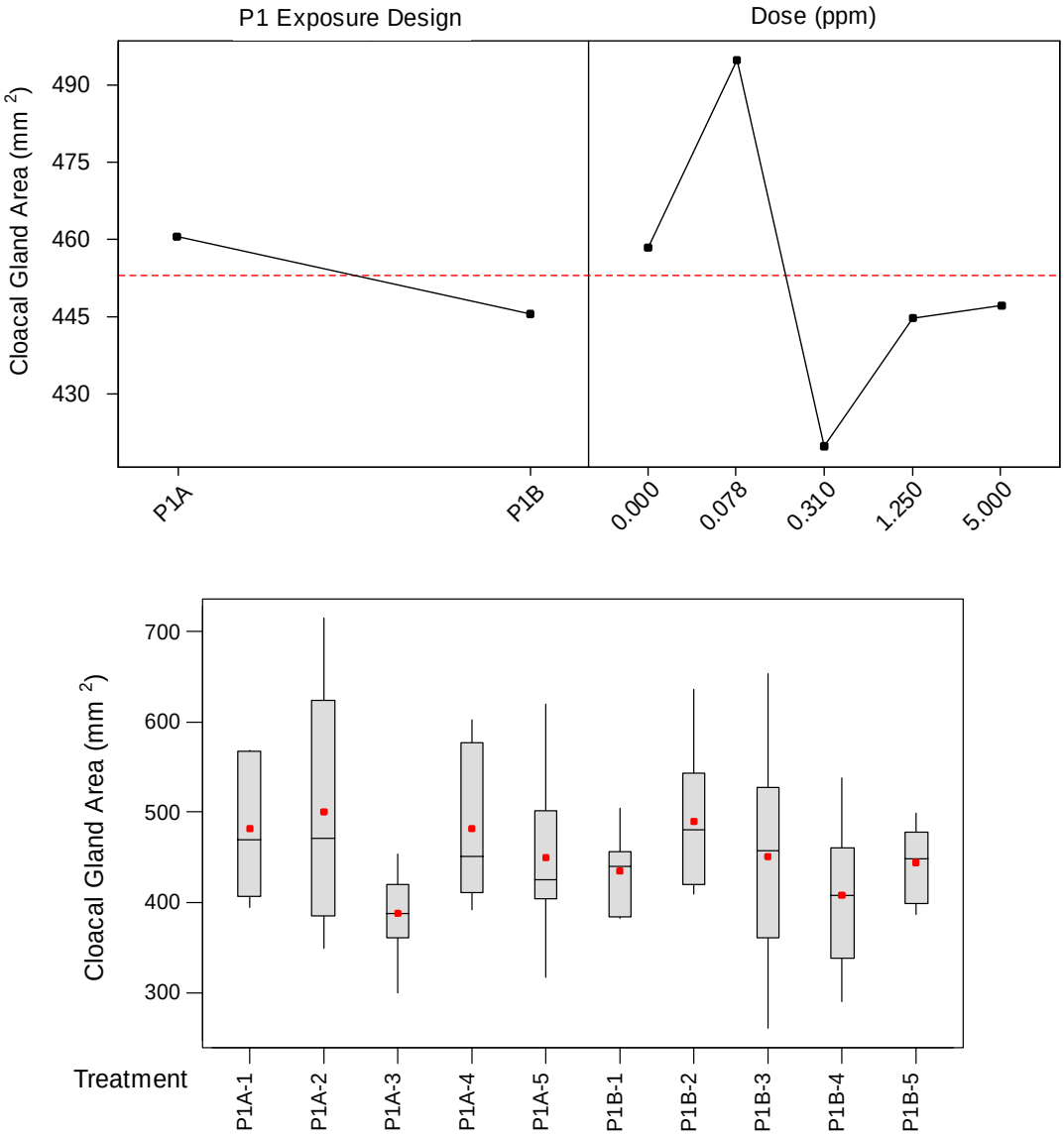


Figure 4.9-1. General Linear Model analysis (above) of the effect of dietary concentration of E2 and the pre-maturation (P1A) or post maturation (P1B) exposure scenarios on cloacal gland surface area (mm^2) in male Japanese quail and box plots (below) of the cloacal gland area (mm^2) of P1 males at necropsy (116 days old). Dietary concentrations of E2: 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm. No significant effect of exposure design (p=0.441) or dietary concentration (p>0.178).

The surface area of the cloacal gland was also measured prior to and during maturation. To reduce disturbance to the birds, the P1A and P1B birds were weighed and their cloacal gland measured on separate days. Because one day of separation during maturation when the gland is enlarging rapidly could affect direct comparison of the surface areas of the two exposure designs, gland size data for the control males from each design were compared over time (Figure 4.9-2).

As seen in Figure 4.9-2, a difference in cloacal gland size between the two exposure designs was observed, particularly at puberty (days 43 and 44), but gland enlargement over time for the two control populations was not different ($p>0.05$), indicating that the rapid change in gland size can occur over a single day during maturation. Therefore, growth curves of cloacal gland size were developed for each of the five dietary treatments within each exposure scenario (P1A and P1B) by plotting the mean surface areas of the cloacal gland as a function of age. The resulting exponential functions (Figure 4.9-3) for each diet concentration were compared using an F statistic ($\alpha = 0.05$).

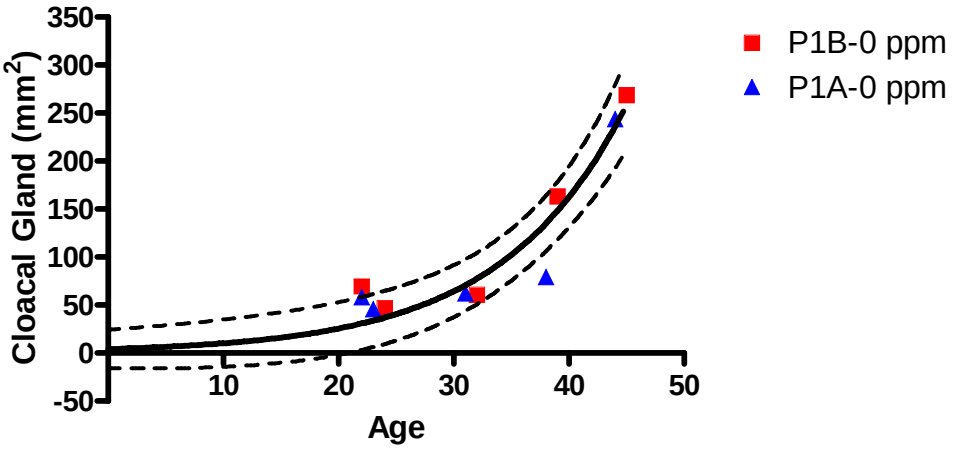


Figure 4.9-2. Comparison of the enlargement over time (days) of the cloacal gland of control males from the P1A (pre-maturation exposure to E2) and the P1B (post-maturation exposure to E2) exposure scenarios. Cloacal gland measurements for the P1B males were collected 1 day after measurements were obtained for the P1A birds. The exponential growth curves of cloacal gland size over time for the two exposure designs were not statistically different ($p>0.05$).

For males exposed to E2 from 3 weeks of age (P1A), the growth curves of the cloacal gland were not statistically different across dietary concentrations of E2 ($p>0.05$). The R^2 values of these growth curves ranged from 0.79 to 0.86. Growth in cloacal gland size over time for P1B males was also unaffected by dietary concentration ($p> 0.05$) (Figure 4.9-3). R^2 values for dietary treatment curves within the P1B design ranged between 0.89 and 0.98. When the data sets for the two exposure designs were compared, no significant difference was detected in cloacal gland enlargement over time between the P1A and P1B exposure scenarios ($p>0.05$) (Figure 4.9-4).

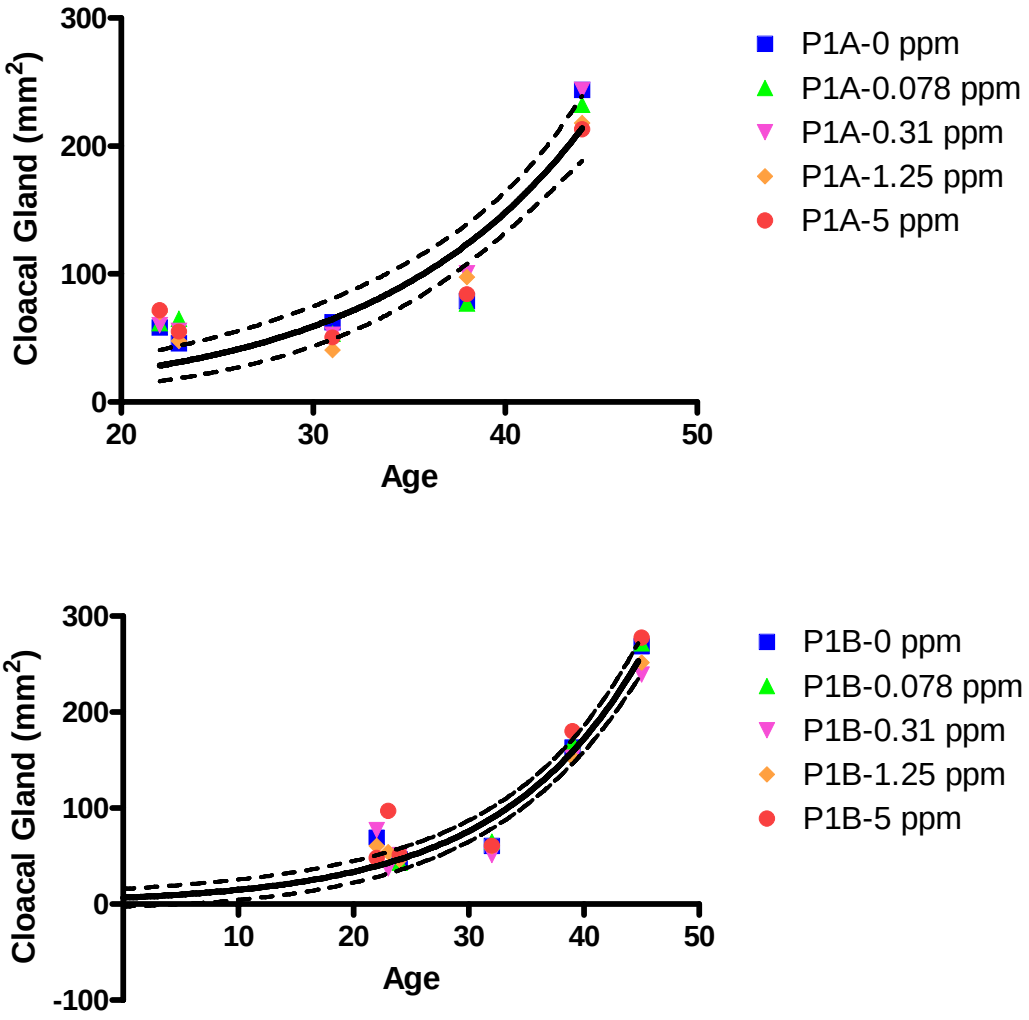


Figure 4.9-3. Comparison of the enlargement over time (days) of the cloacal gland of males from each dietary treatment group within the P1A (above) and (P1B) exposure scenarios. (P1A, pre-maturation exposure to E2; P1B, post-maturation exposure to E2.) The exponential growth curves of cloacal gland size over time across dietary concentrations within each exposure design were not statistically different ($p>0.05$).

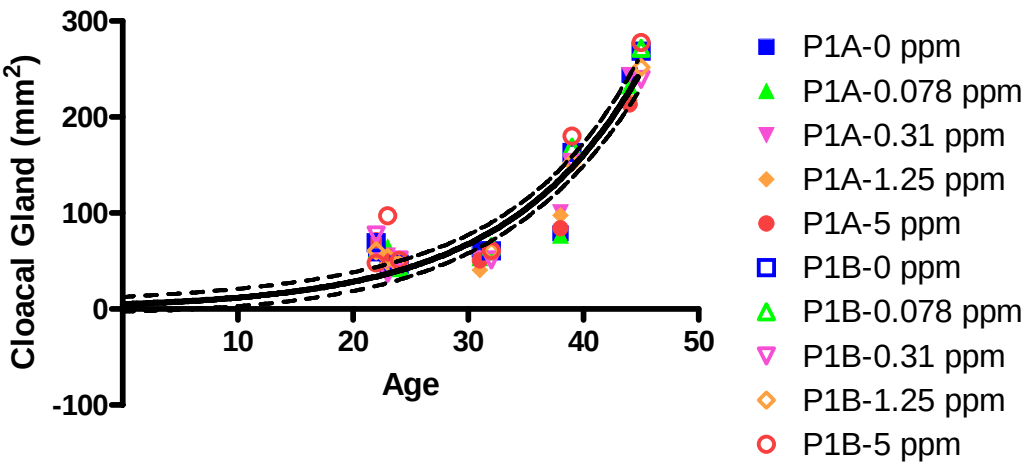


Figure 4.9-4. Comparison of the enlargement over time (days) of the cloacal gland of males from the P1A and P1B exposure scenarios. (P1A, pre-maturation exposure to E2; P1B, post-maturation exposure to E2.) The exponential growth curves of cloacal gland size over time between the two exposure designs were not statistically different ($p>0.05$).

4.10 Reproductive Parameters–Egg Counts (P1)

4.10.1 Egg Production and Viability

Total Eggs Produced

Total eggs laid per hen were not significantly different between exposure scenarios or between dietary treatments ($p > 0.49$). The cumulative number of eggs per hen was regressed against hen age and the slopes compared between dietary treatments and between the P1A and P1B scenarios. As seen in Figures 4.10-1 and 4.10-2, slopes of the mean cumulative number of eggs per hen were not affected by dietary treatment ($p=0.74$) or length of exposure to E2 ($p=0.27$).

A nearly significant difference ($p=0.08$) was observed in which the total eggs laid per hen divided by the maximum number of eggs laid was greater in birds exposed after the onset of egg laying (P1B). However, the difference in the proportions was small between the two exposure scenarios and appeared to be largely influenced by the low mean of the 0.078 ppm group of the P1A birds. Dietary treatment did not have a significant effect ($p = 0.84$) on this proportion (Figures 4.10-3 and 4.10-4).

Day 8 Viability of Eggs

The proportion of viable eggs at day 8 out of the number of eggs set was not significantly different between exposure scenarios or dietary treatments ($p>0.25$). However, the proportion of viable eggs regressed against age indicated a trend for the intercept of the regressions to decrease and slope of the regressions to increase ($p < 0.06$ and $p < 0.08$, respectively) with increasing dietary concentration of E2 (Figures 4.10-5 and 4.10-6). There were no significant differences between exposure designs ($p>0.69$).

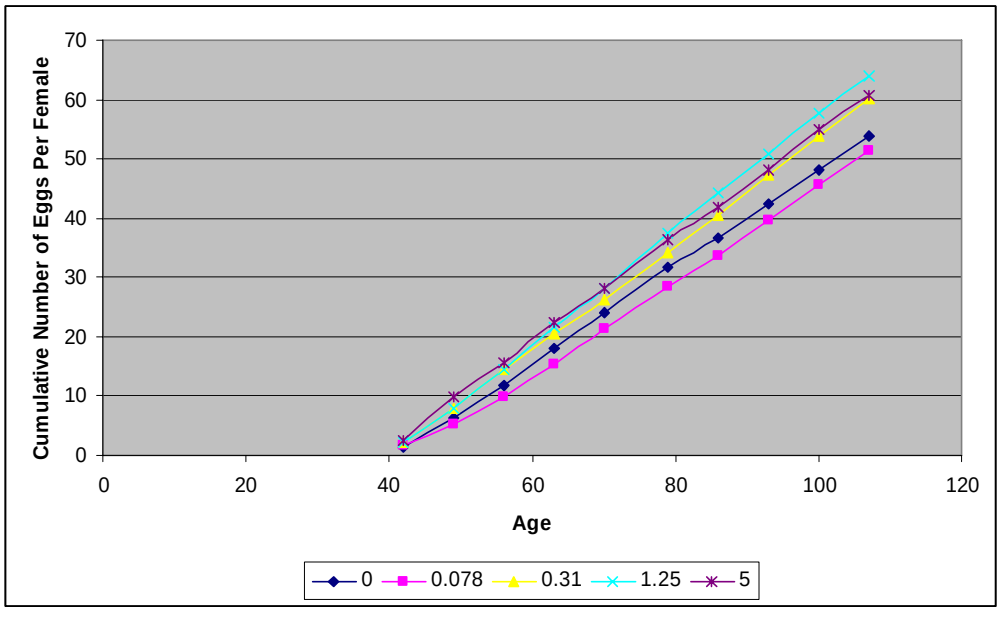


Figure 4.10-1. Cumulative number of eggs per female over age (days) by dietary treatment in hens exposed from 3 weeks of age through egg laying (P1A).

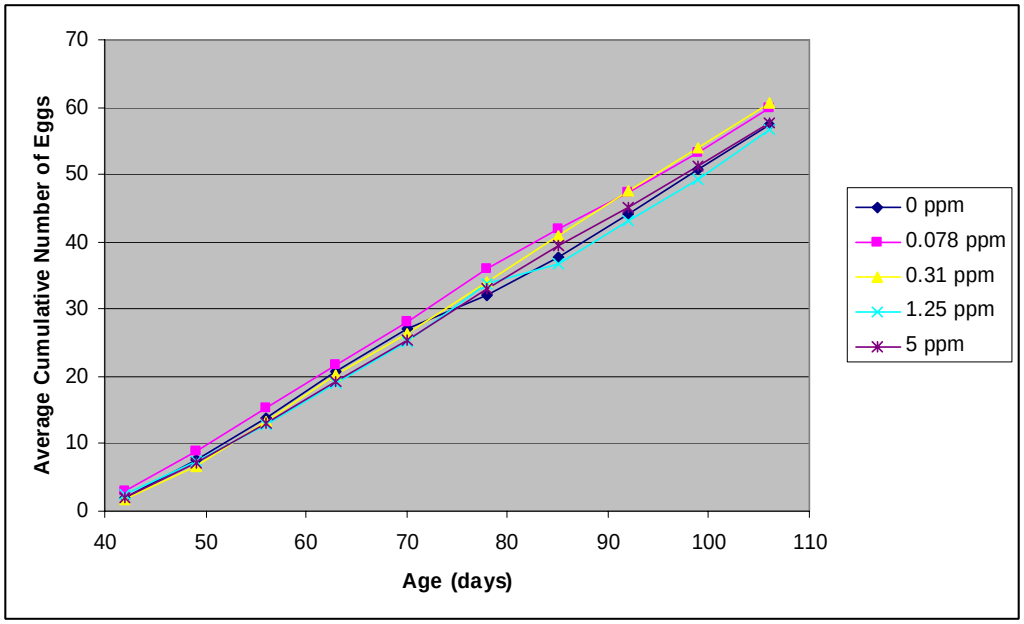


Figure 4.10-2. Average cumulative number of eggs over age by dietary treatment in hens exposed from after the onset of egg laying (P1B). P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

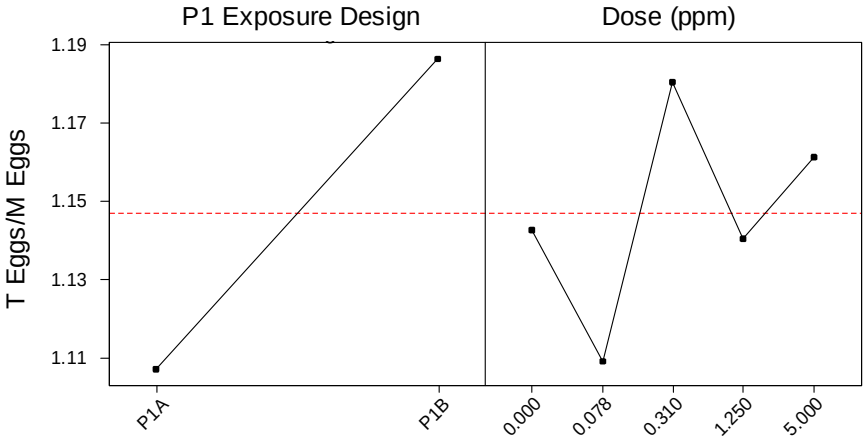


Figure 4.10-3. Main effects of the General Linear Model analysis of the arcsine-transformed proportions of total number of eggs laid per hen divided by the maximum number of eggs laid by a hen (T Eggs/M Eggs). No significant difference was observed across dietary treatments (p=0.84); a nearly significantly (p=0.08) greater total eggs per maximum number of eggs laid was found for P1B hens as compared to P1A hens.

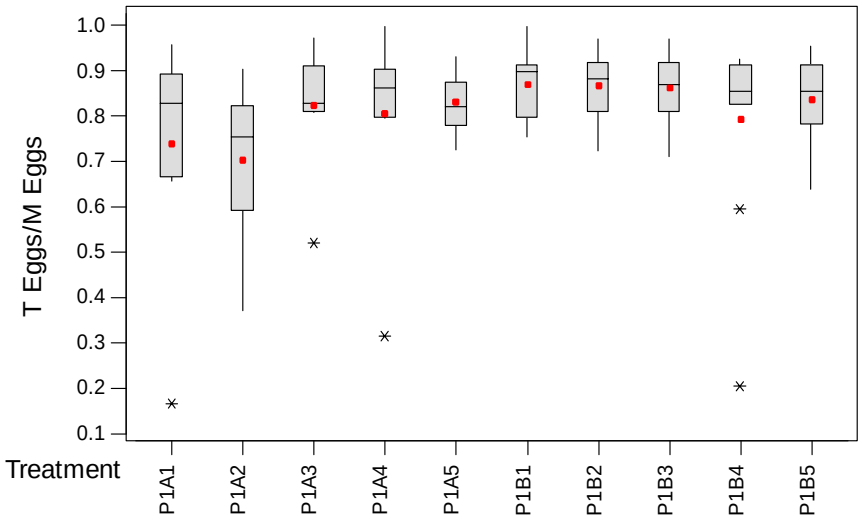


Figure 4.10-4. Box plots of the proportions of total number of eggs laid per hen divided by the maximum number of eggs laid by a hen. A slight increase in proportions for P1B hens was observed (p=0.08). P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

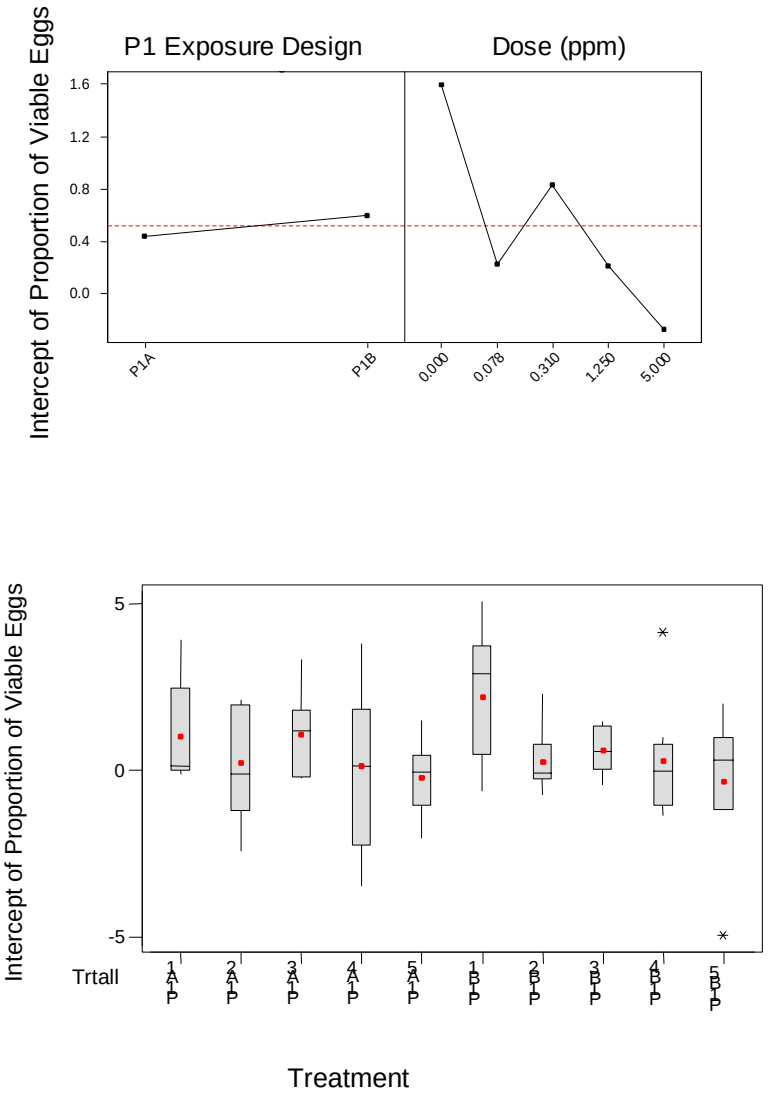


Figure 4.10-5. Main effects of the General Linear Model analysis of the intercepts of the proportion of viable eggs at day 8 out of the number of eggs set regressed against time (top). Box plots of the intercepts by dietary treatment in each of the two exposure designs are also shown (bottom). The mean intercept of the proportion of viable eggs at day 8 out of the number of eggs set decreased with dietary treatment ($p<0.06$). The effect of exposure scenario was insignificant ($p>0.69$). P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

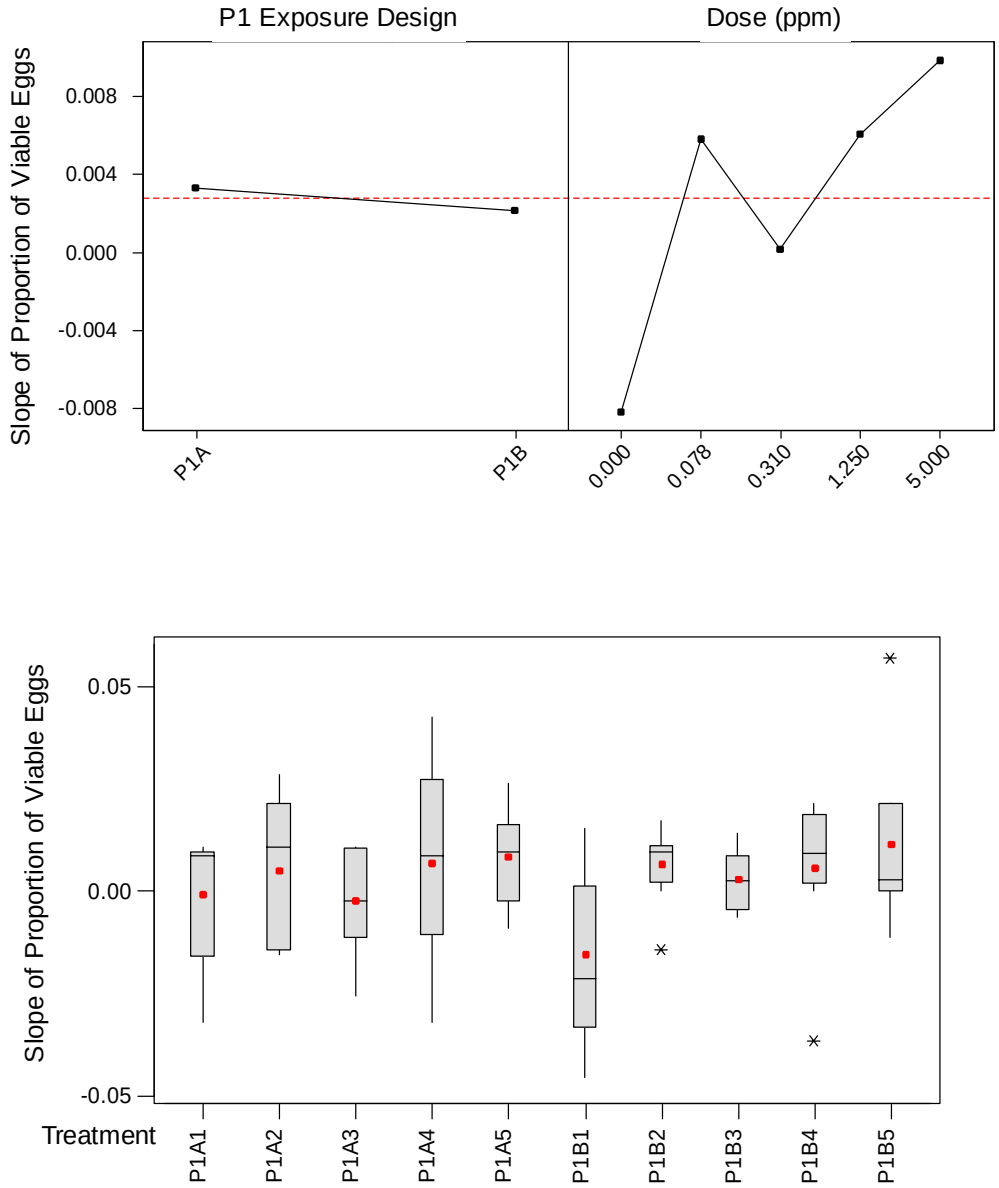


Figure 4.10-6. Main effects of the General Linear Model analysis of the slopes of the proportion of viable eggs at day 8 out of the number of eggs set regressed against time (top). Box plots of the slopes by dietary treatment in each of the two exposure designs are also shown (bottom). The mean slope of the proportion of viable eggs at day 8 out of the number of eggs set increased with dietary treatment ($p<0.08$). The effect of exposure scenario was insignificant ($p>0.69$). P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

Day 15 Viability

The main effects of exposure scenario (p=0.605) and dietary treatment (p=0.762) did not have a significant effect on the proportion of viable eggs at Day 15 out of the number of eggs set. However, they appeared to interact (p=0.108) in their effect on Day 15 viability, but this may be a random occurrence as the interaction is a result of the mean proportion of viable eggs for the P1B controls being lower (p = 0.04) than all other design concentration combinations (Figures 4.10-7 and 4.10-8).

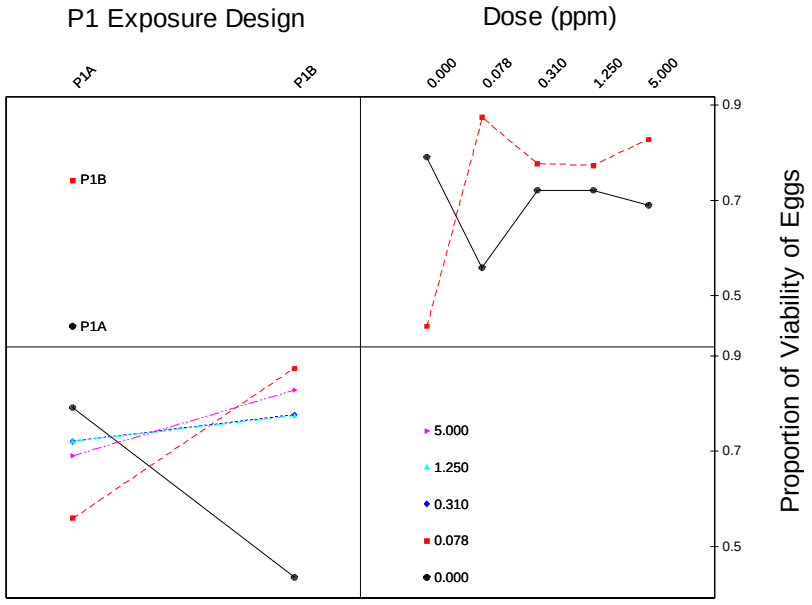


Figure 4.10-7. Interaction between the exposure scenarios and the dietary treatments affecting (p=0.108) the proportion of the viability of eggs at day 15 of the number set. P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty.

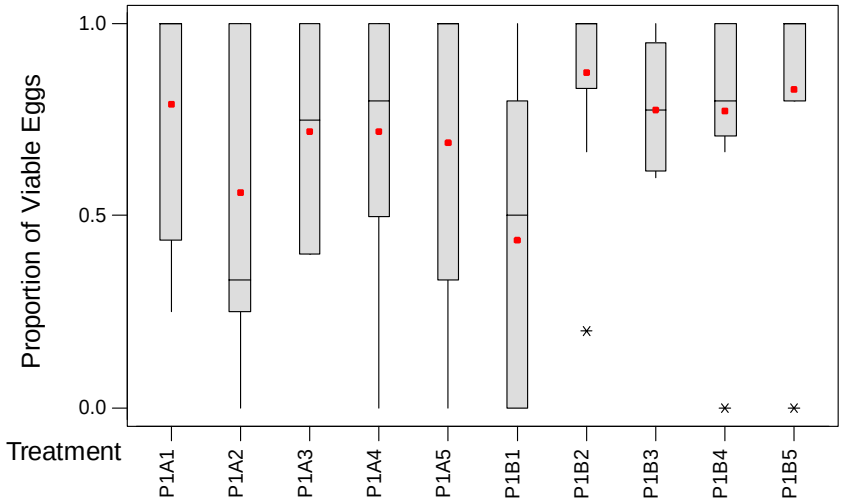


Figure 4.10-8. Box plots of the proportion of eggs viable at 15 days out of the total number set. P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

4.10.2 Hatchlings Produced

Because only the last batch of eggs collected (Week 10) were incubated to hatch, hatch data are limited to this single hatch. The surviving hatchlings made up the F1 breeding population. Hatchling data are summarized in Table 4.10-2.

Neither the number of hatchlings per number set, nor the proportion of hatchlings out of the maximum set (6), differed significantly between exposure scenarios or dietary treatment ($p \geq 0.53$ and $p > 0.60$, respectively). Hatchling number per number of viable eggs at Day 8 was also unaffected by dietary concentration of E2 ($p = 0.74$) or exposure scenario ($p = 0.36$) (Figure 4.10-9).

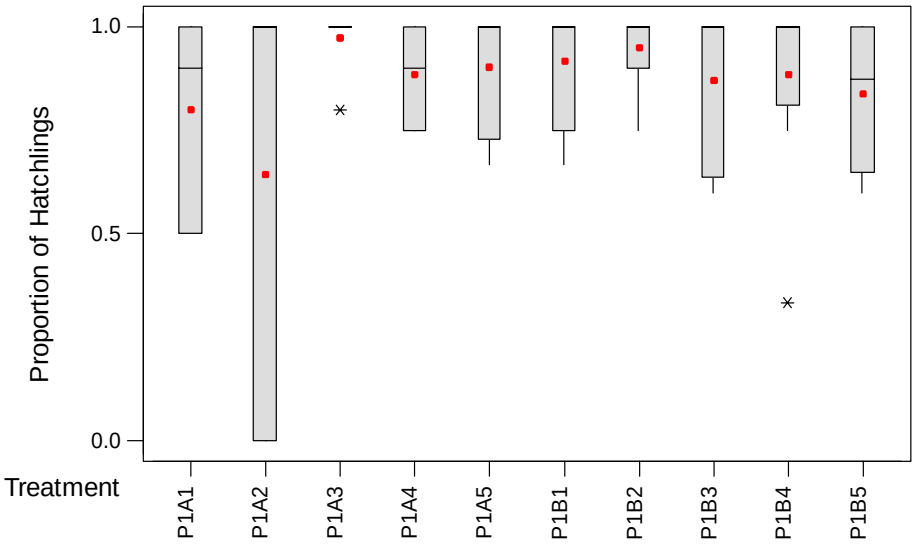


Figure 4.10-9. Box plots of the proportion of hatchlings out of the number viable at day 8 by dietary treatment group in each of the two exposure scenarios. P1A, 13 weeks of exposure to E2 starting prior to puberty; P1B, 5 weeks of exposure after puberty. Diet concentrations of E2 were 1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm.

The sex ratios of the birds hatched to form the F1 generation are shown by parental dietary concentration in Table 4.10-1. The sex ratios were increased in the low doses of the offspring of the P1A birds and then were greatly reduced at the two high concentrations. In the offspring of the P1B birds, this same pattern is repeated except only offspring of the 5 ppm concentration group exhibited a reduced male to female ratio. Reduced sex ratios combined with fewer surviving pairs (aggression) greatly reduced the number of pairs that could be made from P1A birds that did not result in sibling crosses for the F1 generation.

Table 4.10-1 Male to Female Sex Ratios of the F1 Generation by Dietary Treatment Group and Exposure Scenario of Their Parents

P1A					P1B				
0 ppm	**0.078 ppm**	**0.31 ppm**	**1.25 ppm**	**5 ppm**	**0 ppm**	**0.078 ppm**	**0.31 ppm**	**1.25 ppm**	**5 ppm**
0.5	1.0	1.4	0.2	0.3	0.9	1.2	1.2	1.1	0.4

Table 4.10-2. Percentage of eggs laid by P1 generation during week 10 of egg laying that hatched.

Variable	Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
PH-V8[a]	P1A-0 ppm	6	0.80	0.90	0.25	0.50	1.00	0.50	1.00	31%
	P1A-0.078 ppm	7	0.64	1.00	0.48	0.00	1.00	0.00	1.00	74%
	P1A-0.31 ppm	7	0.97	1.00	0.08	0.80	1.00	1.00	1.00	8%
	P1A-1.25 ppm	6	0.88	0.90	0.13	0.75	1.00	0.75	1.00	15%
	P1A-5 ppm	6	0.90	1.00	0.15	0.67	1.00	0.73	1.00	17%
	P1B-0 ppm	7	0.92	1.00	0.14	0.67	1.00	0.75	1.00	16%
	P1B-0.078 ppm	9	0.95	1.00	0.10	0.75	1.00	0.90	1.00	11%
	P1B-0.31 ppm	8	0.87	1.00	0.19	0.60	1.00	0.64	1.00	22%
	P1B-1.25 ppm	8	0.89	1.00	0.24	0.33	1.00	0.81	1.00	27%
	P1B-5 ppm	6	0.84	0.88	0.19	0.60	1.00	0.65	1.00	22%
PH-TSet[b]	P1A-0 ppm	6	0.63	0.65	0.35	0.25	1.00	0.25	1.00	55%
	P1A-0.078 ppm	7	0.44	0.33	0.42	0.00	1.00	0.00	1.00	96%
	P1A-0.31 ppm	7	0.69	0.75	0.26	0.40	1.00	0.40	1.00	38%
	P1A-1.25 ppm	7	0.69	0.75	0.35	0.00	1.00	0.50	1.00	51%
	P1A-5 ppm	7	0.66	0.75	0.39	0.00	1.00	0.33	1.00	60%
	P1B-0 ppm	11	0.42	0.33	0.41	0.00	1.00	0.00	0.80	97%
	P1B-0.078 ppm	9	0.82	1.00	0.27	0.20	1.00	0.71	1.00	33%
	P1B-0.31 ppm	8	0.70	0.63	0.14	0.60	1.00	0.60	0.79	20%
	P1B-1.25 ppm	9	0.72	0.80	0.37	0.00	1.00	0.46	1.00	51%
	P1B-5 ppm	7	0.69	0.75	0.34	0.00	1.00	0.60	1.00	49%
PH-Mset[c]	P1A-0 ppm	6	0.50	0.50	0.33	0.20	0.80	0.20	0.80	66%
	P1A-0.078 ppm	6	0.33	0.30	0.33	0.00	0.80	0.00	0.65	98%
	P1A-0.31 ppm	7	0.54	0.40	0.19	0.40	0.80	0.40	0.80	35%
	P1A-1.25 ppm	7	0.46	0.60	0.28	0.00	0.80	0.20	0.60	60%
	P1A-5 ppm	7	0.43	0.40	0.27	0.00	0.80	0.20	0.60	63%
	P1B-0 ppm	9	0.35	0.33	0.33	0.00	0.83	0.00	0.67	93%
	P1B-0.078 ppm	9	0.59	0.67	0.24	0.17	0.83	0.42	0.83	40%
	P1B-0.31 ppm	8	0.54	0.50	0.15	0.33	0.83	0.50	0.63	27%
	P1B-1.25 ppm	9	0.52	0.67	0.29	0.00	0.83	0.25	0.75	57%
	P1B-5 ppm	7	0.50	0.50	0.27	0.00	0.83	0.33	0.67	54%

[a] Percentage hatched of eggs viable at Day 8 of incubation.
[b] Percentage hatched of total eggs set.
[c] Percentage hatched of maximum eggs set

4.11 Egg Shell Quality (P1)

Egg shell quality was evaluated by candling for cracks and by measuring the thickness and strength of the shell.

4.11.1 Eggs with Cracked Shells

The numbers of cracked eggs per number of eggs laid were not significantly different between dosing strategies or between dietary treatments ($p > 0.16$). However, from the regression of the time series of the proportion of eggs cracked out of the number of eggs laid, it was found that the intercept and slope of the time series were significantly different across dietary treatments ($p < 0.01$). The mean intercept of the proportion of cracked eggs increased with dietary treatment ($p<0.02$) (Figure 4.11-1). The mean slope of the proportion of cracked eggs decreased slightly with increasing dietary concentration of E2 ($p<0.01$), with eggs from control birds having a positive slope (increased proportion of cracked eggs with age) and the birds treated above the 0.078 ppm E2 level having negative slopes (decreased proportion of cracked eggs with age) (Figure 4.11-2). No difference in the intercepts and slopes of the proportion of eggs cracked out of the number laid over time was detected between the exposure scenarios ($p>0.51$). A summary of the intercepts and slopes of the proportion of cracked eggs of those laid over time is found in Table 4.11-1.

Table 4.11-1. Slopes and Intercepts of the Time Series of the Proportion of Eggs Cracked of Eggs Laid

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Intercept, Proportion Cracked									
P1A-0 ppm	8	0.106	0.050	0.168	-0.032	0.425	-0.029	0.244	158%
P1A-0.078 ppm	8	0.152	0.103	0.286	-0.296	0.594	-0.032	0.388	188%
P1A-0.31 ppm	8	0.246	0.148	0.297	-0.014	0.771	-0.008	0.521	121%
P1A-1.25 ppm	8	0.323	0.277	0.324	-0.011	0.886	0.018	0.602	100%
P1A-5 ppm	7	0.441	0.304	0.361	0.000	0.997	0.156	0.808	82%
P1B-0 ppm	11	0.108	0.095	0.117	-0.046	0.368	0.029	0.178	108%
P1B-0.078 ppm	9	0.186	0.244	0.154	-0.014	0.411	0.020	0.303	83%
P1B-0.31 ppm	9	0.113	0.003	0.219	-0.072	0.548	-0.037	0.258	194%
P1B-1.25 ppm	10	0.290	0.144	0.287	0.028	0.780	0.060	0.571	99%
P1B-5 ppm	9	0.338	0.333	0.322	-0.177	0.817	0.132	0.601	95%
Slope, Proportion Cracked									
P1A-0 ppm	8	0.001	0.001	0.002	-0.003	0.003	-0.001	0.002	252%
P1A-0.078 ppm	8	0.000	0.000	0.004	-0.005	0.008	-0.003	0.002	2063%
P1A-0.31 ppm	8	-0.002	0.000	0.003	-0.008	0.001	-0.005	0.000	160%
P1A-1.25 ppm	8	-0.002	-0.002	0.002	-0.004	0.001	-0.004	0.000	109%
P1A-5 ppm	7	-0.004	-0.003	0.004	-0.011	0.001	-0.007	0.000	105%
P1B-0 ppm	11	0.000	0.000	0.001	-0.003	0.001	-0.002	0.001	453%
P1B-0.078 ppm	9	-0.002	-0.002	0.002	-0.005	0.001	-0.003	0.000	105%
P1B-0.31 ppm	9	0.000	0.000	0.002	-0.005	0.002	-0.002	0.001	552%
P1B-1.25 ppm	10	-0.003	-0.002	0.003	-0.007	0.000	-0.005	-0.001	99%
P1B-5 ppm	9	-0.003	-0.003	0.005	-0.009	0.008	-0.006	-0.001	188%

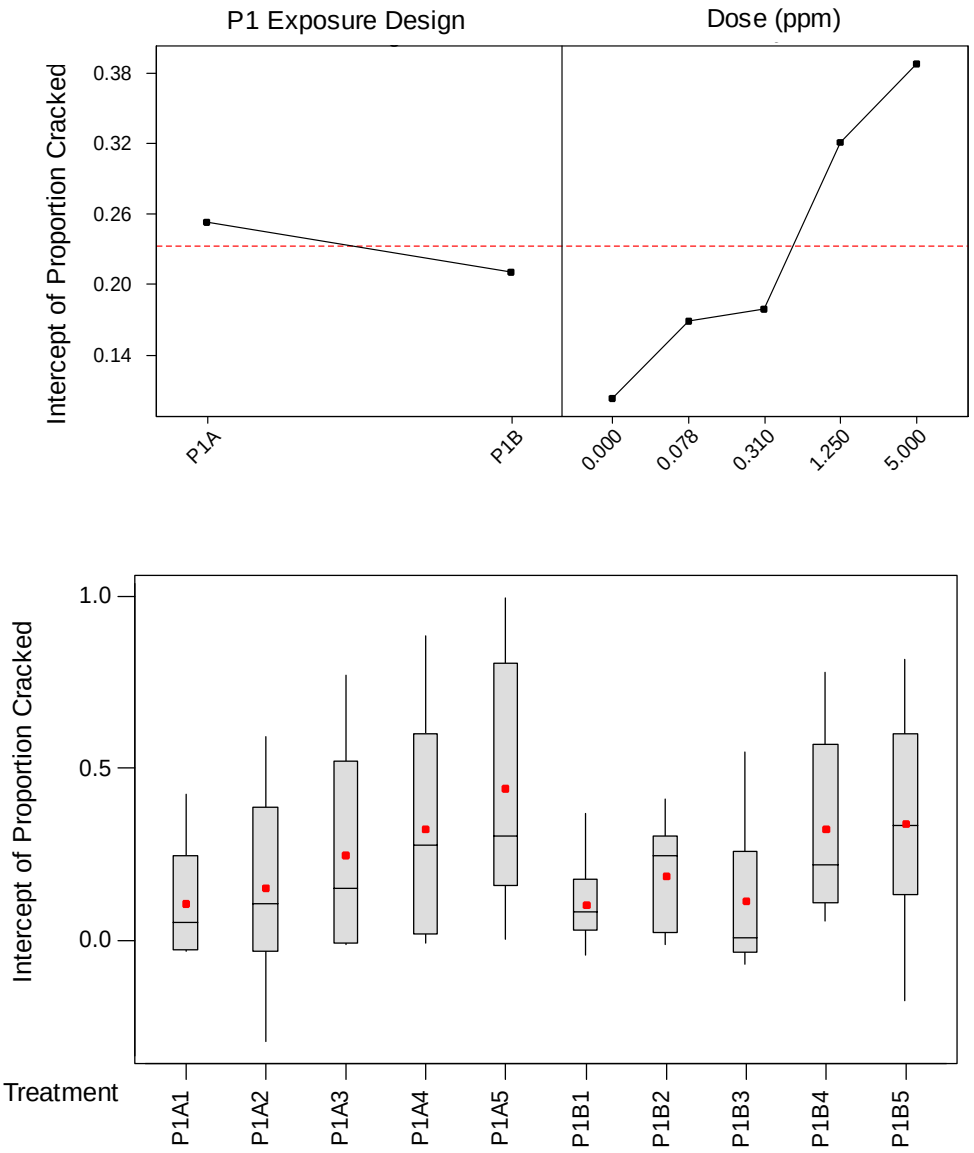


Figure 4.11-1. Main effects of the General Linear Model analysis and box plots of the intercepts of the proportion of cracked eggs out of the number of eggs laid regressed against time. The mean intercept of the proportion of cracked eggs increased with diet treatment ($p<0.01$). The effect of exposure scenario was insignificant ($p>0.44$). Means are indicated by solid circles.

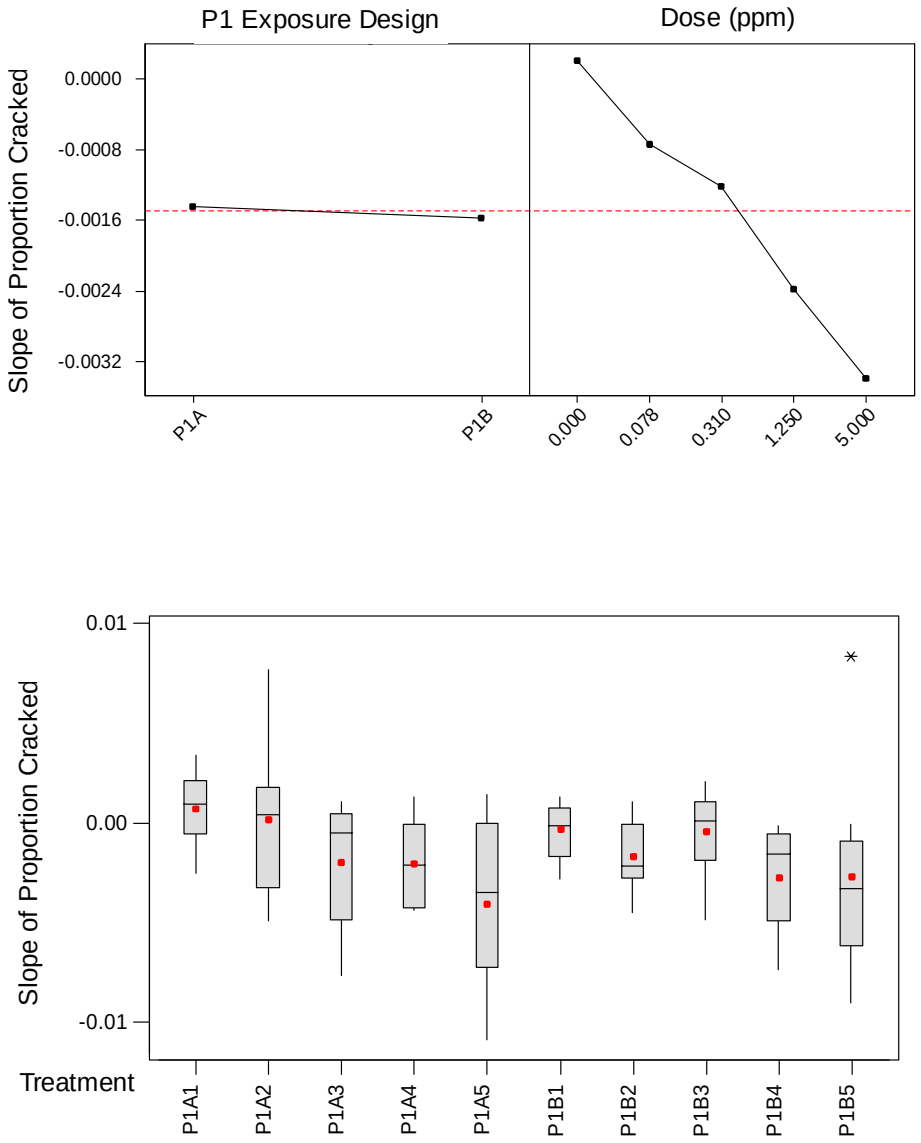


Figure 4.11-2. Main effects of the General Linear Model analysis and box plots of the slopes of the proportion of cracked eggs out of the number of eggs laid regressed against time. The mean slope of the proportion of cracked eggs decreased with diet treatment ($p<0.01$). The effect of exposure scenario was not significant ($p>0.84$). Means are indicated by solid circles.

4.11.2 Thickness

Egg shell thickness by dietary concentration and exposure scenario was also evaluated prior to and after pairing of the adults, as summarized in Table 4.11-2 (at the end of this section). Eggshell thickness was not significantly different between P1 exposure scenarios (p=0.507) or dietary concentrations (p=0.247). Differences in eggshell thickness prior to and post pairing were also not detected (p=0.755; Figure 4.11-3). However, the slopes of the natural log-transformed shell thickness regressed against time was nearly significantly (p=0.052) increased with dietary treatment in eggs laid by birds in the P1B exposure scenario (Figure 4.11-4). No significant effect on shell thickness over time was observed for eggs laid by birds in the P1A exposure scenario.

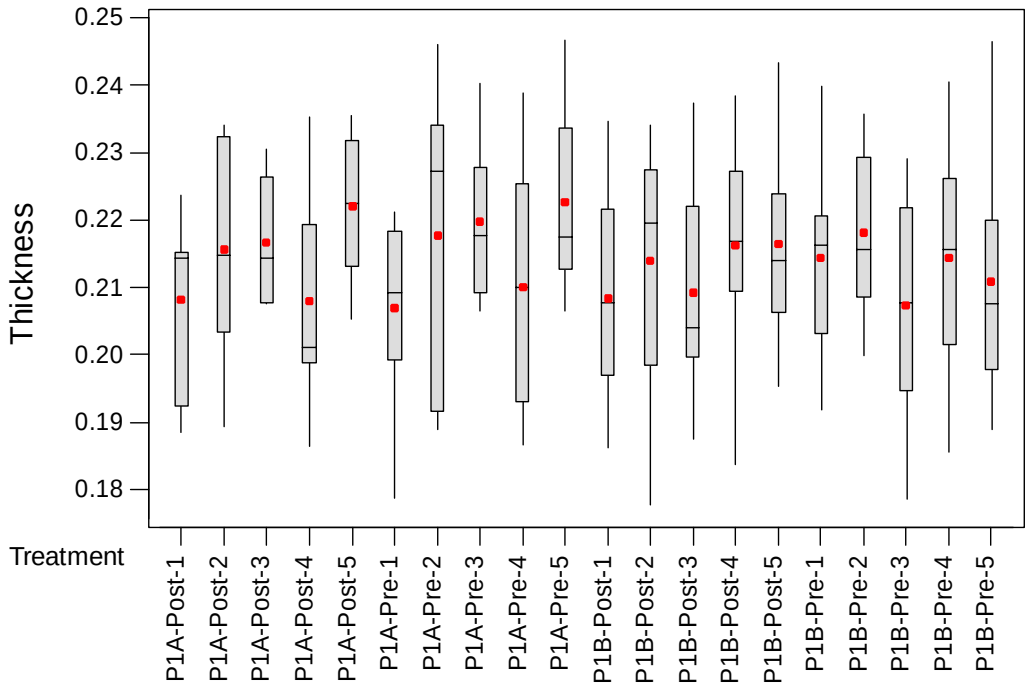


Figure 4.11-3. Box plots of the eggshell thickness (mm) of Japanese quail eggs. Thickness shown by dietary treatment (1, 0.0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm); parental exposure scenario (P1A, exposure for 13 weeks from 3 weeks of age; P1B exposure for 5 weeks from 11 weeks of age); and pre- or post pairing of the parents. Means are indicated by solid circles.

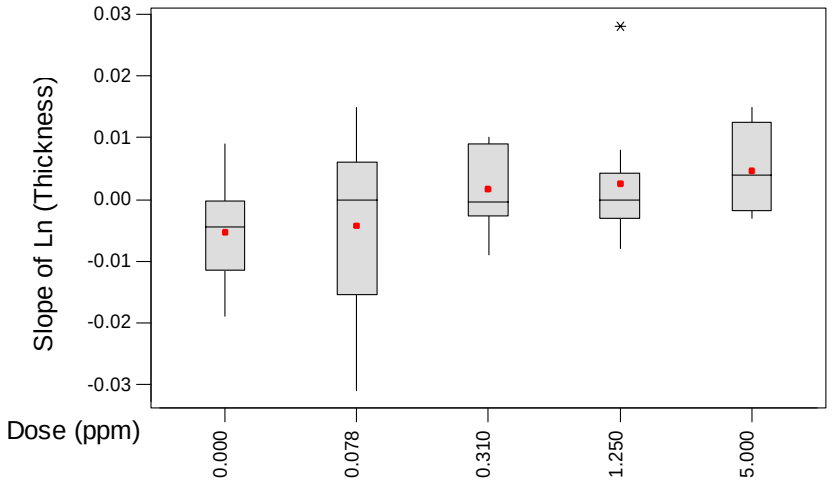


Figure 4.11-4. Box plots of the slopes of the natural log-transformed eggshell thickness values (mm) of eggs from birds in the P1B exposure scenario regressed against time. Slopes increased (p=0.052) with dietary treatment. Means are indicated by solid circles.

4.11.3 Mechanical Measures of Shell Strength

While both the maximum load prior to rupture and the load to rupture (breaking force) were measured (Table 4.11-2), only the breaking force is reported here because the two values were highly correlated. Eggshell strength measures by dose and exposure scenarios both prior to and after pairing of the adults are summarized in Table 4.11-2.

The breaking force needed to crack an egg (load to rupture) tended to increase with dietary concentration of E2 (p=0.13). There was also a nearly significant increase (p=0.057) in breaking strength detected in eggs laid prior to pairing of the adults (Figure 4.11-5). P1 exposure scenario did not have a significant effect (p=0.43) on eggshell strength using this measure (Figure 4.11-5). A slightly increased dose-response in breaking force (p=0.077) was detected in eggs laid by P1A hens prior to pairing than after pairing and the mean breaking strength tended to increase with dietary concentration (p=0.082) (Figure 4.11-6). There was no significant difference in eggshell breaking strength related to pre- or post-pairing in eggs laid by birds in the P1B exposure scenario (p=0.199).

Table 4.11-2. P1 eggshell quality measurements pre- and post-pairing by exposure scenario and dietary concentration.

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Thickness, mm									
P1A-Pre-0 ppm	8	0.21	0.21	0.01	0.18	0.22	0.20	0.22	7%
P1A-Pre-0.078 ppm	8	0.22	0.23	0.02	0.19	0.25	0.19	0.23	10%
P1A-Pre-0.31 ppm	8	0.22	0.22	0.01	0.21	0.24	0.21	0.23	5%
P1A-Pre-1.25 ppm	8	0.21	0.21	0.02	0.19	0.24	0.19	0.23	9%
P1A-Pre-5 ppm	7	0.22	0.22	0.01	0.21	0.25	0.21	0.23	6%
P1A-Post 0 ppm	7	0.21	0.21	0.01	0.19	0.22	0.19	0.22	6%
P1A-Post-0.078 ppm	8	0.22	0.21	0.02	0.19	0.23	0.20	0.23	8%
P1A-Post-0.31 ppm	8	0.22	0.21	0.01	0.21	0.23	0.21	0.23	4%
P1A-Post-1.25 ppm	7	0.21	0.20	0.02	0.19	0.24	0.20	0.22	8%
P1A-Post-5 ppm	7	0.22	0.22	0.01	0.21	0.24	0.21	0.23	5%
P1B-Pre-0 ppm	10	0.21	0.22	0.01	0.19	0.24	0.20	0.22	6%
P1B-Pre-0.078 ppm	9	0.22	0.22	0.01	0.20	0.24	0.21	0.23	6%
P1B-Pre-0.31 ppm	8	0.21	0.21	0.02	0.18	0.23	0.19	0.22	8%
P1B-Pre-1.25 ppm	10	0.21	0.22	0.02	0.19	0.24	0.20	0.23	8%
P1B-Pre-5 ppm	9	0.21	0.21	0.02	0.19	0.25	0.20	0.22	8%
P1B-Post 0 ppm	10	0.21	0.21	0.02	0.19	0.23	0.20	0.22	7%
P1B-Post-0.078 ppm	9	0.21	0.22	0.02	0.18	0.23	0.20	0.23	9%
P1B-Post-0.31 ppm	8	0.21	0.20	0.02	0.19	0.24	0.20	0.22	8%
P1B-Post-1.25 ppm	10	0.22	0.22	0.02	0.18	0.24	0.21	0.23	7%
P1B-Post-5 ppm	9	0.22	0.21	0.01	0.20	0.24	0.21	0.22	6%
Maximum Load, N									
P1A-Pre-0 ppm	8	10.1	10.8	1.66	6.82	12.0	9.18	11.2	16%
P1A-Pre-0.078 ppm	8	10.9	11.7	2.35	8.02	13.8	8.28	12.8	22%
P1A-Pre-0.31 ppm	8	11.5	11.0	1.69	9.93	15.1	10.3	12.2	15%
P1A-Pre-1.25 ppm	8	11.1	10.8	1.42	9.57	13.8	10.1	12.3	13%
P1A-Pre-5 ppm	7	12.2	12.3	1.04	11.2	14.1	11.2	12.9	8%
P1A-Post 0 ppm	7	10.0	10.3	0.80	8.80	10.6	8.81	10.6	8%
P1A-Post-0.078 ppm	8	10.7	11.0	1.49	8.10	12.5	9.54	12.0	14%
P1A-Post-0.31 ppm	8	10.9	10.8	0.91	9.00	11.9	10.6	11.7	8%
P1A-Post-1.25 ppm	7	10.5	9.86	1.51	9.13	13.4	9.34	11.5	14%
P1A-Post-5 ppm	7	10.8	10.4	1.67	8.59	13.1	9.41	12.6	16%
P1B-Pre-0 ppm	10	11.3	11.5	1.53	8.72	13.3	10.2	12.2	14%
P1B-Pre-0.078 ppm	9	11.9	11.2	1.83	10.00	15.6	10.4	13.2	15%

Table 4.11-2. P1 eggshell quality measurements pre- and post-pairing by exposure scenario and dietary concentration (continued).

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Maximum Load , N (continued)									
P1B-Pre-0.31 ppm	8	10.5	10.2	1.37	9.20	13.0	9.47	11.8	13%
P1B-Pre-1.25 ppm	10	11.5	11.5	2.84	7.38	15.5	9.22	14.9	25%
P1B-Pre-5 ppm	9	11.4	12.2	1.80	7.51	13.3	10.5	12.7	16%
P1B-Post 0 ppm	10	10.5	11.2	1.50	8.06	11.9	9.01	11.6	14%
P1B-Post-0.078 ppm	9	11.1	11.3	1.45	8.50	13.5	10.0	11.8	13%
P1B-Post-0.31 ppm	8	9.76	9.67	1.00	8.58	11.3	8.84	10.6	10%
P1B-Post-1.25 ppm	10	11.3	10.6	2.16	8.51	14.8	9.58	13.0	19%
P1B-Post-5 ppm	9	11.6	11.8	1.56	8.82	13.9	10.6	13.0	13%
Stiffness, N/m									
P1A-Pre-0 ppm	8	60169	60419	8469	46534	73247	54182	67184	14%
P1A-Pre-0.078 ppm	8	65258	65098	8779	54115	77841	57201	72715	13%
P1A-Pre-0.31 ppm	8	86139	68219	36316	56769	151589	62222	121905	42%
P1A-Pre-1.25 ppm	8	76021	67015	30306	58746	150154	61260	71526	40%
P1A-Pre-5 ppm	7	68307	64530	9173	58212	84334	61159	74048	13%
P1A-Post 0 ppm	7	58974	59310	4459	53119	64471	53568	63590	8%
P1A-Post-0.078 ppm	8	61558	63056	7931	47820	72062	55892	68352	13%
P1A-Post-0.31 ppm	8	61084	59450	3308	58116	66120	58541	65052	5%
P1A-Post-1.25 ppm	7	61881	63106	6975	52310	73541	56643	65749	11%
P1A-Post-5 ppm	7	67132	67552	9857	52680	79908	55810	73831	15%
P1B-Pre-0 ppm	10	105462	70530	121009	53165	448960	60886	75364	115%
P1B-Pre-0.078 ppm	9	67996	69011	7585	58683	78351	60507	75167	11%
P1B-Pre-0.31 ppm	8	65629	63183	11260	54505	86771	56497	73734	17%
P1B-Pre-1.25 ppm	10	68824	66370	9246	57353	83406	61324	77970	13%
P1B-Pre-5 ppm	9	71860	72480	7689	59669	83515	66858	78117	11%
P1B-Post 0 ppm	10	58804	59376	3204	53589	62617	56131	61700	5%
P1B-Post-0.078 ppm	9	61563	62608	7506	49162	72336	54926	67652	12%
P1B-Post-0.31 ppm	8	57420	55904	6949	49921	68368	51730	64844	12%
P1B-Post-1.25 ppm	10	59821	57921	8641	47916	73197	54024	69840	14%
P1B-Post-5 ppm	9	63873	62734	10838	54219	90321	56031	65457	17%

Table 4.11-2. P1 eggshell quality measurements pre- and post-pairing by exposure scenario and dietary concentration (continued).

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Load at Rupture[a], N									
P1A-Pre-0 ppm	8	8.12	8.62	1.33	5.46	9.60	7.34	8.94	16%
P1A-Pre-0.078 ppm	8	8.74	9.34	1.88	6.42	11.1	6.63	10.3	22%
P1A-Pre-0.31 ppm	8	9.20	8.80	1.35	7.94	12.1	8.22	9.74	15%
P1A-Pre-1.25 ppm	8	8.92	8.63	1.13	7.65	11.0	8.06	9.84	13%
P1A-Pre-5 ppm	7	9.78	9.86	0.83	8.92	11.3	8.95	10.3	8%
P1A-Post 0 ppm	7	7.96	8.25	0.64	7.04	8.51	7.05	8.45	8%
P1A-Post-0.078 ppm	8	8.56	8.81	1.19	6.48	10.0	7.63	9.63	14%
P1A-Post-0.31 ppm	8	8.70	8.68	0.73	7.20	9.52	8.47	9.34	8%
P1A-Post-1.25 ppm	7	8.40	7.89	1.21	7.31	10.7	7.47	9.19	14%
P1A-Post-5 ppm	7	8.61	8.28	1.34	6.88	10.5	7.53	10.1	16%
P1B-Pre-0 ppm	10	9.00	9.22	1.22	6.97	10.6	8.16	9.80	14%
P1B-Pre-0.078 ppm	9	9.56	8.96	1.46	7.97	12.5	8.36	10.5	15%
P1B-Pre-0.31 ppm	8	8.42	8.12	1.10	7.36	10.4	7.58	9.41	13%
P1B-Pre-1.25 ppm	10	9.22	9.16	2.27	5.90	12.4	7.37	11.9	25%
P1B-Pre-5 ppm	9	9.11	9.74	1.44	6.01	10.6	8.41	10.2	16%
P1B-Post 0 ppm	10	8.38	8.97	1.20	6.45	9.53	7.21	9.31	14%
P1B-Post-0.078 ppm	9	8.87	9.04	1.16	6.80	10.8	7.97	9.47	13%
P1B-Post-0.31 ppm	8	7.81	7.73	0.80	6.86	9.06	7.07	8.49	10%
P1B-Post-1.25 ppm	10	9.02	8.47	1.73	6.81	11.8	7.66	10.4	19%
P1B-Post-5 ppm	9	9.29	9.47	1.25	7.05	11.1	8.46	10.4	13%

[a] Breaking force or breaking strength.

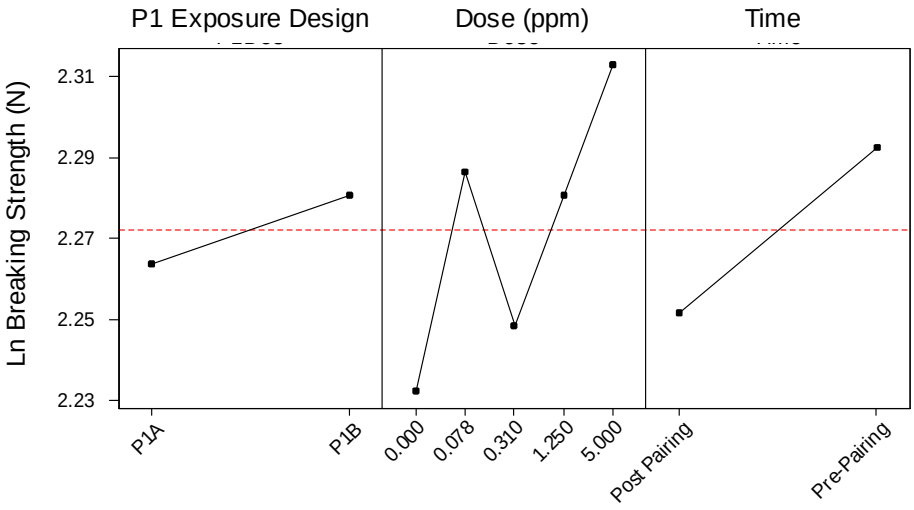


Figure 4.11-5. Main effects of the General Linear Model analysis of the natural log-transformed breaking strength (N). The effect of exposure scenario was insignificant (p=0.43); increased breaking strength with increasing dietary concentration of E2 (p=0.13) and increased shell strength prior to pairing (p=0.057) were observed.

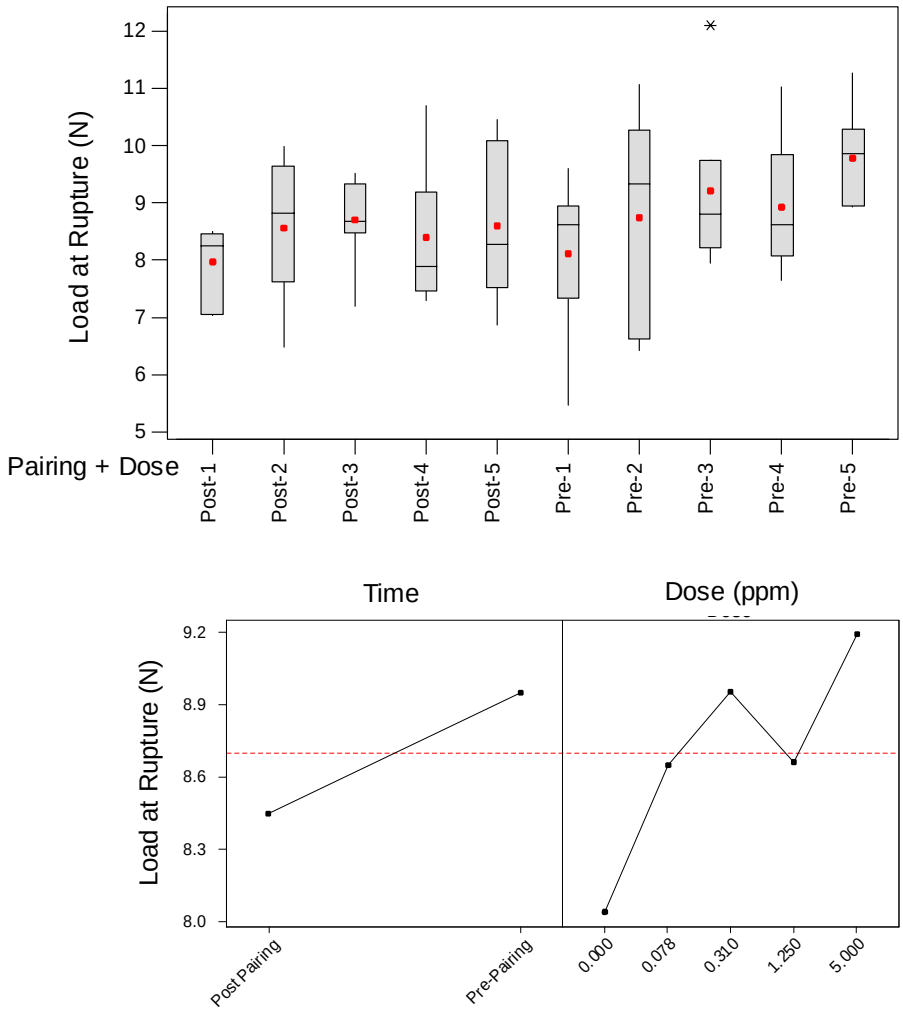


Figure 4.11-6. Box plots (above) of the breaking strength by dietary treatment of eggs laid prior to and post pairing. Main effects of the General Linear Model analysis of the breaking strength (N) of eggs laid by hens in the P1A exposure scenario (below). Nearly significant increase in breaking strength pre-pairing (p=0.077). Means are indicated by solid circles.

4.11.4 Shell Stiffness

No significant effect of exposure scenario or dietary treatment (p 0.89) on shell stiffness was found. The nearly significantly greater shell strength of eggs laid prior to pairing as measured by breaking force was found to be highly significant (p<0.001) using the shell stiffness measure. (An extreme value of an order of magnitude greater than remaining values was found in the controls, but without the high observation, pairing was still highly significant, p < 0.001) (Figure 4.11-7). Although mean shell stiffness was

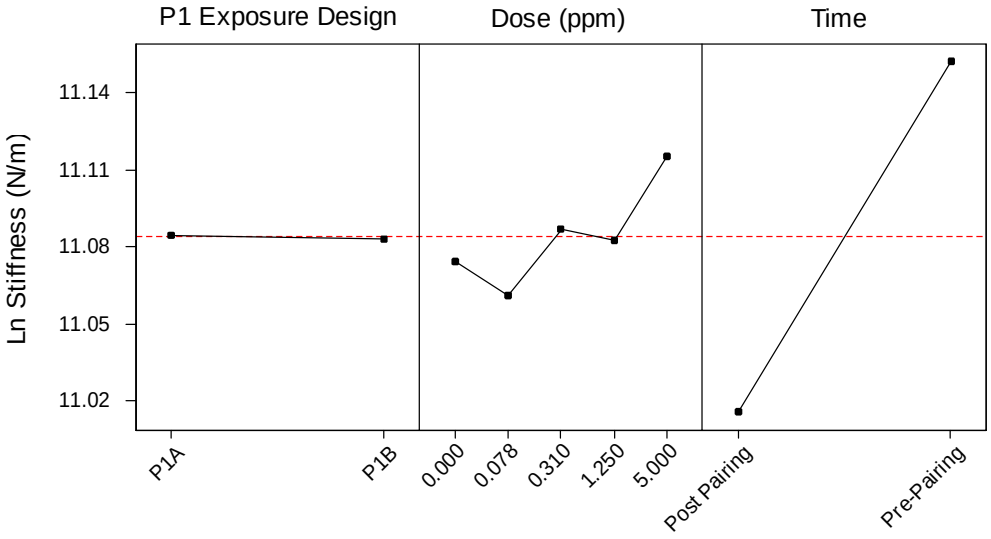


Figure 4.11-7. Main effects of the General Linear Model analysis of the natural log-transformed weights of quasi-static shell stiffness (N/m). The effects of exposure scenario and dietary treatment were not significant ($p \geq 0.89$); shell stiffness was increased significantly ($p < 0.001$) during pre-exposure pairing as compared to the period after pairing of the adults.

increased in eggs from both P1A and P1B hens during the pre-pairing period, the increase in shell stiffness in P1B eggs had no clear relationship to dietary concentration ($p=0.56$). However, eggs laid by P1A hens tended ($p=0.13$) to increase in shell stiffness with increasing dietary concentration of E2 prior to and post pairing.

4.12 Steroid Content in Eggs Laid by P1 Birds

Measurements were made of both 17ß-estradiol and testosterone in pooled egg yolks.

4.12.1 17ß-Estradiol

The E2 content of eggs from both the P1A and P1B birds increased significantly ($p<0.001$ and $p=0.018$, respectively) with dietary exposure to E2 (Tables 4.12-1 and 4.12-2). E2 was significantly greater in eggs from the 5 ppm dietary treatment group of the P1A birds ($p=0.04$) compared to the control mean and had a significant linear regression with dietary concentration ($p<0.001$) (Figures 4.12-1 and 4.12-2). The more lengthy exposure period (13 weeks) of the P1A birds to the treated diets resulted in higher egg burdens of E2 than were found in eggs laid by P1B hens exposed for 5 weeks (Figure 4.12-1). The mean concentration of the E2 in eggs from P1A hens consuming the 5 ppm diet was almost twice as high as that found in eggs laid by P1B hens consuming the same diet (Tables 4.12-1 and 4.12-2).

Table 4.12-1. Steroid content of eggs laid by P1A birds (n=4)[a].

	Estradiol (ppb)							
Dose, ppm	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
0	0.14	0.16	0.030	0.10	0.17	0.11	0.16	21%
0.078	0.22	0.19	0.082	0.16	0.34	0.17	0.31	37%
0.31	0.18	0.17	0.056	0.13	0.24	0.13	0.23	31%
1.25	0.19	0.18	0.058	0.12	0.26	0.13	0.24	31%
5	0.36	0.34	0.090	0.27	0.49	0.29	0.45	25%
	Testosterone (ppb)							
Dose, ppm	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
0	2.03	1.93	0.38	1.73	2.53	1.73	2.42	19%
0.078	2.75	2.82	0.35	2.29	3.09	2.40	3.05	13%
0.31	3.92	4.13	1.80	1.97	5.45	2.18	5.45	46%
1.25	2.37	2.48	0.30	1.94	2.59	2.05	2.59	13%
5	2.57	2.58	0.056	2.51	2.63	2.52	2.63	2%

[a] Four composite samples per dietary concentration; 3 or 4 eggs per composite. E2 significantly increased with dietary concentration ($p<0.001$; Least Squares Linear Regression)

Table 4.12-2. Steroid content of eggs laid by P1B birds (n=4)[a].

	Estradiol (ppb)							
Dose, ppm	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
0	0.11	0.09	0.053	0.08	0.19	0.08	0.17	48%
0.078	0.08	0.08	0.023	0.06	0.11	0.07	0.10	27%
0.31	0.13	0.11	0.035	0.10	0.18	0.10	0.16	28%
1.25	0.16	0.17	0.056	0.08	0.21	0.10	0.20	36%
5	0.19	0.18	0.087	0.10	0.28	0.11	0.27	46%
	Testosterone (ppb)							
Dose, ppm	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
0	2.53	2.49	0.20	2.35	2.81	2.37	2.75	8%
0.078	1.72	1.73	0.065	1.65	1.80	1.66	1.79	4%
0.31	2.36	2.25	0.60	1.85	3.11	1.87	2.98	25%
1.25	2.32	2.44	0.75	1.34	3.09	1.55	2.98	32%
5	2.23	2.19	0.31	1.94	2.61	1.96	2.55	14%

[a] Four composite samples per dietary concentration; 3 or 4 eggs per composite. E2 significantly increased with dietary concentration (p=0.018; Regression Analysis)

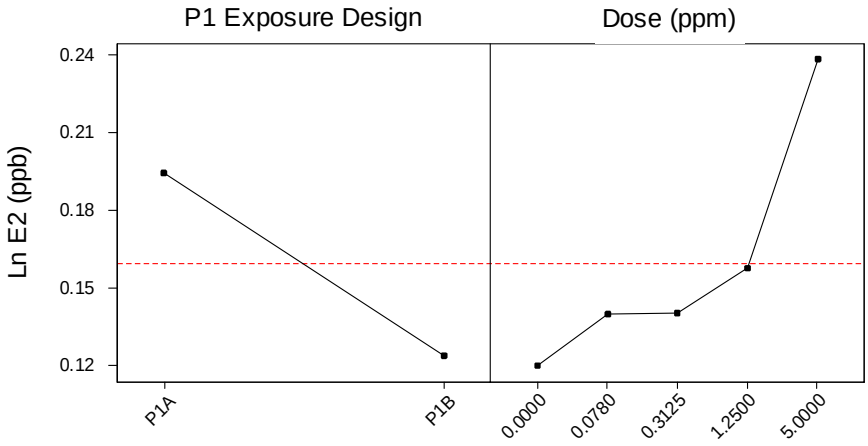


Figure 4.12-1. Main effects of the General Linear Model analysis of the natural log-transformed concentrations (ppb) of E2 in eggs. P1A exposure produced eggs with greater (p<0.001) E2 burdens. E2 increased in eggs as function of dietary treatment (p<0.001).

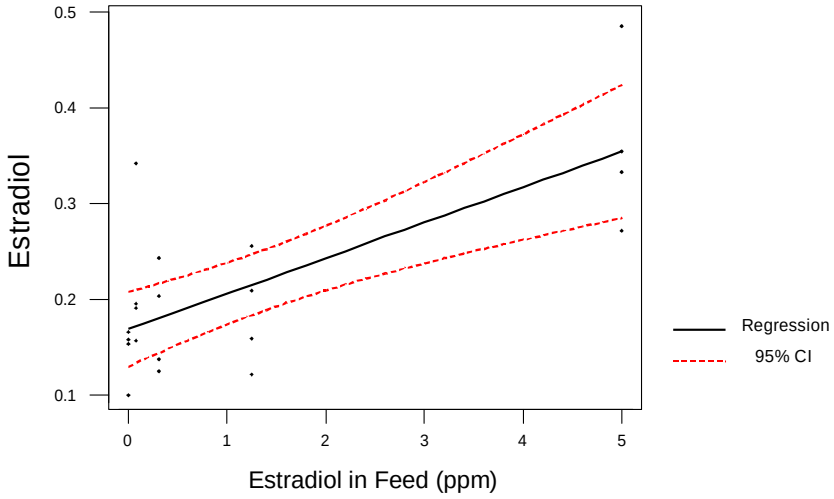


Figure 4.12-2. Linear regression of estradiol content in egg yolk (ppb) by dietary concentration (ppm) in eggs laid by P1A hens.

4.12.2 Testosterone

No consistent dose-response relationship was observed for testosterone content in eggs (Tables 4.12-1 and 4.12-2). A significant interaction (p=0.034) between exposure scenario and treatment concentrations was observed, wherein, testosterone levels in eggs laid by P1A hens were greater than levels detected in control eggs, but the increases were not dose-linear (Figure 4.12-3). When the P1 scenarios were analyzed separately, testosterone concentrations in eggs laid by P1A parents consuming E2-treated diet tended to be elevated above testosterone levels in eggs laid by control hens (p=0.073), while testosterone content in eggs from treated P1B birds tended to be lower than control egg values (p=0.112) (Figure 4.12-4).

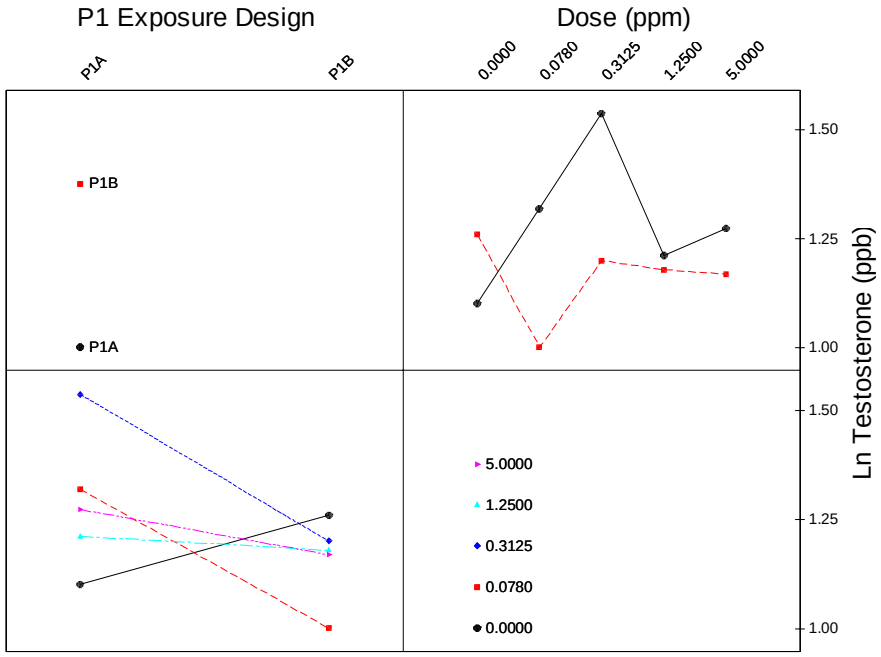


Figure 4.12-3. **Interaction (p=0.034) between exposure scenario and E2 treatment concentrations in the General Linear Model analysis of natural log transformed testosterone levels (ppb) in eggs laid by birds exposed to E2 for 13 weeks (P1A) or 5 weeks (P1B).**

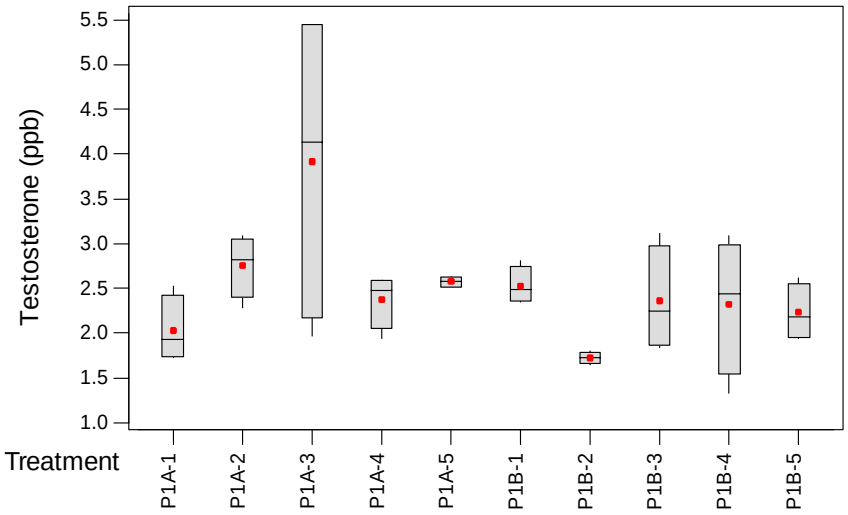


Figure 4.12-4. **Box plots of testosterone concentrations (ppb in yolk) in eggs laid by birds exposed to E2 in diet under two exposure scenarios (P1A, exposure from pre-maturation, P1B exposure after onset of egg laying).** Means are indicated by solid circles.

4.13 Analytical Characterization of Test Material and Endocrine Active Compounds in Test Diets

4.13.1 Estradiol Concentration, Stability, and Cross-Contamination in Test Diets

Estimation of purity of the test substance (estradiol), determination of method detection limit (MDL) and quantitation limits, and estimations of stability of estradiol in the basal diet of the quail are reported in detail in Appendix B. Comparison of nominal and measured test diet concentrations and feed trough stability from the in-life study are summarized below. A complete report by feed batch is found in Appendix D.

Samples from 16 batches of dosed feed were analyzed for one or more of the following parameters: feed dosing concentration verification and spiking uniformity, stability over the exposure period, cross-contamination during the exposure period, and determination of endogenous E2 in unspiked control feed.

E2 concentrations of the test diets over the study period are shown in Figures 4.13-1 and 4.13-2 for the P1 and F1 generations, respectively. Average E2 concentrations in control diets of Layena® and Startena® were 0.04 ppm or less. E2 levels in the 0.078, 0.31, 1.25, and 5 ppm nominal diets averaged between 0.02 and 0.12; 0.13 and 0.3; 0.72 and 1.13; and 3.3 and 4.81 ppm, respectively.

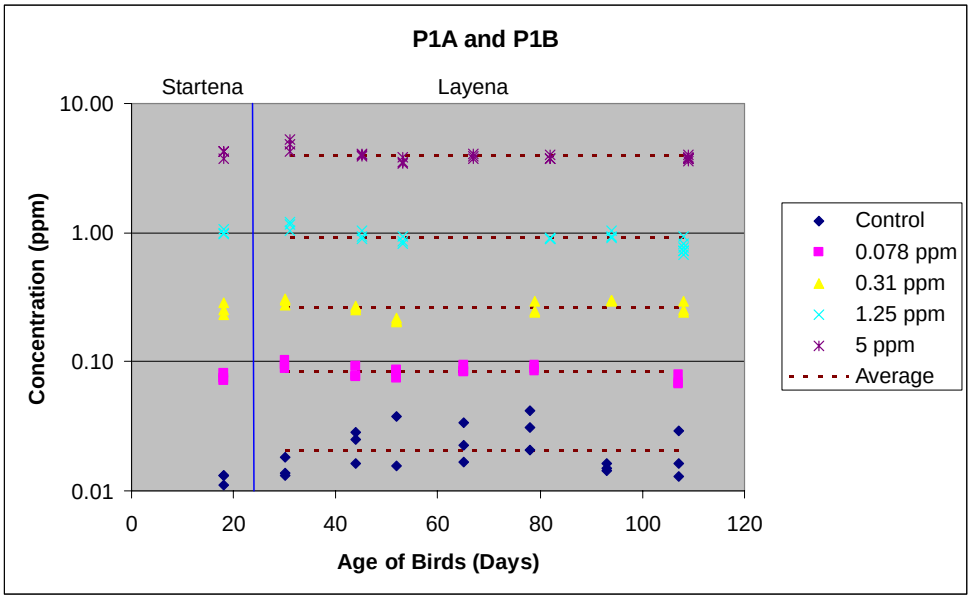


Figure 4.13-1. Overall and observed concentration of quail feed over time for the P1 generation.

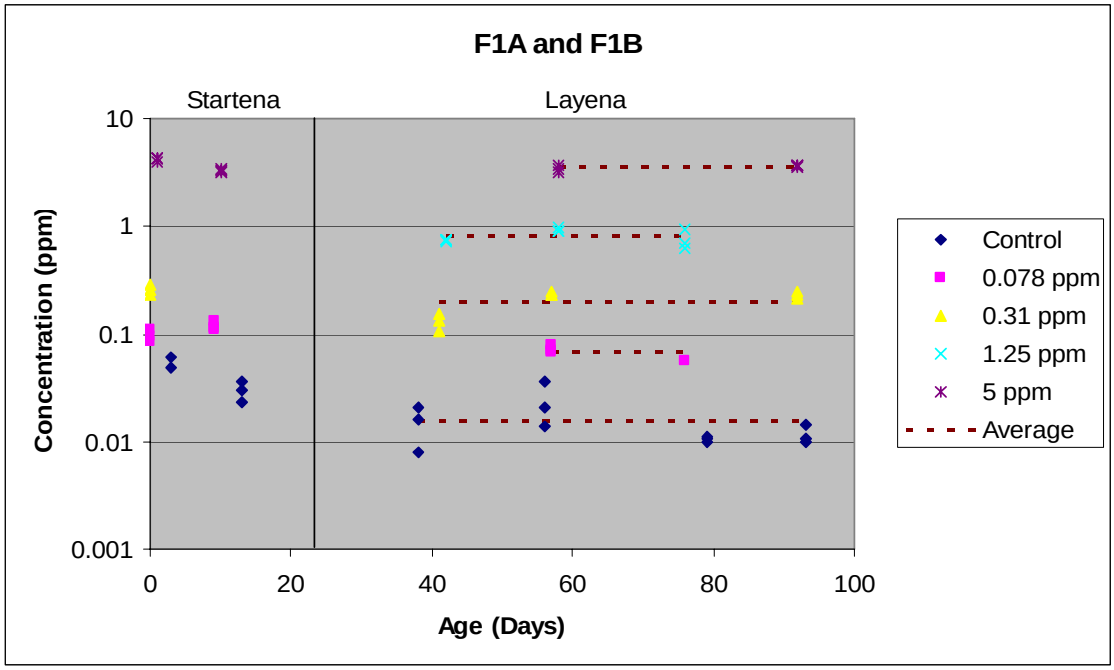


Figure 4.13-2. Overall and observed concentration of quail feed over time for the F1 generation.

Stability of feed during storage and during the exposure period in the feed troughs for P1 and F1 Generations is summarized in Table 4.13-1. Also reported in this table are the results of the cross-contamination analyses from feed trough samples.

Only one feed batch (feed batch 10) showed any significant change between the initial measurement immediately following preparation of the test diet and the final measurement of E2 content from feed trough samples at the end of an exposure period. For this batch, the 0.078 µg estradiol/g feed sample showed a major reduction of about 83% in estradiol. However, the reduction in test substance occurred over a period of 32 days with the feed at ambient temperature in the feed troughs on the 18th through 20th day and refrigerated all other days until extracted. Also for batch 10, this same sample pair (target of 0.078 µg/g estradiol) estimated at 154% of target at day zero. The matrix blanks and spikes and ICV and CCV values for that day were normal save the CCV, which was at 128% recovery for the estradiol and 148% for the surrogate recovery. The remaining batches tested for stability held within a range of 83% and 131% recovery over the stability test period.

Feed cross-contamination samples were analyzed for batches 1, 4, 8, and 10 and were all below the MDL indicating contamination of the test diets in the feed troughs was undetectable.

Table 4.13-1. Contamination and stability of feed during storage and exposure for P1 and F1 generations.

Stability Performance – Batch 1 (µg/g)			Contamination	Stability
Target Conc.	10/30/2003	11/7/2003	% Difference	% Difference
0	U	U	Below MDL	
0.078	0.08	0.08		0%
0.31	0.26	0.23		-11%
1.25	1.00	0.97		-3%
5	4.10	4.66		14%
Stability Performance – Batch 4 (µg/g)			**Contamination**	**Stability**
Target Conc.	12/3-12/4/03	12/16/2003	% Difference	% Difference
0	U	U	Below MDL	
0.078	0.081	0.076		-6%
0.31	0.209	0.263		26%
1.25	0.872	0.831		-5%
5	3.572	2.975		-17%
Stability Performance – Batch 8 (µg/g)			**Contamination**	**Stability**
Target Conc.	1/27-1/29/04	2/02,03/04	% Difference	% Difference
0	U	U	Below MDL	
0.078	0.072	0.071		-1.4%
0.31	0.263	0.264		1.5%
1.25	0.723	0.948		31.7%
5	3.760	4.318		14.8%
Stability Performance – Batch 10 (µg/g)			**Contamination**	**Stability**
Target Conc.	2/19,20,23/2004	3/10/2004 [a]	**Target Conc.**	% Difference
0	U	U	Below MDL	
0.078	0.12	0.02U		-83.3% [b]
0.31	Not analyzed	0.11		
1.25	Not analyzed	0.64		
5	3.30	2.50		-24.2%

[a] Diet was placed in the feed trough on 3/07/04 for an exposure period of 3 days.
[b] Day 3 result below MDL

A test for endogenous estradiol in blank feed showed levels below the MDL (nothing detected in 2 of 3 replicate samples).

There were two commercial feed formulations used during the study, Purina Game Bird Layena® (for reproductive birds) and Purina Game Bird Startena® (for growing chicks).

4.13.2 Endocrine-Active Compounds in Basal Diet

Concentrations of three major phytoestrogen contaminants in the basal diet of the quail are shown in Table 4.13-2 by lot number. Total phytoestrogen content, determined as the sum of the three phytoestrogen concentrations, in the basal diet of the breeding pairs (Purina Game Bird Layena®) averaged 329 $\pm$ 180 ppm over the study. The average phytoestrogen content of basal diet of the chicks (Purina Game Bird Startena®) was over 2.5 times the amount found in the adult diet (833 $\pm$ 152 ppm). Only one lot of Startena®

Table 4.13-2. Endocrine-active compounds in the basal diet.

Feed Type Lot No.	Phytoestrogens (ppm in feed)			Mycotoxin	Bioassay
	Daidzein	Glycitein	Genestein	Zearalenone	Total Estrogenic[a]
Startena®					
03AUG12SPK2	250	45	260	0.5	4.48
03NOV25	341	60	450	ND[d]	--[b]
03DEC04TRL3	397	71	489	ND	--
2:6:5 MIX[c] of 03DEC04TRL3 03OCT07SPK2 03NOV24SPK2	308	50	419	ND	--
04FEB19	381	33	540	ND	--
04MAY13SPK2	359	54	492	ND	--
Layena®					
03OCT09	155	23	168	ND	12.9
03NOV10	56	ND	76	ND	0.82
03DEC09SPK1	98	14	138	ND	--
1:1 MIX[e] 03DEC09SPK1 03DEC04TRL3	270	34	361	ND	--
04FEB26	112	11	165	ND	--
04APR01SPK1	123	26	144	ND	--

[a] Determined by Estrogen-Dependent Cell Proliferation (Welshons et al. 1990), values expressed in ppm zearalenone equivalents; 0.006 ppm = Limit of Detection, < 0.064 = Very Low, 0.064 to <1 ppm = Low, ≥1 to < 10 ppm = Moderate, ≥10 ppm = High.
[b] Not determined
[c] 2:6:5 mix of three lots of feed.
[d] Not detectable; detection limit for zearalenone was 0.5 ppm.
[e] 1:1 mix Startena® and Layena® transition diet at 3 weeks of age, consumed for 4 days.

had measurable zearalenone. The estrogenic mycotoxin was not found in any of the lots of the adult basal diet. Of the three lots analyzed for total estrogenic content by bioassay, the lot of Startena® diet had a moderate level of estrogenic contaminants and one of the lots of Layena® had a high total estrogenic content.

The analytical and quality control data and parameters for the determination of the endocrine-active compound content of the basal diet are reported in Appendix E.

4.14 Summary of the Results of the Parental Generation (P1)

Few fitness or endocrine endpoints were affected by E2 dietary exposure in the parental birds. For females, only the incidence of a right oviduct, length of the spotted area of female-type plumage, shell thickness over time, and estrogen content of egg yolks showed a difference between exposure scenario. No clear difference in the histological response of the reproductive organs of hens exposed prior to maturation and those exposed after the onset of egg laying were apparent. A greater number of 5 ppm E2 treated hens from the P1B exposure had diffuse hypertrophy of cortical and medullary cells of the adrenals.

Degenerative changes of the testis were observed with greater frequency and severity in the E2 treated P1A males. In males exposed to E2 post-puberty, only those fed the high E2 concentration diet had the testicular lesion. Hypospermia was also observed in treated P1A birds. Few incidences of hypospermia occurred in the epididymis of P1B males. There appeared to be somewhat of an increase with E2 concentration in the atrophy of submucosal glands of the cloacal gland in males from the pre-puberty exposure scenario; the lesion was not found in any of the P1B males. Cellular hypertrophy of the adrenal glands occurred in the high concentration group of the P1A males and was absent in males from the P1B exposure scenario. Pre-puberty growth rate increased in P1A over the P1B males which had yet to receive dietary treatments under their exposure scenario. Over all sexual maturation occurred about 1.4 days earlier in P1A males and 2 days earlier in P1A males with non-male plumage. Plumage dimorphism was altered in P1 males, with a significant increase in the incidence of non-male plumage with dietary concentration detected in males under the P1A design and a similar, less pronounced dietary effect in the P1B exposure scenario.

Tables 4.14-1 (females) and 4.14-2 (males) summarize the results of the endpoint measurements obtained for the parental generation (P1).

Table 4.14-1. Summary of results for P1 females.

Parameter	P1 Effect	Dietary Concentration Effect
Terminal Adult Body Weight	None	None
Growth Rate	None	None
Tibiotarsus		
Length	None	None
Diameter	None	None
Weight	None	None
Tarsometatarsus Length	P1B greater (but control also increased)	None
Aggression		
Feather Loss	None	None
Pecking Injury	P1B greater (unrelated to E2 treatment)	None
Organ Weight		
Thyroid-Gross	P1B increased (but control elevated)	None
Thyroid/Body Weight	P1B increased (but control elevated)	None
Thyroid/Brain Weight	P1B increased (but control elevated)	None
Adrenal Gland-Gross	P1B increased	None
Adrenal Gland/Body Weight	P1B increased	None
Adrenal Gland/Brain Weight	P1B increased	None
Liver-Gross	None	None
Liver/Body Weight	None	None
Liver/Brain Weight	None	None
Brain-Gross	None	None
Brain/Body Weight	None	None
Ovary-Gross	None	None
Ovary/Body Weight	None	None
Ovary/Brain Weight	None	None

Table 4.14-1. Summary of results for P1 females (continued).

Parameter	P1 Effect	Dietary Concentration Effect
Oviduct-Gross	None	None
Oviduct/Body Weight	None	None
Oviduct/Brain Weight	None	None
Active Oocytes	None	None
Gross Abnormalities		
Incidence of Rt. Ovary	None	None
Incidence of Rt. Oviduct	P1B greater	None
Neck Curvature	NA	NA
Foot/Leg	None	None
Sexual Maturation	None	None
Plumage Dimorphism		
Female Phenotype	None	None
Length of Spotted Area	Interaction P1A-5ppm diet greater[a]	P1A-5 ppm (trend when combined with P1B)[a]
Reproductive Parameters		
Total Eggs	None	None
Total Eggs/Max	P1B greater (but result of low P1A-0.078 ppm response)[a]	None
Eggs Viable on Day 8	None	None
production over time	None	Increased slopes for both scenarios[a]
Eggs Viable on Day 15	Interaction P1B-0 ppm diet (< all other design-concentration combinations)[a]	None
Hatchlings/Eggs Set	None	None
Hatchlings/Viable Day 8	None	None
Hatchlings/Max Eggs Set	None	None
Hatchling Prod.OverTime	None	None
Shell Quality		
Proportion Cracked Eggs	None	None
Prod. Cracked Over Time	None	Controls increased, treated decreased
Shell Thickness	None	None
Shell Thickness Over Time	P1B increase[a]	Controls and low concentration decrease, higher concentrations increase[a]

Table 4.14-1. Summary of results for P1 females (continued).

Parameter	P1 Effect	Dietary Concentration Effect
Breaking Strength	None	Increase[a]
Breaking Strength Over Time	None	None
Breaking Strength Pre/Post Pairing	P1A slight increase[a]	P1A Increase pre-pairing[a]
Shell Stiffness	None	None
Shell Stiffness Over Time	None	None
Shell Stiffness Pre/Post Pairing	P1A and P1B greater pre, but not different	P1A increase pre- and post-pairing[a]
Egg Steroid Content		
Estrogen	P1A greater	Increase
Testosterone	Interaction-P1A-0 ppm diet, E2 treated higher than control; P1B-0 ppm diet, E2 treated lower than control	See P1 effect

Note a. $p \leq 0.15$

Table 4.14-2. Summary of results for P1 males.

Parameter	P1 Effect	Dietary Concentration Effect
Terminal Adult Body Weight	None	None
Growth Rate	P1A increased pre-puberty[a]	None
Tibiotarsus		
Length	P1A smaller (P1A control smaller, P1B-0.31 ppm response greatest)	Not linear[a]
Diameter	None	None
Weight	None	None
Tarsometatarsus Length	P1B greater (but control also increased, P1A 1.25 ppm smallest)	None
Aggression		
Feather Loss	None	None
Pecking Injury	P1B greater (unrelated to E2 treatment)	None
Organ Weight		
Thyroid-Gross	None	None
Thyroid/Body Weight	None	None
Thyroid/Brain Weight	None	None
Adrenal Gland-Gross	P1B increased (P1B control high)	None
Adrenal Gland/Body Weight	P1B increased (P1B control high)[a]	None
Adrenal Gland/Brain Weight	P1B increased (P1B control high)[a]	None
Liver-Gross	None	None
Liver/Body Weight	None	None
Liver/Brain Weight	None	None
Brain-Gross	None	None
Brain/Body Weight	None	None
LeftTestis-Gross	None	None
Left Testis/Body Weight	None	None
LeftTestis/Brain Weight	None	None

Table 4.14-2. Summary of results for P1 males (continued).

Parameter	P1 Effect	Dietary Concentration Effect
Right Testis-Gross	None	None
Right Testis/Body Weight	None	None
Right Testis/Brain Weight	None	None
Testes Asymmetry	None	None
Cloacal Gland-Gross	None	None
Cloacal Gland/Body Weight	None	None
Cloacal Gland/Brain Weight	None	None
Gross Abnormalities-Organ Lesions	P1A greater (few, unrelated to E2 concentration)	None
Sexual Maturation-Day to First Foam		
All Males	P1A matured sooner (~1.4 day)	None
Males with Non-Male Plumage	P1A matured sooner (~2 days)	None
Plumage Dimorphism		
Incidence of Non-Male Phenotype	None	P1A increased; P1B increased[a]
Length of Spotted Area	None	P1A-5 ppm (None when combined with P1B)
Cloacal Gland Size	None	None

Note a. $p \leq 0.15$

5.0 FIRST GENERATION OFFSPRING (F1) RESULTS

5.1 Adult Body Weight, Growth, and Tibiotarsus Length (F1)

5.1.1 Body Weight and Growth

Females

The body weight of male and female F1 birds was measured on the day of hatch (day 1) and days 23, 27, 34, 41, 51, and 93/94 (at necropsy).

There appeared to be a highly significant difference in hatch weights between female offspring of P1A and P1B parents ($p<0.001$), with hatchlings of parents that were exposed for 13 weeks to E2 (P1A) weighing less (about 10% overall) than those from parents that were exposed for 5 weeks (P1B) to the steroid (Figure 5.1-1). The differences in hatchling weights tended ($p=0.077$) to depend upon the dietary treatment the parents received; however, this dose response was nonlinear (Figure 5.1-2).

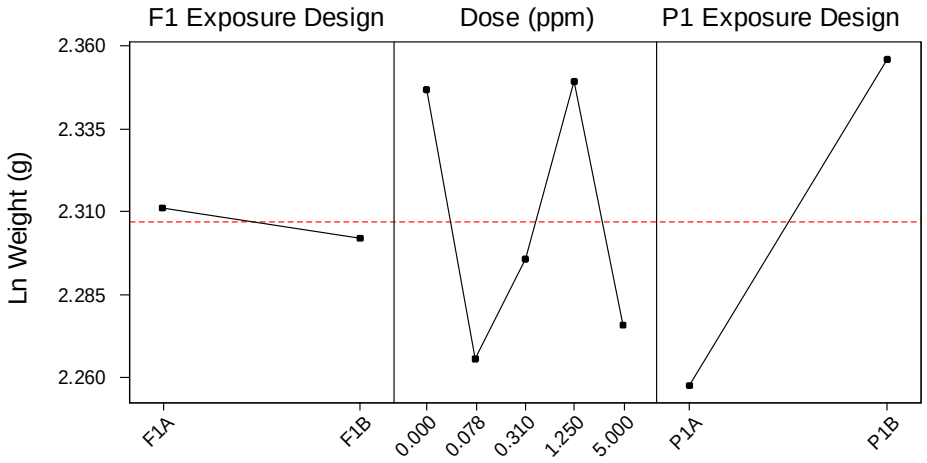


Figure 5.1-1. General Linear Model analysis of the effects of parental exposure scenario and parental dietary treatment on offspring (F1) hatchling weight (g). Natural-log transformed hatch weights were used. Significant differences in hatch weight due to parental exposure ($p<0.001$) scenario and a nearly significant ($p=0.077$) parental dietary treatment effect on hatch weight of offspring were observed.

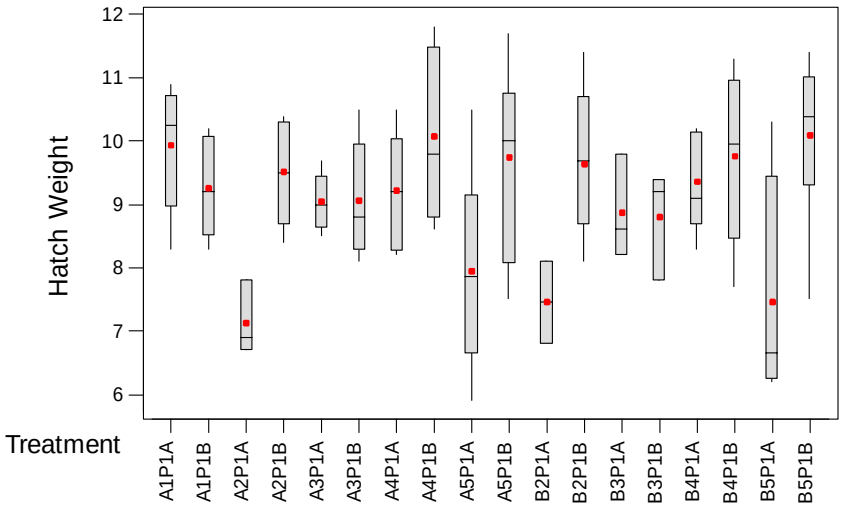


Figure 5.1-2. Box plots of the hatch weights (g) of the female F1 offspring of parents exposed to E2-dosed diets from 3 weeks of age for 13 weeks (P1A) or from 11 weeks of age for 5 weeks (P1B). F1a females are exposed from hatch to the same dietary concentration as their parents; F1b females were not treated. Means are indicated by solid circles.

Consumption of treated diet did not affect the necropsy weight (p=0.52) of the adult F1 offspring of the P1 populations. There was, however, a significant difference in body weight between female offspring from the different parental dosing scenarios (p= 0.001) with offspring of the P1B parental group (5 weeks of treatment) exhibiting greater necropsy weights than comparable offspring from the P1A parental group (11 weeks of treatment). The overall difference in the mean F1 adult body weights between those from P1A parents and those from P1B parents was small, about 15 grams (6%).

Also statistically significant was the effect of parental dietary treatment (*in ovo* exposure) on the adult body weight of their offspring (p=0.004). Although not concentration-linear, the weight differences were similar to those observed when the offspring were hatchlings (Figure 5.1-3).

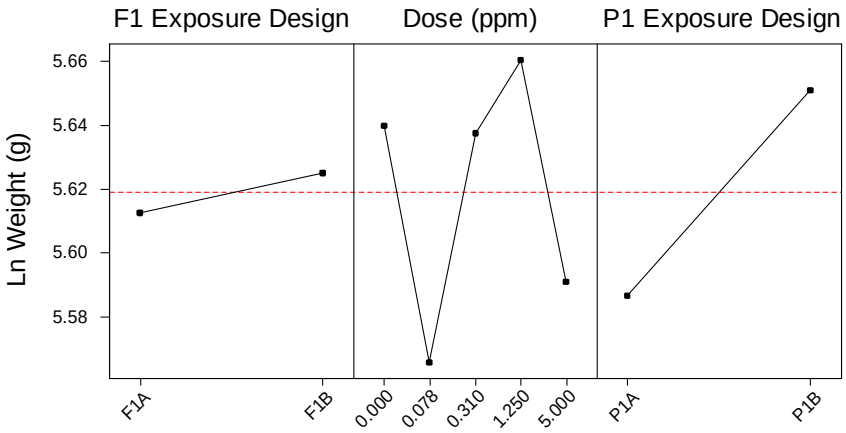
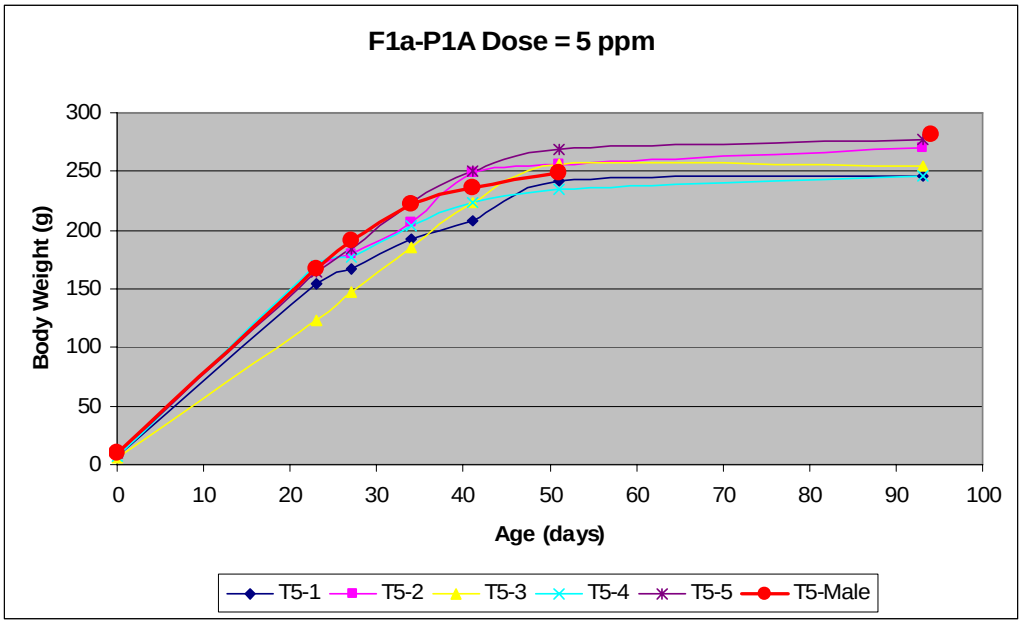


Figure 5.1-3. General Linear Model analysis of the effects of F1 dietary treatment, parental exposure scenario and parental dietary treatment on adult F1 body weight (g). Natural log- transformed body weights were used. Significant differences in body weight due to parental exposure (p=0.001) scenario; a parental dietary treatment (p=0.004) were observed.

In female F1 chicks, mean body growth rates between 23 and 34 days of age (p=0.090) and between 51 and 94 days of age (p=0.086) were nearly significantly less (about 10 to 11%) in those fed treated diet (F1a) compared to those receiving no additional exposure above *in ovo* (F1b). No difference in body weight growth was observed between 34 and 51 days of age (p=0.351). Figure 5.1-4 shows the body weight growth of F1 chicks with the greatest combined direct and *in ovo* exposure (F1a-P1A) and the least combined exposure (F1b-P1B) from hatch through 93 or 94 days of age, respectively.



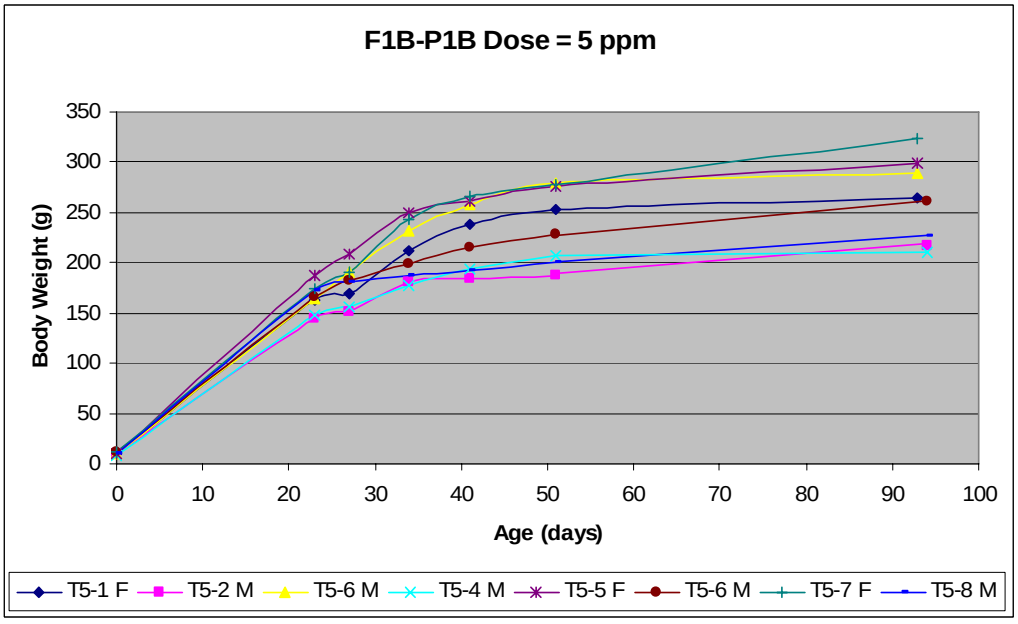


Figure 5.1-4. Body weight of F1 chicks with the combined greatest direct and *in ovo* exposure (F1a-P1A) and least combined exposure (F1b-P1B) from hatch through 93 or 94 days of age, respectively. Both male (M) and female (F) chick weights are shown.

Males

Unlike females, male F1 hatch weights were not statistically different between P1 exposure scenarios (p=0.98) or parental dietary treatments (p>0.64). Mean hatch weights of the males were also unaffected by F1 exposure design (p=0.93). Although no P1 dietary concentration effects on the terminal body weight of the adult F1 males were observed (p=0.48), a nearly

significant (p=0.08) interaction between the F1 and P1 exposure designs affecting necropsy body weight was detected. When the F1 exposure strategies were analyzed separately, the mean body weights of treated (F1a) males were significantly affected by P1 exposure scenario (p=0.02) whereas, the mean body weights of the untreated (F1b) males were not (p=0.26)(Figure 5.1-5). The effect of the P1 design was increased body weights in F1a male offspring of parents who received dietary exposure to E2 for 13 weeks as compared to those whose parents were treated for only 5 weeks.

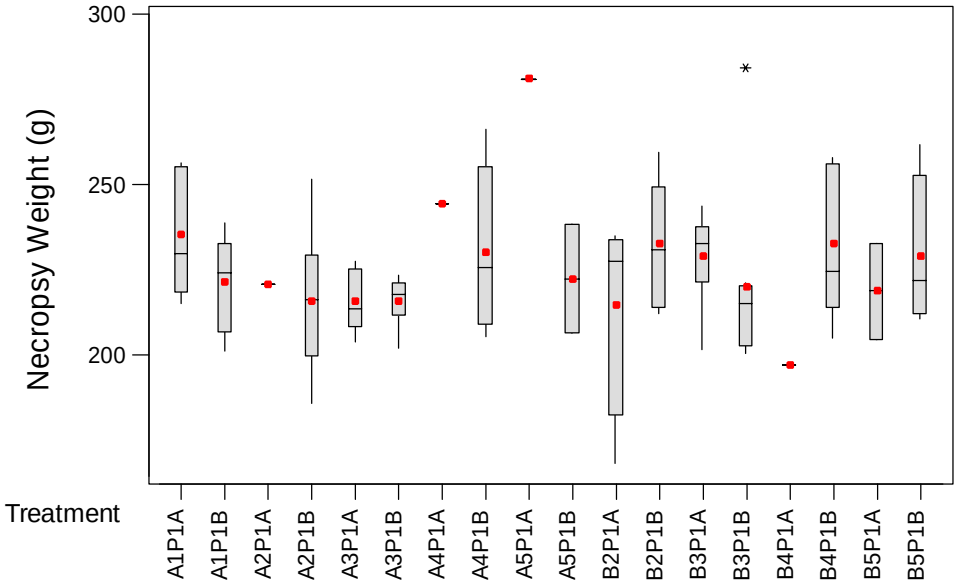


Figure 5.1-5. Box plots of the necropsy (Day 93/94) body weight (g) of male F1 offspring of parents exposed to E2-dosed diets from 3 weeks of age for 13 weeks (P1A) or from 11 weeks of age for 5 weeks (P1B). F1a males are exposed from hatch to the same dietary concentration as their parents; F1b males are not treated.

Growth rates of F1 male offspring were significantly affected by parental dietary treatment (p<0.001) for one growth segment (34 to 51 days of age); however, the slope differences are inconsistent across treatment groups and exposure scenarios (Figure 5.1-6).

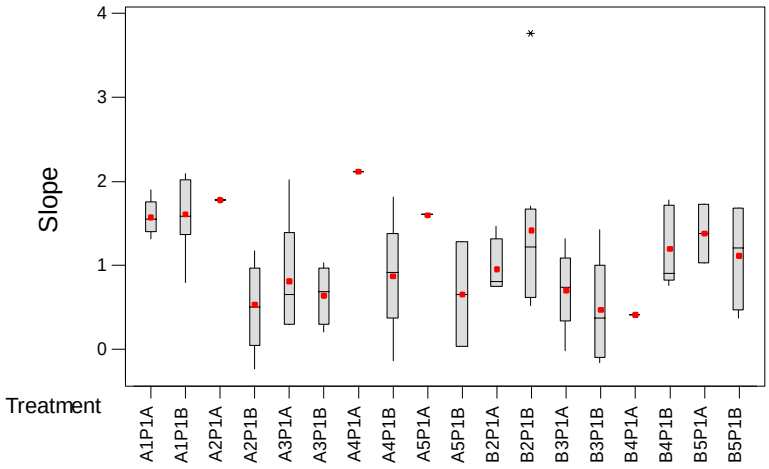


Figure 5.1-6. Box plots of the slopes (g/day) of the growth curve segment between 34 and 51 days of age of the male F1 offspring of parents exposed to E2-dosed diets for 13 weeks (P1A) or 5 weeks (P1B). F1a males are exposed from hatch to the same dietary concentration as their parents; F1b males are not treated. Means are indicated by solid circles. (* = extreme value).

5.1.2 Measurements of the Tibiotarsus and Tarsometarsus of Adult Quail at Necropsy (F1)

Females

Tibiotarsus

In females, the F1 and P1 exposure designs did not have a significant effect on length of the tibiotarsus ($p>0.63$). A tendency ($p=0.060$) for dietary treatments to induce differences in tibiotarsus lengths was observed, but the effect was non-concentration linear with the median tibiotarsus length of the 1.25 ppm E2 treatment group of untreated (F1b) birds of P1B parents significantly ($p=0.014$) greater than all other treatment design combinations (Figure 5.1-7.). Significant differences in tibiotarsus diameter were observed between the two F1 exposure strategies ($p=0.001$). Treated (F1a) birds had reduced diameters compared to their F1b counter parts, regardless of P1 exposure scenario (Figure 5.1-8). P1 exposure design and dietary treatment effects were not significant ($p>0.22$). Parental dietary treatment significantly affected the weight of the tibiotarsus ($p=0.003$), but the effect was not linear (Figure 5.1-9). It also appeared that an interaction between the F1 and P1 exposure designs ($p=0.147$) may be affecting tibiotarsus weight (Figure 5.1-10), with reduced bone weights in untreated (F1b) female offspring of parents from the P1A exposure scenario compared to F1b offspring from P1B parents. Treated females (F1a) were not affected by the exposure scenario of their parents (Figure 5.1-10).

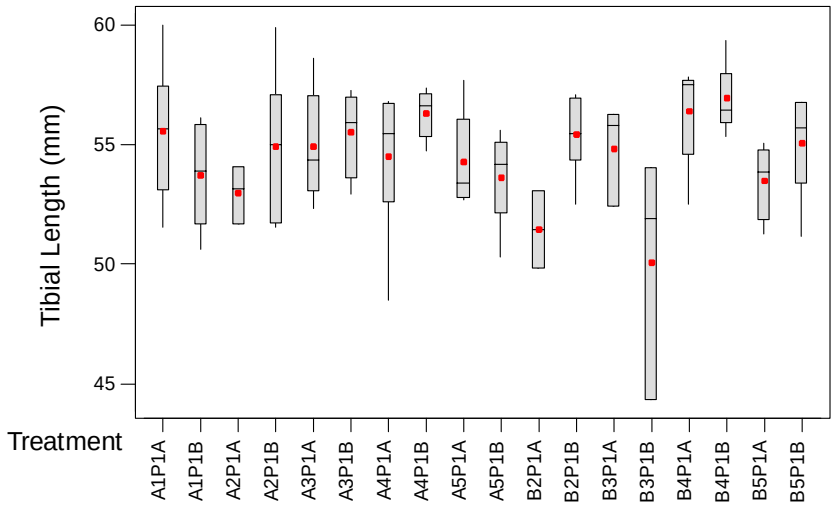


Figure 5.1-7. Box plots of tibiotarsus length in mm of F1 females by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

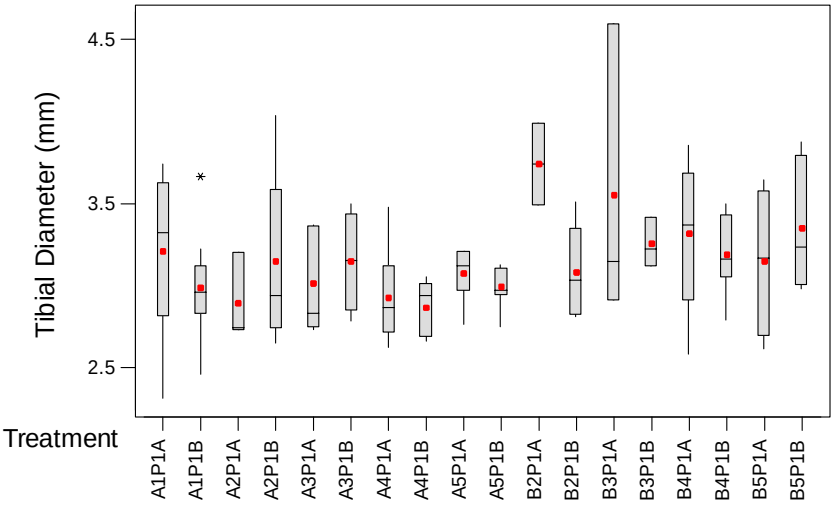


Figure 5.1-8. Box plots of tibiotarsus diameter in mm of F1 females by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

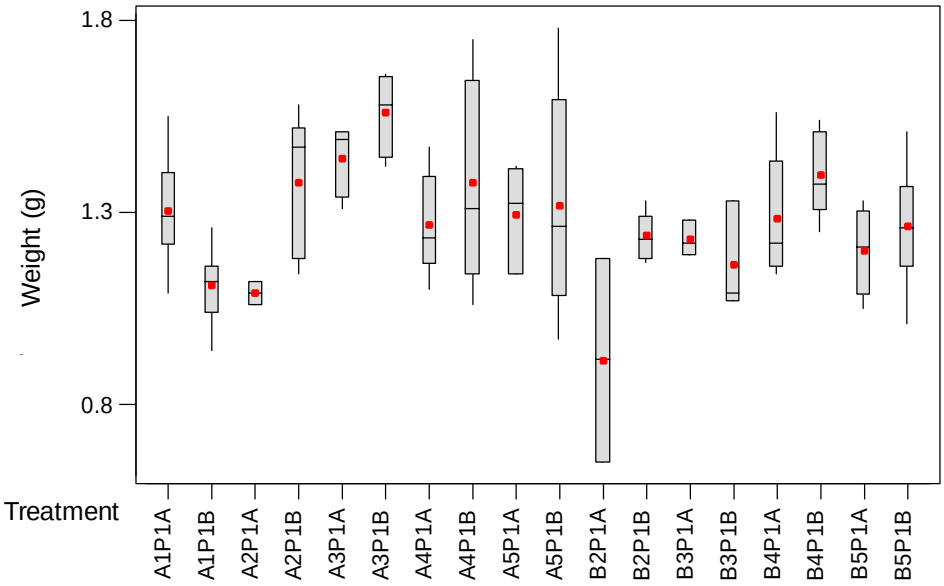


Figure 5.1-9. Box plots of tibiotarsus weight in g of F1 females by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

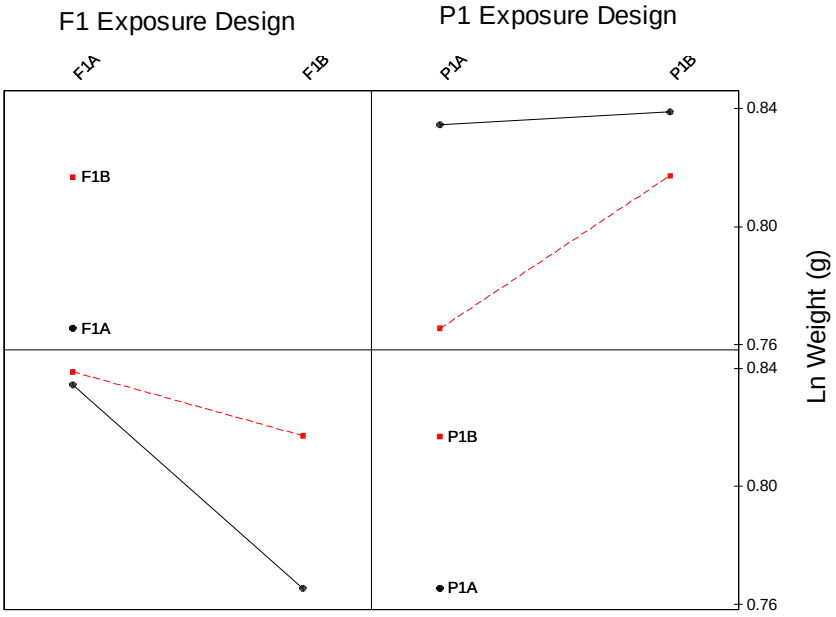


Figure 5.1-10. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed tibiotarsus weight (g) in F1 females (General Linear Model analysis; p=0.147).

Tarsometatarsus

No significant differences in tarsometarsus length were observed between F1 exposure strategy (p=0.896), parental dietary treatment (p=0.190), or P1 exposure scenario (p=0.728) in F1 female quail.

Males

Tibiotarsus

Tibiotarsus length in males from the F1 generation was not affected by parental dietary concentration of E2, F1 exposure design, or parental exposure scenario (p>0.48). However, there was a trend (p=0.11) for the tibiotarsus diameter of male offspring of P1A parents exposed for 13 weeks to E2 to be greater than tibiotarsus diameter of the offspring of the P1B (5 week exposure) birds (Figure 5.1-11). The distribution of tibiotarsus length and diameter in F1 males by dietary treatment within F1 and P1 exposure designs is shown in Figure 5.1-12. There was also a nearly significant (p=0.09) interaction between the F1 and P1 exposure designs affecting the weight of the tibiotarsus in F1 male birds (Figures 5.1-13 and 5.1-14). Treated (F1a) offspring of P1A parents appeared to have heavier tibias than treated offspring of P1B parents, whereas, tibiotarsus weight of the F1b males appeared unaffected by parental exposure scenario.

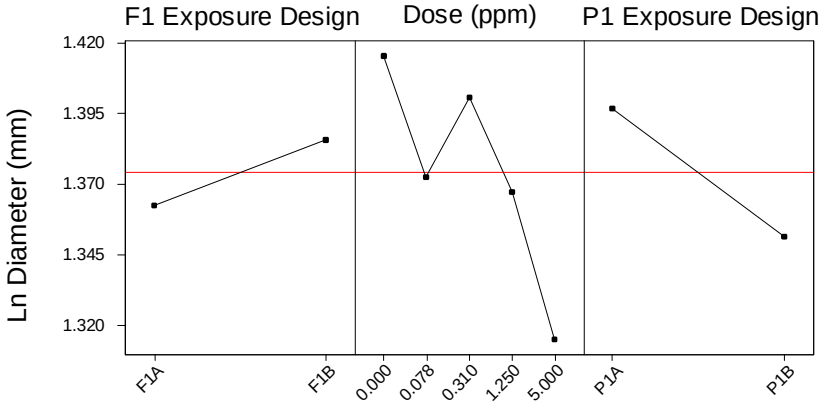


Figure 5.1-11. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed tibiotarsus diameter (mm) of F1 males (General Linear Model analysis; nearly significant differences between F1 exposure scenarios, p=0.11).

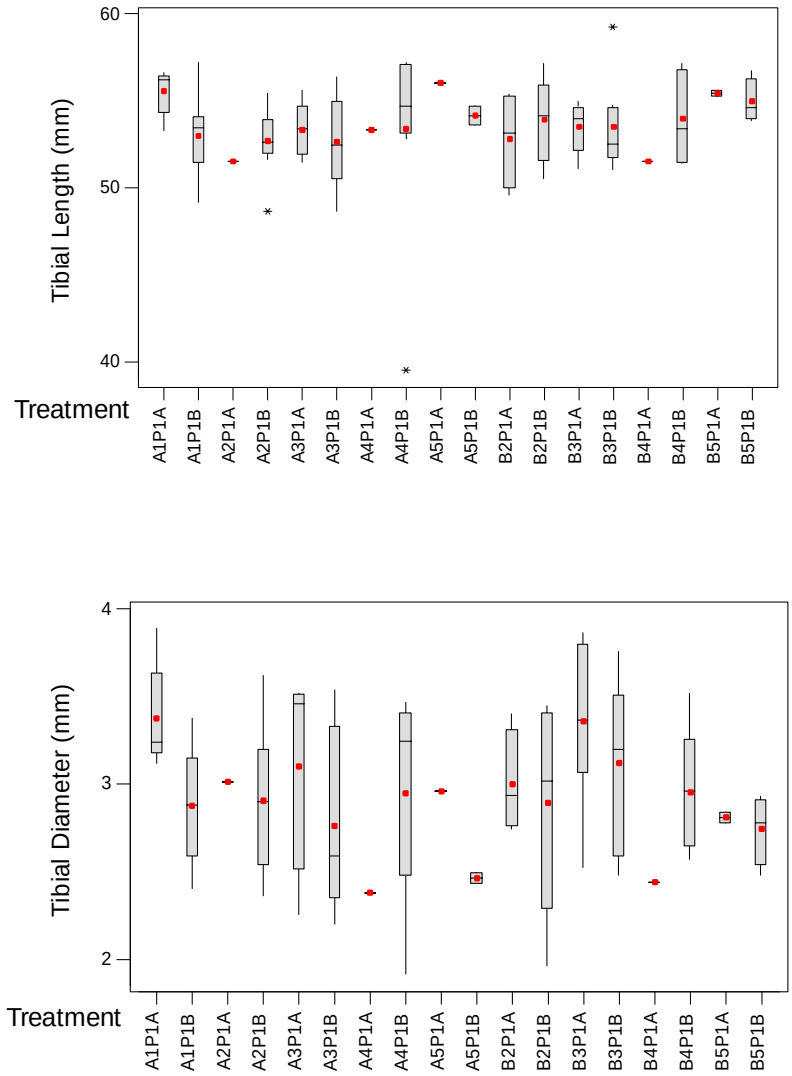


Figure 5.1-12. Box plots of tibiotarsus length in mm (above) and tibiotarsus diameter in mm (below) of F1 males by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

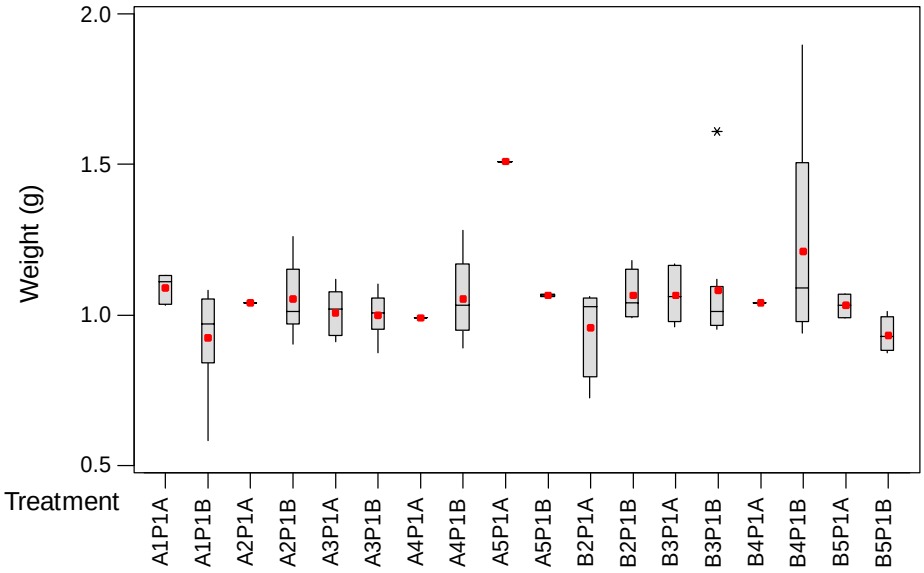


Figure 5.1-13. Box plots of tibiotarsus weight in g of F1 males by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

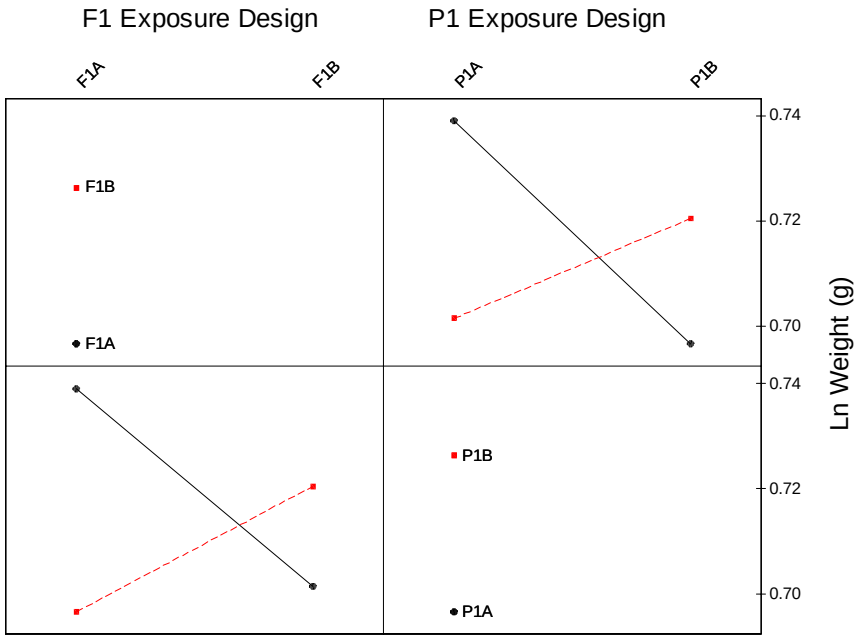


Figure 5.1-14. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed tibiotarsus weight (g) in F1 males (General Linear Model analysis; p=0.09).

Tarsometatarsus

A nearly significant (p=0.07) F1 exposure design effect on the tarsometatarsus lengths in F1 males was detected, but as seen in Figure 5.1-15 , treated (F1a) males had overall mean tarsometatarsus lengths only about 2% greater than those of F1 males receiving no treatment. No effect of dietary treatment (p=0.91) or parental exposure scenario (p=0.88) on the bone length was observed (Figure 5.1-15). The distribution of tarsometatarsus lengths in F1 males by dietary treatment and F1 and P1 exposure design is shown in Figure 5.1-16.

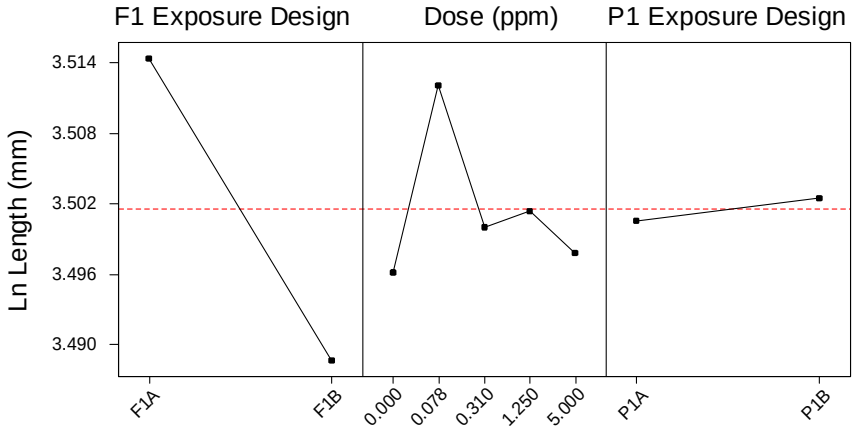


Figure 5.1-15. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed tarsometatarsus lengths (mm) of F1 males (General Linear Model analysis; nearly significant differences between F1 exposure scenarios, p=0.07).

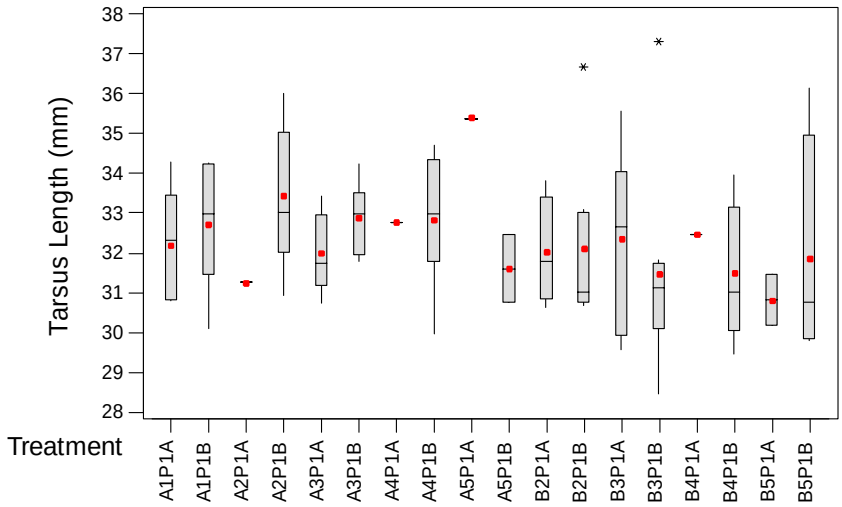
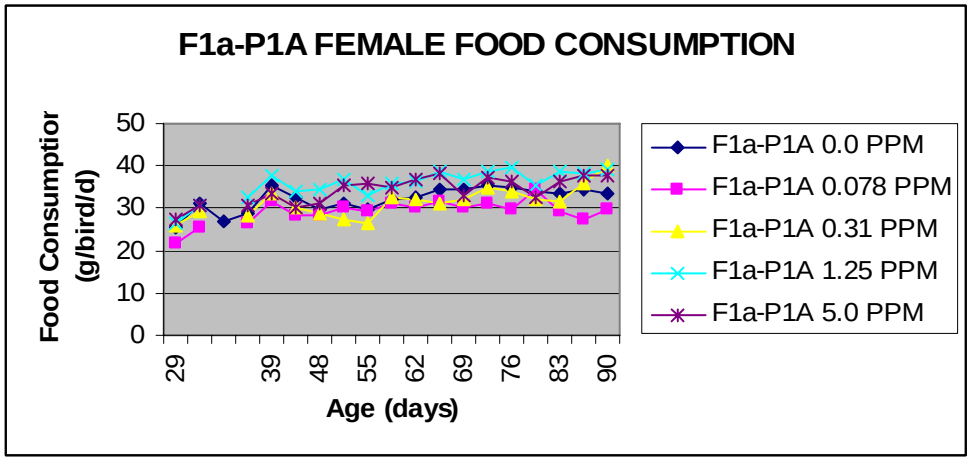


Figure 5.1-16. Box plots of tarsometatarsus length in mm of F1 male quail by parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) within F1 exposure strategy (F1a, treated with same diets as parents; F1b, untreated) and parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying). Means are indicated by solid circles.

5.2 Food Consumption (F1)

Females

Average food consumption over time for the F1a and F1b female quail is shown in Figures 5.2-1 and 5.2-2, respectively. Average food consumption rates of treated (F1a) females did not differ significantly from that of untreated (F1b) hens (p=0.26), nor did *in ovo* exposure from the two different P1 exposure scenarios result in significantly different (p=0.95) food consumption rates. However, there was a significant (p=0.003), though nonlinear, effect of treatment concentration on food consumption (Figure 5.2-3).



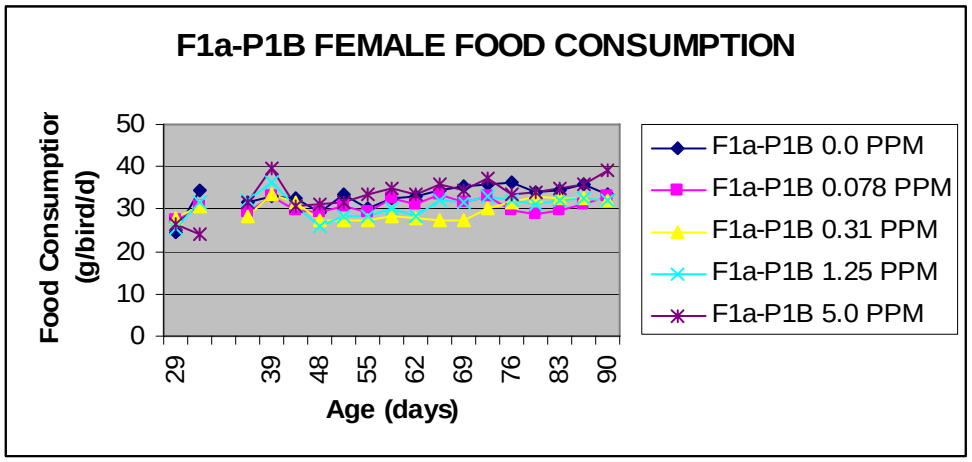
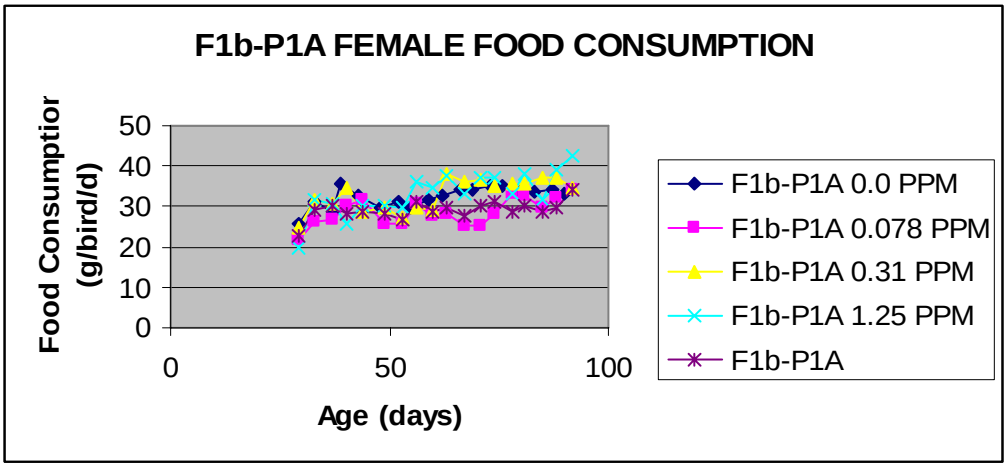


Figure 5.2-1. Average food consumption (g/bird/day) of F1 females fed E2 treated feed from hatch (F1a). F1a hens were fed treated feed at the same concentration as their parents. In addition to their dietary exposure, F1a hens received an *in ovo* dose from parents fed E2 from either 3 weeks of age (F1a-P1A) or 11 weeks of age (F1a-P1B).



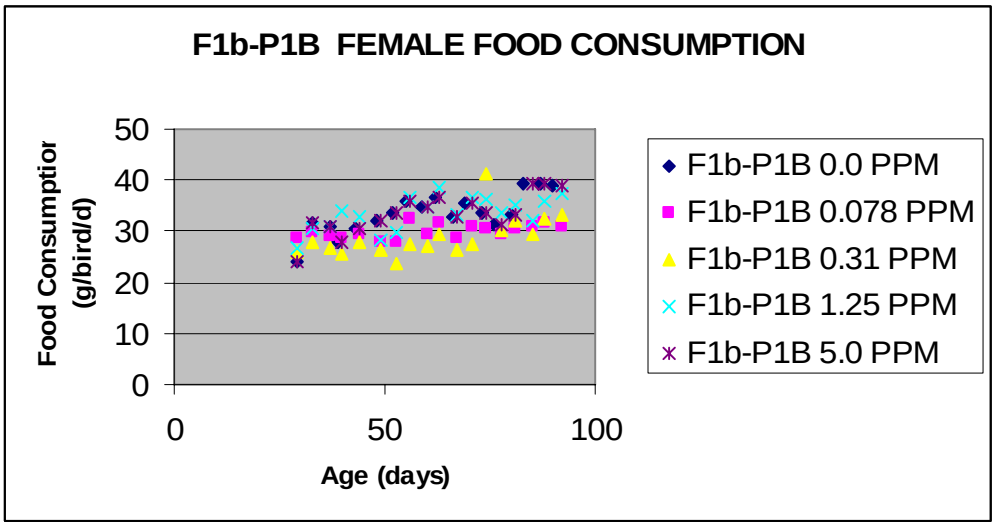


Figure 5.2-2. Average food consumption (g/bird/day) in untreated F1 females (F1b). F1b hens received an *in ovo* dose from parents fed E2 from either 3 weeks of age (F1b-P1A) or 11 weeks of age (F1b-P1B).

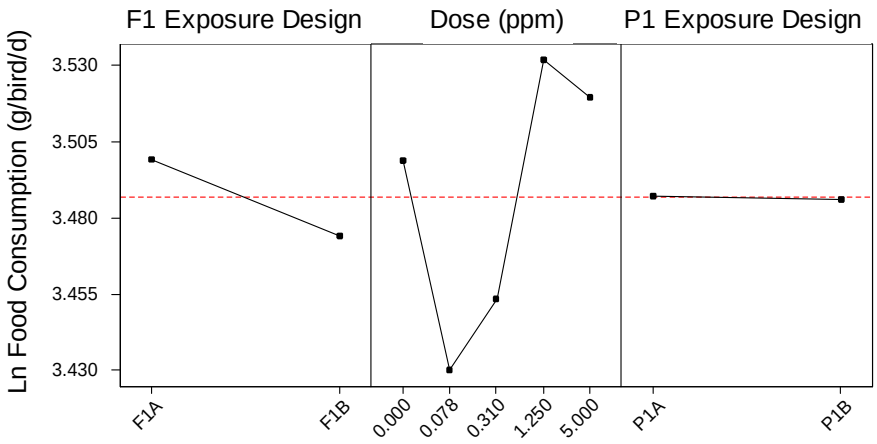


Figure 5.2-3. General Linear Model analysis of the effect of F1 exposure strategy, P1 exposure scenario and dietary concentration on the natural log-transformed average food consumption rate (g/bird/d) in female quail fed E2 treated diets. F1a females were treated from hatch with the same dietary concentration of E2 given to their parents. F1b birds were not fed treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from 3 weeks of age; in the P1B design, breeding quail were exposed to E2 for 5 weeks. Significant (p=0.003) for a non-concentration linear P1 dietary treatment effect.

Food consumption was regressed against age from 3 weeks of age through 13 weeks of age and the slopes were compared between dietary treatments and between the F1a and F1b exposure designs and the P1A and P1B exposure scenarios by General Linear Model analysis. For female quail, no significant difference in food consumption rate over time (age) between the two F1 or P1 exposure designs (p=0.337 and p=0.151, respectively) was observed. However, there was a nearly significant (p=0.108) dietary treatment effect on the slope of the food consumption rate over time. The nonlinear response was due to a nearly significant food consumption rate decrease with age in the 0.078 ppm E2 treatment groups (p=0.074, Kruskal-Wallis) (Figure 5.2-4).

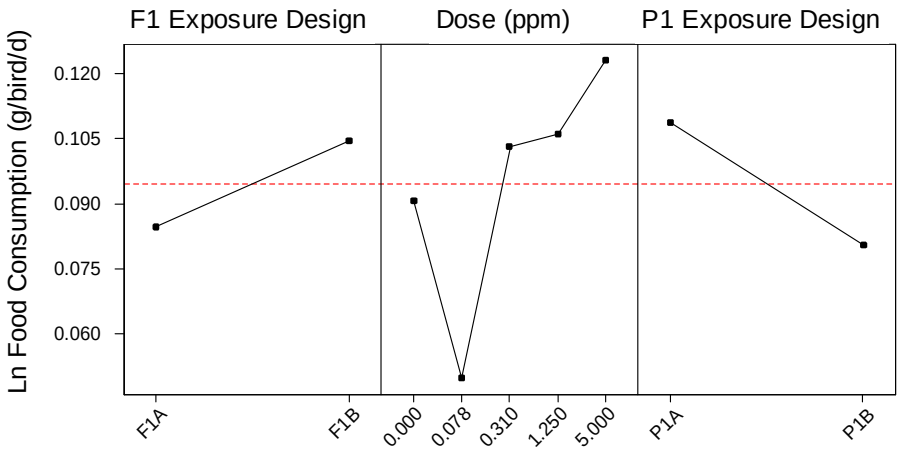
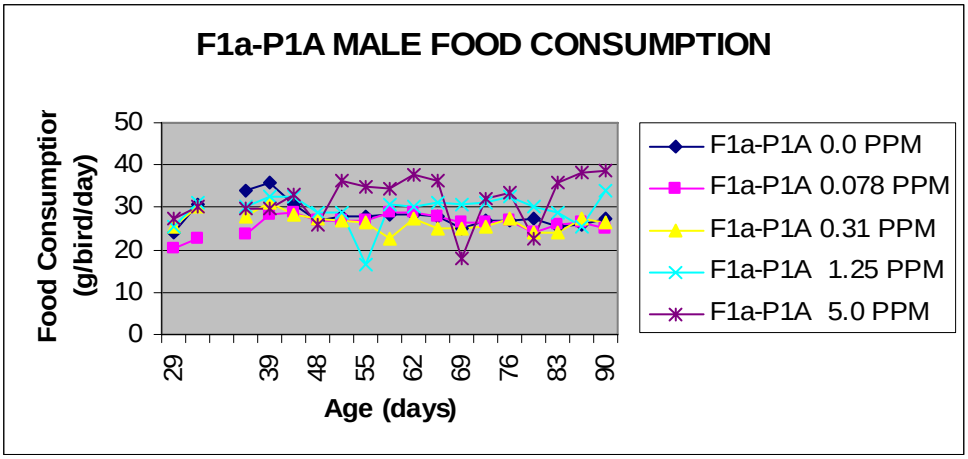


Figure 5.2-4. General Linear Model analysis of the effect of F1 exposure strategy, P1 exposure scenario and dietary concentration on the slopes of food consumption regressed against age (g/bird/d) in female quail fed E2 treated diets. F1a females were treated from hatch with the same dietary concentration of E2 given to their parents. F1b birds were not fed treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from pre-maturation; in the P1B design, breeding quail were exposed to E2 for 5 weeks. Nearly significant (p=0.108) dietary treatment effect (non-dose linear).

Males

Average food consumption over time for the F1a and F1b male quail is shown in Figures 5.2-5 and 5.2-6, respectively. Average food consumption rates for males were not affected by F1 treatment (p=0.470). However, nearly significant differences in food consumption as a result of dietary treatment (p=0.120) and of P1 exposure scenario (p=0.125) were detected (Figure 5.2-7).

When food consumption was regressed against age, F1 exposure and dietary treatment did not have an effect on the slopes of the food consumption curves of the male offspring. Only P1 exposure design showed a nearly significant effect (p=0.148) on the slope of the food consumption curve (Figure 5.2-8); however, the difference in the mean slope values between the two designs (0.02) is unlikely to be biologically important.



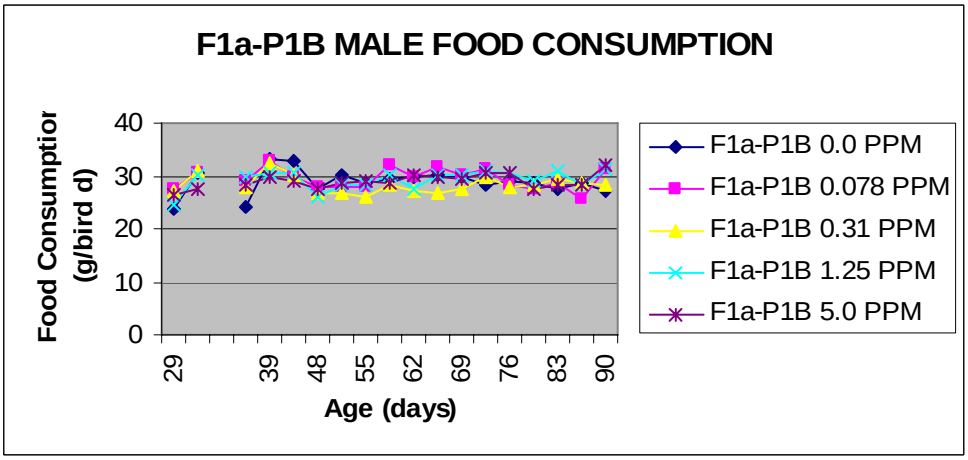
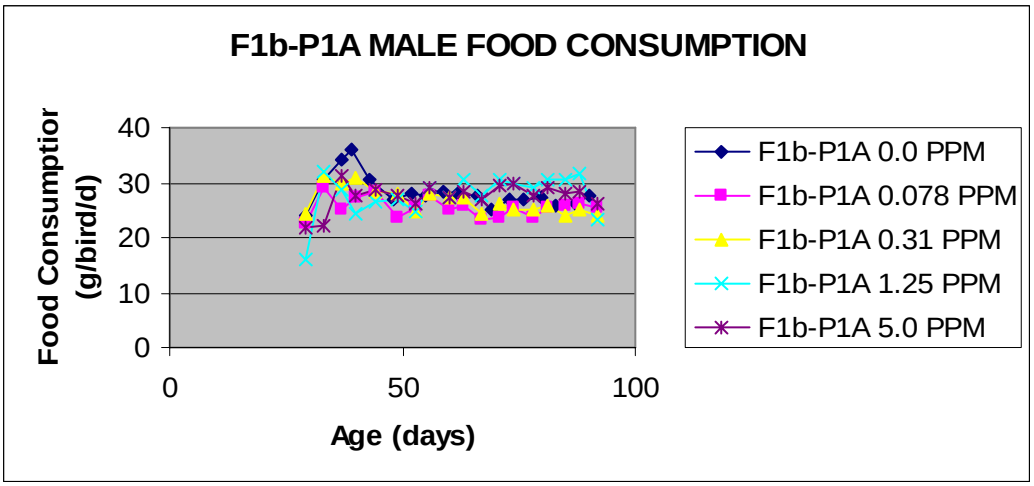


Figure 5.2-5. Average food consumption (g/bird/day) of F1 males fed E2 treated feed from hatch (F1a). F1a birds were fed treated feed at the same concentration as their parents. In addition to their dietary exposure, F1a males received an *in ovo* dose from parents fed E2 from either 3 weeks of age (F1a-P1A) or 11 weeks of age (F1a-P1B).



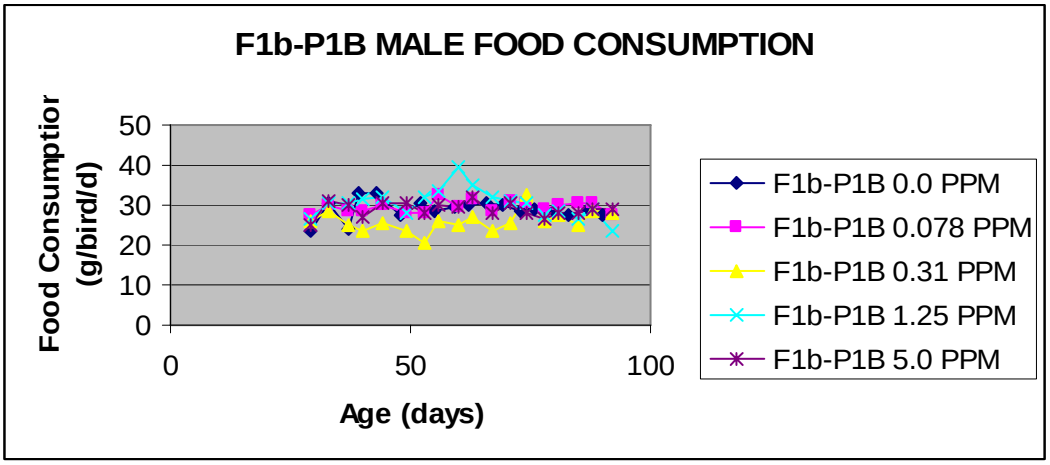
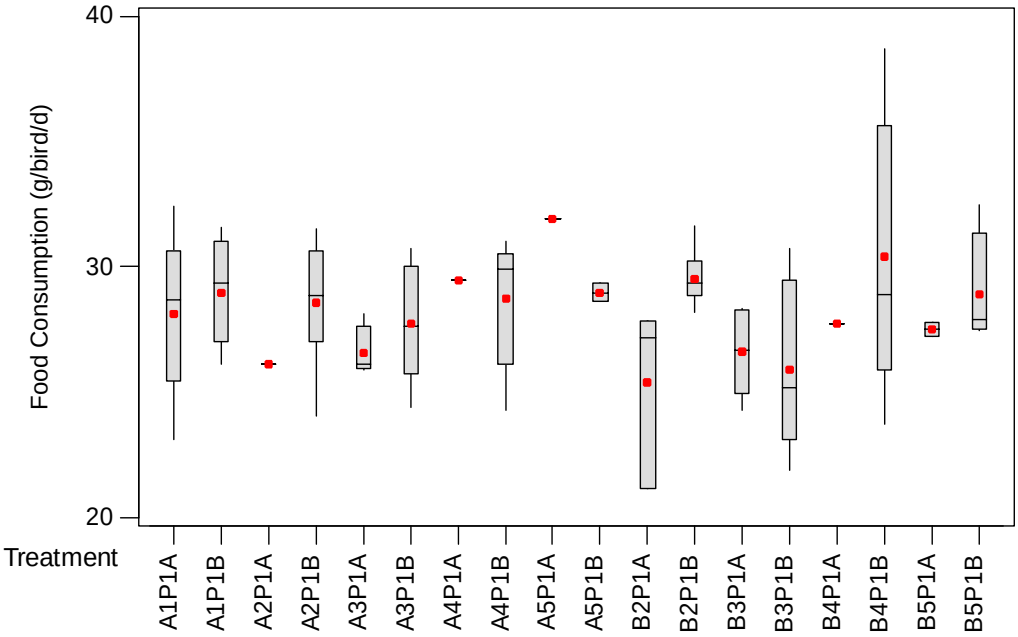


Figure 5.2-6. Average food consumption (g/bird/day) in untreated F1 males (F1b). F1b males received an *in ovo* dose from parents fed E2 from either 3 weeks of age (F1b-P1A) or 11 weeks of age (F1b-P1B).



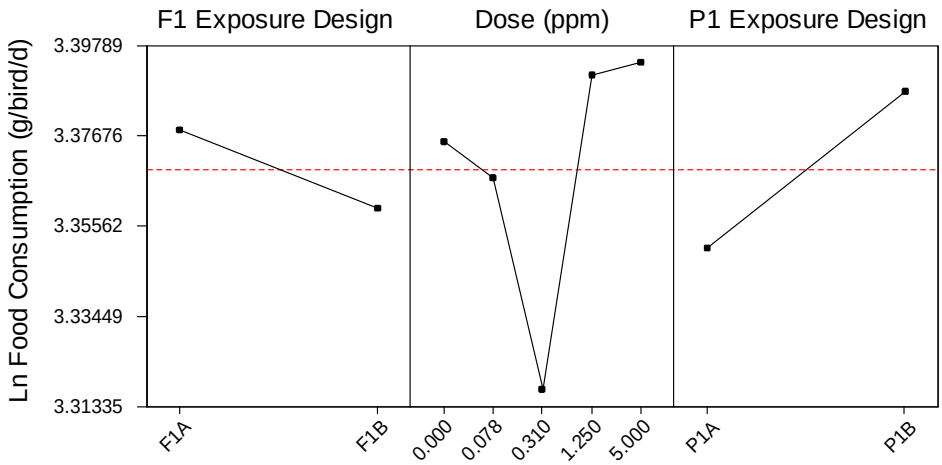


Figure 5.2-7. Box plots (above) of average food consumption (g/bird/d) by male F1 offspring. General Linear Model analysis (below) of the effect of F1 exposure strategy, P1 exposure scenario and dietary concentration on the natural log of food consumption (g/bird/d) in male quail fed E2 treated diets. F1a birds were treated from hatch with the same dietary concentration of E2 given to their parents. F1b birds were not fed treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from pre-maturation; in the P1B design, breeding quail were exposed to E2 for 5 weeks. Nearly significant non-concentration linear dietary treatment effect (p=0.120) and a P1 exposure scenario effect (p=0.125). Means are indicated by solid circles.

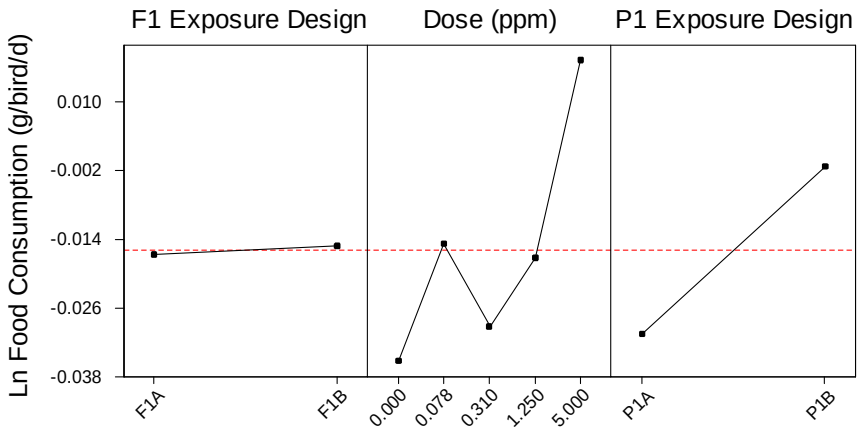


Figure 5.2-8. General Linear Model analysis of the effect of F1 exposure strategy, P1 exposure scenario and dietary concentration on the slopes of log transformed food consumption regressed against age (g/bird/d) in male quail fed E2 treated diets. F1a birds were treated from hatch with the same dietary concentration of E2 given to their parents. F1b birds were not fed treated diets. In the P1A exposure scenario, birds were exposed to E2 for 13 weeks from pre-maturation; in the P1B design, breeding quail were exposed to E2 for 5 weeks. A P1 exposure design effect detected (p=0.148).

5.3 Clinical Observations, Aggression, Unscheduled Deaths, and Abnormalities Observed at Necropsy (F1)

5.3.1 Clinical Observations

As found in the P1 generation, clinical signs were limited to aggressive behavior and associated feather damage and pecking injury. No egg binding was observed in the F1 generation. One case of rectal prolapse in a 0.078 ppm E2 treated female was noted. Birds were paired at 3 weeks of age and no aggression deaths of males by their female pen mate occurred. Aggression or de-feathering increased post-puberty within pen pairs and all pairs were separated to equalize mating opportunity and stress across the pairs.

5.3.2 Aggression (Injuries)

Females

Pecking Injury and Feather Loss

The occurrence of pecking injuries was nearly significantly greater in F1b (non-treated) birds than F1a birds ($p = 0.068$) and significantly greater in P1B offspring than P1A offspring ($p = 0.040$) (Figure 5.3-1), although the incidence of pecking injury was low and unrelated to E2 concentration. Little incidence of aggressive territorial behavior monitored by loss of feathers from the front of neck and chest area from females trying to reach females in adjoining pens was observed ($p>0.483$) (Table 5.3-1). Occurrence of feather loss on the head and back of neck of females as a result of mounting attempts by the male were counted as a potential measure of copulation activity. It appeared that offspring of P1B parents tended ($p=0.108$) to have a greater proportion of females with feather loss than did female offspring of P1A parents (Figure 5.3-2). Feather loss was unaffected by F1 exposure design or P1 dietary concentration ($p>0.194$).

Table 5.3-1. Incidence of pecking injury and feather loss in females of the F1 generation.

Treatment	Count	Pecking Injury	Feather Loss, Chest Feathers Rubbed Off	Feather Loss, Head and Neck Area
F1A-P1A-0 ppm	10	0	2	4
F1A-P1A-0.078 ppm	3	0	1	1
F1A-P1A-0.31 ppm	5	0	1	1
F1A-P1A-1.25 ppm	6	0	0	1
F1A-P1A-5 ppm	6	0	0	0
F1B-P1A-0.078 ppm	2	0	0	2
F1B-P1A-0.31 ppm	3	0	0	2
F1B-P1A-1.25 ppm	5	0	0	1
F1B-P1A-5 ppm	4	0	0	3
F1A-P1B-0 ppm	7	1	2	4
F1A-P1B-0.078 ppm	7	0	0	4
F1A-P1B-0.31 ppm	4	0	0	4
F1A-P1B-1.25 ppm	4	0	0	4
F1A-P1B-5 ppm	8	0	1	2
F1B-P1B-0.078 ppm	6	1	0	6
F1B-P1B-0.31 ppm	3	0	0	3
F1B-P1B-1.25 ppm	7	1	2	2
F1B-P1B-5 ppm	6	2	0	2

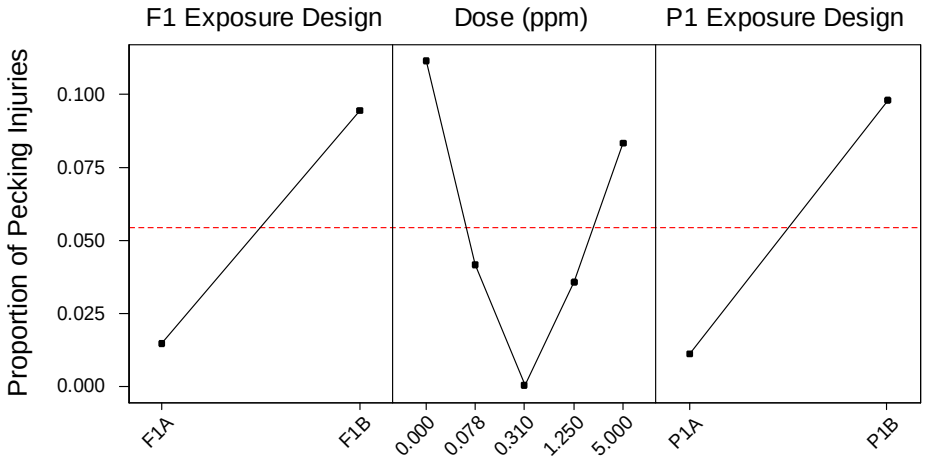


Figure 5.3-1. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on proportion of F1 females with pecking injuries. General Linear Model analysis; nearly significant difference between F1 exposure strategy, p=0.068; significant difference between P1 exposure scenarios, p=0.040.

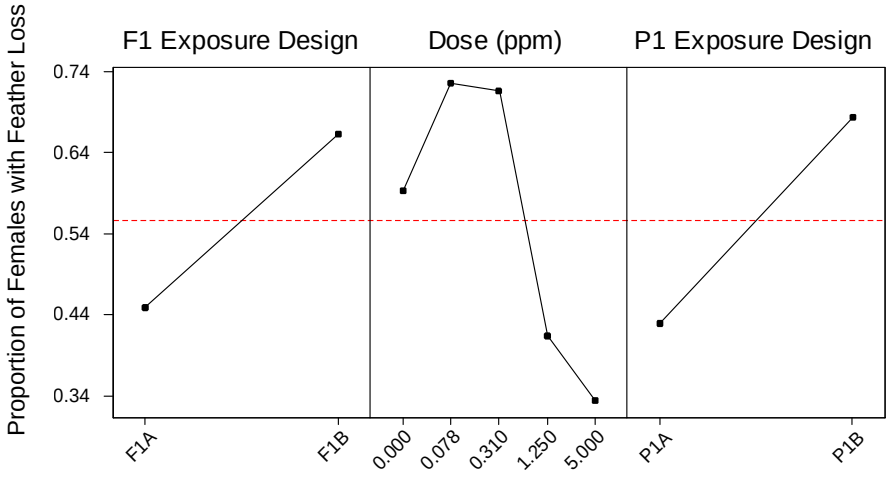


Figure 5.3-2. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on proportion of F1 females with feather loss associated with mounting attempts by males. General Linear Model analysis; nearly significant difference between P1 exposure scenario, p=0.108.

Non-Pecking Injuries

In the F1 generation, a total of 6 females had a non-pecking injury during the course of their development from hatchling to laying hen. Three of the females suffered a broken bill tip, 2 had chest impact injuries, and one injured her head on a valve of the auto-water system.

Males

Pecking Injury and Feather Loss

Feather damage and loss of chest, and neck feathers—largely from attempting to reach males in adjacent cages—was not affected by P1 dietary concentration or F1 or P1 exposure design ($p>0.25$; Figure 5.3-3). Feather loss from female aggression was slightly greater in the 5 ppm and 0.31 ppm dosed birds than in the controls or other dietary treatment groups ($p = 0.117$) (Figure 5.3-4.) As shown in Table 5.3-2, no difference in feather condition was attributable to F1 or P1 exposure designs ($p>0.205$).

Table 5.3-2. Incidence of pecking injury and feather loss in males of the F1 generation.

Treatment	Count	Pecking Injury	Feather loss, Chest feathers rubbed off	Feather Loss Head and neck area	Non-pecking Injuries
F1A-P1A-0 ppm	5	0	0	0	1[a]
F1A-P1A-0.078 ppm	1	0	0	0	0
F1A-P1A-0.31 ppm	5	1	1	3	0
F1A-P1A-1.25 ppm	1	0	0	0	0
F1A-P1A-5 ppm	1	0	0	1	0
F1B-P1A-0.078 ppm	4	0	1	0	0
F1B-P1A-0.31 ppm	6	0	1	1	0
F1B-P1A-1.25 ppm	1	0	0	0	0
F1B-P1A-5 ppm	2	0	0	0	0
F1A-P1B-0 ppm	7	0	0	0	0
F1A-P1B-0.078 ppm	8	1	1	1	0
F1A-P1B-0.31 ppm	5	0	0	0	0
F1A-P1B-1.25 ppm	7	0	1	0	0
F1A-P1B-5 ppm	2	1	0	1	0
F1B-P1B-0.078 ppm	8	0	0	0	1[b]
F1B-P1B-0.31 ppm	7	0	1	2	0
F1B-P1B-1.25 ppm	5	0	0	1	0
F1B-P1B-5 ppm	4	0	0	1	0

[a] Growth in the auricular region
[b] Unknown tissue near spleen

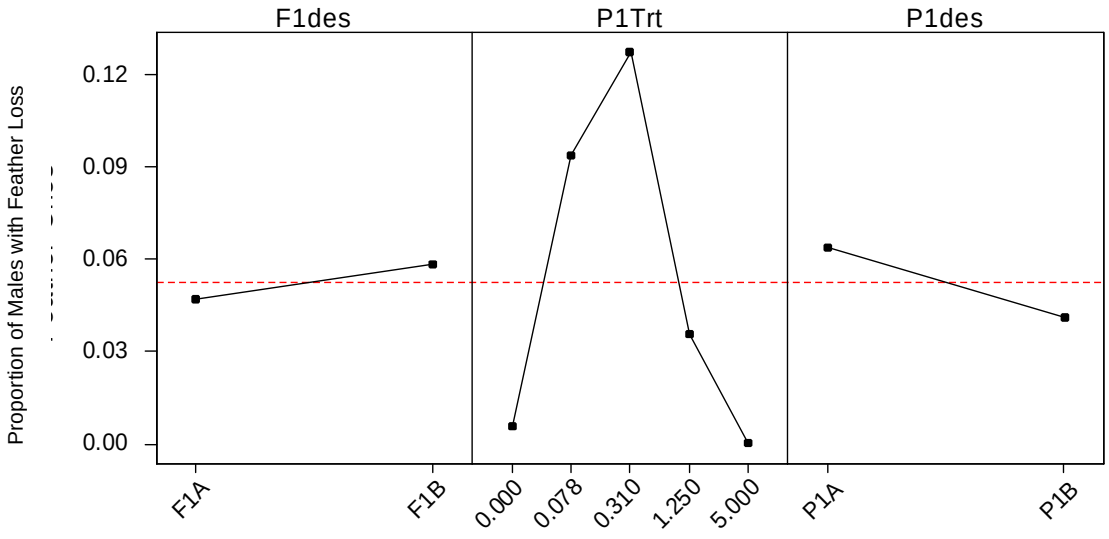


Figure 5.3-3. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on proportion of F1 males with feather loss associated with territoriality. General Linear Model analysis; no significant difference between P1 exposure scenario or dietary treatments, p≥0.25.

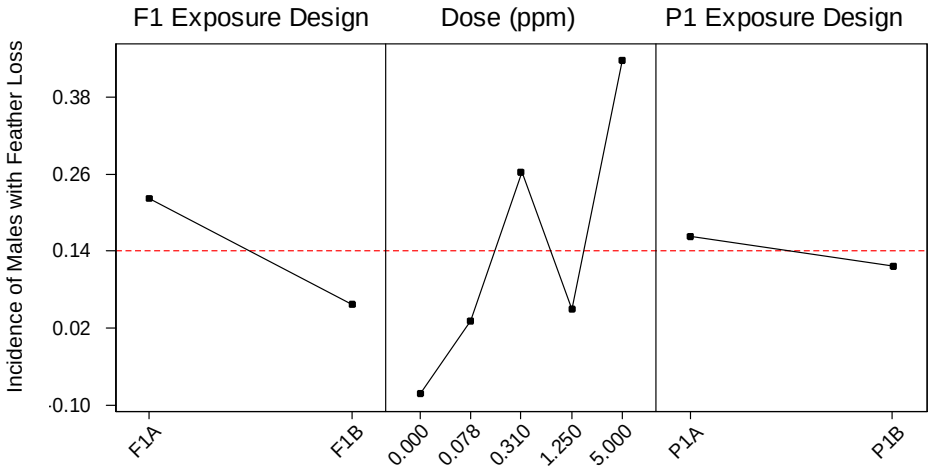


Figure 5.3-4. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on incidence rate of feather loss treatment group in F1 males associated with female aggression. General Linear Model analysis; nearly significant difference between P1 dietary concentrations, p=0.117.

Non-Pecking Injuries

Two non-pecking injuries were reported, a hematoma on the chest of an F1b-P1B male and a broken tip of the upper mandible of a shared control bird from P1A parents.

5.3.3 Early (Unscheduled) Deaths

A total of 16 unscheduled deaths (males, females and undetermined gender) occurred in the F1 generation. Of the 16 deaths, 13 were chicks under 14 days of age and 2 were juveniles (Table 5.3-3). About half (53%) of these young birds had foot and/or leg malformations. All of the young birds that died were offspring of E2-treated parents, the majority (60%) from parents in the 1.25 ppm and 5 ppm treatment groups. Only one adult, a female from a control group, died prior to the termination of the study. She was in good condition and died suddenly of no apparent cause.

Table 5.3-3. Incidence of unscheduled deaths of F1 birds.

Treatment	No. Deaths < 14 Day of Age	No. Deaths Juvenile	No. Deaths Adults
F1aP1A-0 ppm	0	0	0
F1aP1A-0.078 ppm	1[a]	0	0
F1aP1A-0.31 ppm	0	0	0
F1aP1A-1.25 ppm	1[b]	0	0
F1aP1A-5 ppm	1[b]	0	0
F1bP1A-0.078 ppm	0	0	0
F1bP1A-0.31 ppm	0	0	0
F1bP1A-1.25 ppm	1, 1[b]	0	0
F1bP1A-5 ppm	1[b]	0	0
F1aP1B-0 ppm	0	0	1[c]
F1aP1B-0.078 ppm	0	0	0
F1aP1B-0.31 ppm	1	1[b]	0
F1aP1B-1.25 ppm	1[b]	1[b]	0
F1aP1B-5 ppm	1	0	0
F1bP1B-0.078 ppm	1[b]	0	0
F1bP1B-0.31 ppm	2	0	0
F1bP1B-1.25 ppm	1	0	0
F1bP1B-5 ppm	0	0	0

[a] Rectal prolapse
[b] Foot/leg malformation
[c] Female, no apparent cause

5.3.4 Abnormalities Observed at Necropsy

Females

Gross Abnormalities

A total of 20 incidents of right oviduct occurrence were observed in F1 females at necropsy. However, the distribution of the abnormality across treatments was unrelated ($p>0.708$) to dietary concentration or F1 or P1 exposure designs (Table 5.3-4). Only one incident of a right ovary was found; it occurred in the 5 ppm E2 dietary exposure group of the treated (F1a) offspring of P1A parents. Only two other gross tissue abnormalities were observed at necropsy in adult females of the F1 generation, a 5 mm nodule on the median lobe of the liver of an F1a-P1A hen treated with 0.078 ppm E2 diet and a missing right adrenal gland in an untreated (F1b) hen from parents exposed to 5 ppm E2 under the P1A exposure scenario.

Female offspring from P1B parents had nearly significantly ($p=0.069$) greater occurrence of an abnormal neck curvature than did offspring from P1A dosed birds, but the increase in incident rate was small. F1a and F1b mean responses were not significantly different ($p = 0.88$), nor were the mean responses for P1 dietary concentrations ($p = 0.167$) (Figure 5.3-5). The incidence of foot or leg malformations did not show any significant effect of dietary treatment or exposure scenario in female birds.

Table 5.3-4. Incidence of abnormalities observed in F1 females.

Treatment	Count	Abnormal Foot/Leg at Hatch	Abnormal foot developed after hatch (> 3weeks of age)	Abnormal Neck Curvature at >3 weeks	Right Ovary	Right Oviduct	Liver	Abnormal Adrenal Gland	Other Abnormalities
F1a-P1A-0 ppm	10	0	0	0	0	1	0	0	0
F1a-P1A-0.078 ppm	3	0	0	1	0	0	1	0	1
F1a-P1A-0.31 ppm	5	1	0	0	0	2	0	0	0
F1a-P1A-1.25 ppm	6	2	0	1	0	2	0	0	0
F1a-P1A-5 ppm	6	0	0	0	1	1	0	0	0
F1b-P1A-0.078 ppm	2	1	0	0	0	0	0	0	0
F1b-P1A-0.31 ppm	3	0	0	0	0	2	0	0	0
F1b-P1A-1.25 ppm	5	0	0	0	0	1	0	0	0
F1b-P1A-5 ppm	4	0	0	0	0	0	0	1	0
F1a-P1B-0 ppm	7	1	2	0	0	3	0	0	0
F1a-P1B-0.078 ppm	7	1	0	1	0	2	0	0	0
F1a-P1B-0.31 ppm	4	0	0	0	0	0	0	0	0
F1a-P1B-1.25 ppm	4	0	0	1	0	0	0	0	0
F1a-P1B-5 ppm	8	1	0	0	0	1	0	0	1[a]
F1b-P1B-0.078 ppm	6	0	0	0	0	2	0	0	0
F1b-P1B-0.31 ppm	3	0	1	1	0	0	0	0	0
F1b-P1B-1.25 ppm	7	0	0	2	0	2	0	0	0
F1b-P1B-5 ppm	6	0	0	0	0	1	0	0	1[a]

[a] Small nodule on the upper mandible of adult hen

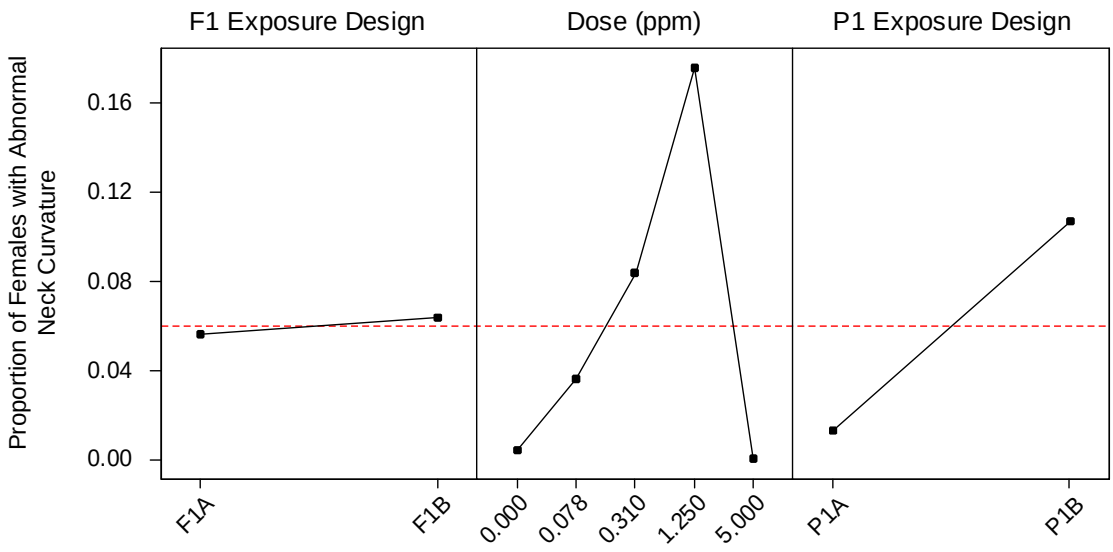


Figure 5.3-5. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on incidence proportion of abnormal neck curvature per group in F1 females. General Linear Model analysis; nearly significant differences between P1 exposure scenarios, p=0.069.

Males

Gross Abnormalities

With the exception of a 6 mm diameter mass of unknown tissue type near the spleen in one male and a growth in auricular region in another, no other soft tissue abnormalities were found in males of the F1 generation. Likewise, foot/leg and neck abnormalities were few, and no significant difference in their occurrence was detected between F1 and P1 exposure designs or across P1 dietary treatments (p> 0.15) (Table 5.3-5).

Table 5.3-5. Incidence of abnormalities observed in F1 males.

Treatment	Count	Abnormal Foot/Leg at Hatch	Abnormal foot developed after hatch (>3weeks of age)	Abnormal Neck Curvature at >3 weeks	Non-pecking Injuries	Other Abnormalities
F1A-P1A-0 ppm	5	0	0	0	1	0
F1A-P1A-0.078 ppm	1	0	0	0	0	0
F1A-P1A-0.31 ppm	5	0	0	0	0	0
F1A-P1A-1.25 ppm	1	0	0	1	0	0
F1A-P1A-5 ppm	1	0	0	0	0	0
F1B-P1A-0.078 ppm	4	0	0	0	0	0
F1B-P1A-0.31 ppm	6	0	0	0	0	0
F1B-P1A-1.25 ppm	1	0	0	0	0	0
F1B-P1A-5 ppm	2	0	0	0	0	0
F1A-P1B-0 ppm	7	1	0	0	0	0
F1A-P1B-0.078 ppm	8	0	1	0	0	0
F1A-P1B-0.31 ppm	5	0	1	1	0	0
F1A-P1B-1.25 ppm	7	0	0	0	0	0
F1A-P1B-5 ppm	2	0	0	0	0	1[a]
F1B-P1B-0.078 ppm	8	0	1	0	1	1[b]
F1B-P1B-0.31 ppm	7	0	0	0	0	0
F1B-P1B-1.25 ppm	5	0	0	0	0	0
F1B-P1B-5 ppm	4	0	0	0	0	0

[a] Growth in the auricular region
[b] Unknown tissue (6 mm diameter) near spleen

5.4 Organ Weights of Adult Quail at Necropsy (F1)

5.4.1 Females

Thyroid

In female quail of the F1 generation, there were significant differences in both absolute thyroid weight and the thyroid-to-body weight ratio. Absolute mean thyroid weight was significantly different between F1 exposure designs (p=0.042), greater in treated (F1a) hens than in untreated hens (F1b) (Figures 5.4-1 and 5.4-2). Likewise, F1a exposures resulted in increased mean thyroid-to-body weight ratios (p=0.023; Figure 5.4-3) and a nearly significant increase in thyroid-to-brain weight ratios (p=0.06) in hens. Absolute thyroid weights also exhibited a significant effect of P1 exposure scenario, with thyroid weight significantly greater (p=0.035) in hens from P1B (5 week exposure) parents than in hens from P1A (13 week exposure) parents (Figure 5.4-2). After normalization to brain weight, the P1 effect was observed, but was reduced to a nearly significant effect (p=0.06). In contrast, thyroid-to-body weight ratios were not affected by P1 exposure design (p=0.19). Absolute thyroid weight (p= 0.52) and both body- and brain-normalized thyroid weights (p=0.49 and p=0.73, respectively) were unaffected by parental dietary treatment.

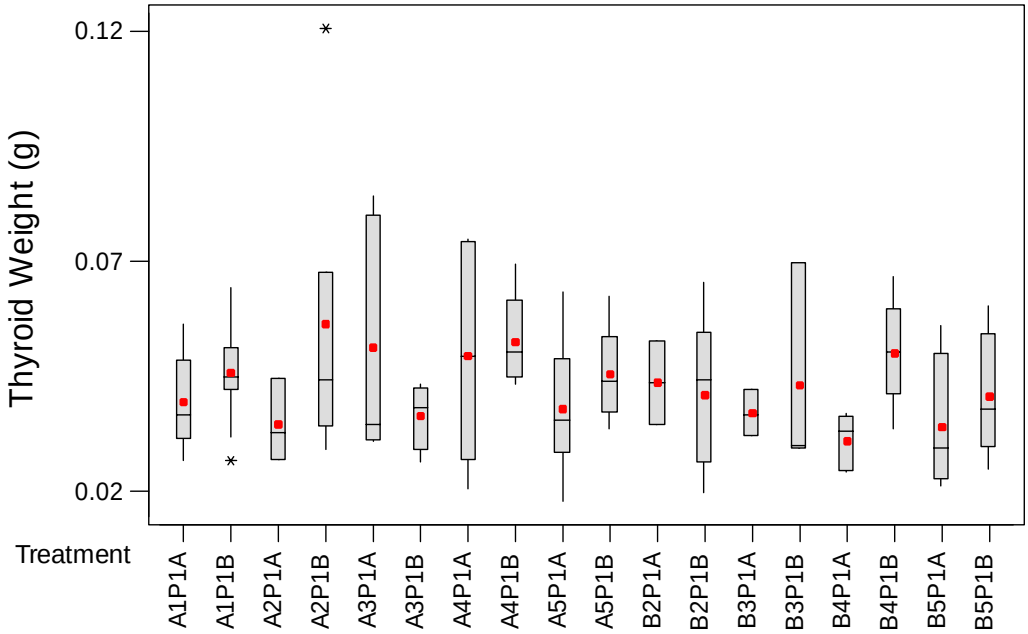


Figure 5.4-1. Box plots of thyroid weight (g) of F1 hens by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

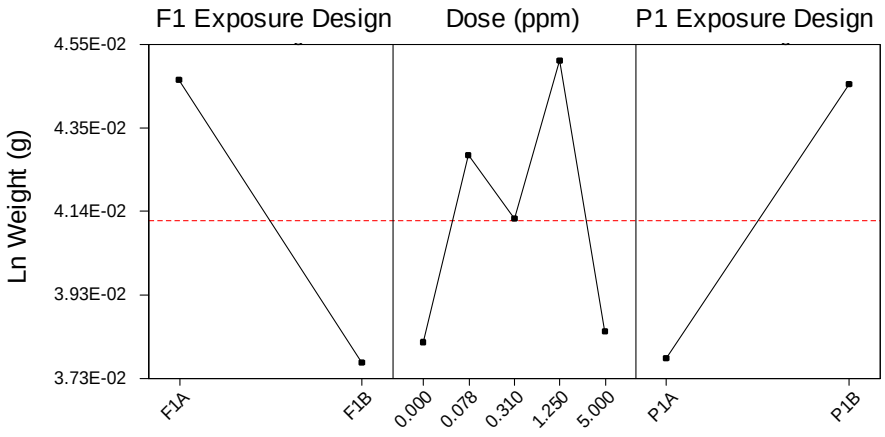


Figure 5.4-2. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed thyroid weights (g) of F1 females. General Linear Model analysis; highly significant differences between F1 exposure strategies, p=0.042, and P1 exposure scenarios, p=0.035. F1a birds were fed the same dietary treatments as their parents.

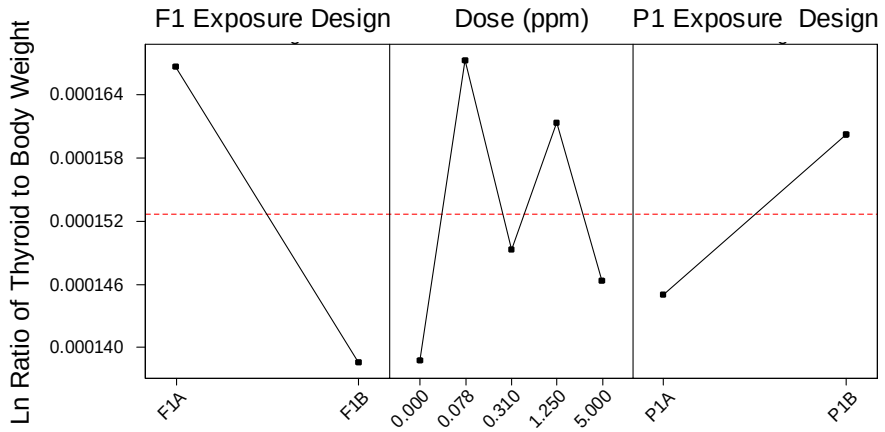


Figure 5.4-3. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed thyroid weight to body weight ratio of F1 females. General Linear Model analysis; significant differences between F1 exposure strategies, p=0.023, and P1 exposure scenarios, p=0.185. F1a birds were fed the same dietary treatments as their parents.

Pancreas

In female quail, gross pancreatic weights, as well as pancreatic weights normalized to body weight and to brain weight, showed highly significant effects of F1 exposure design ($p<0.001$). Both the weight and relative weights of the pancreas were reduced in treated (F1a) hens (Figure 5.4-4). Absolute pancreas weight and pancreas-to-body weight ratio were also significantly different across parental dietary treatment concentrations ($p=0.03$ and $p=0.047$ respectively), but the effects were not dose-linear (Figures 5.4-5 and 5.4-6). Normalization to brain weight reduced the dietary differences to a nearly significant effect ($p=0.14$). When normalized to body weight, a nearly significant interaction ($p = 0.063$) between F1 and P1 exposure designs was detected. As seen in Figure 5.4-7, untreated F1 offspring of parents exposed for 13 weeks to E2 (F1b-P1A females) tended to have larger mean pancreas to body weight ratios than the untreated offspring of parents exposed for only 5 weeks (F1b-P1B females) to E2; whereas, pancreas-to-body weight ratios of F1a hens were unaffected by parental exposure design. The difference in the mean relative pancreatic weights between these two exposure populations (F1b-P1A and F1b-P1B) was about 10%. No interaction was observed when the pancreas weight was normalized to brain weight ($p=0.48$).

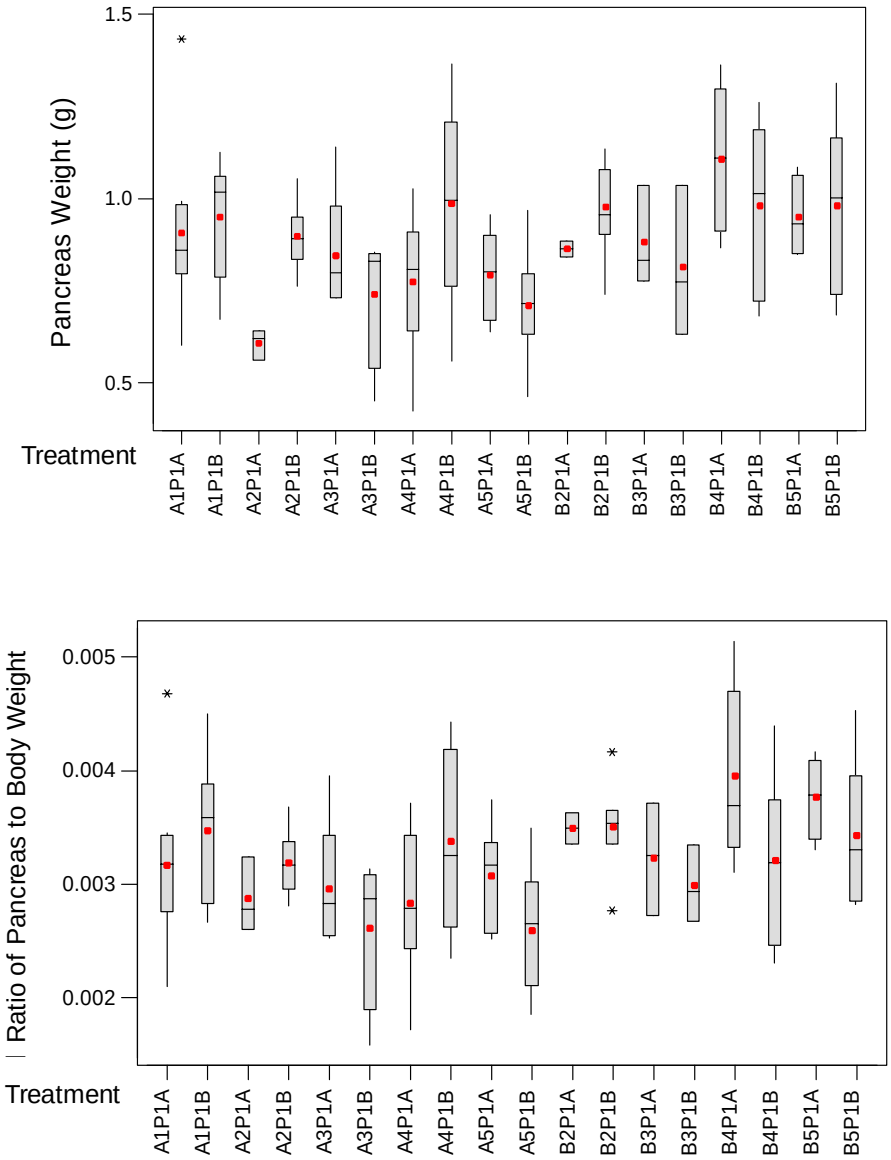


Figure 5.4-4. Box plots of pancreatic weight in grams (above) and pancreas-to-body weight ratio (below) of F1 hens by parental exposure scenario. (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

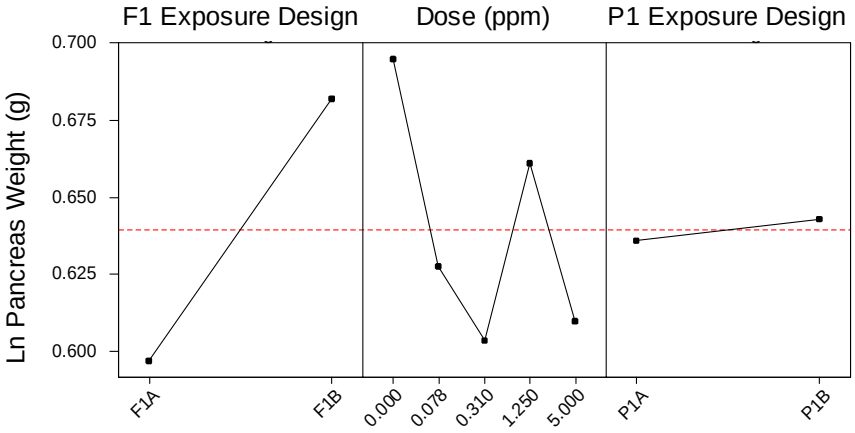


Figure 5.4-5. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed pancreas weight of F1 females. (General Linear Model analysis; highly significant difference between F1 exposure strategies, p<0.001; significant difference across parental dietary treatments, p=0.03.) F1a birds were fed the same dietary treatments as their parents.

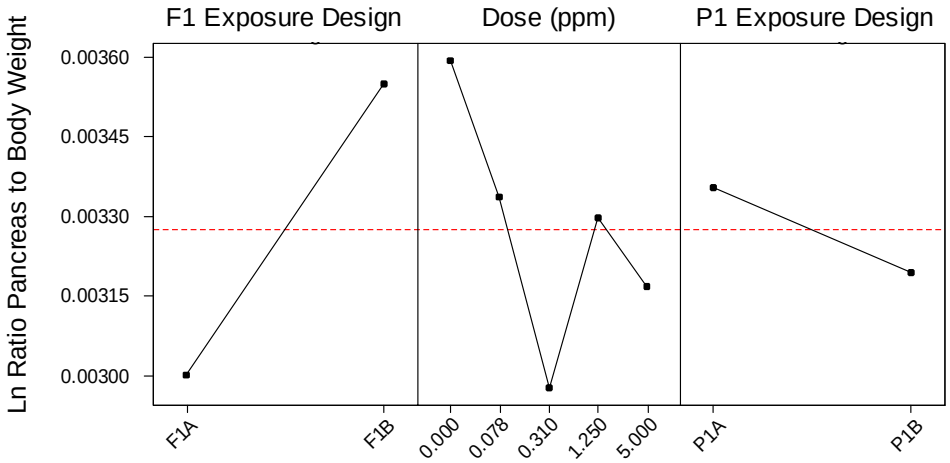


Figure 5.4-6. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed pancreatic weight to body weight ratio of F1 females. (General Linear Model analysis; highly significant differences between F1 exposure strategies, p<0.001, and significant differences across parental dietary treatments, p=0.047.) F1a birds were fed the same dietary treatments as their parents.

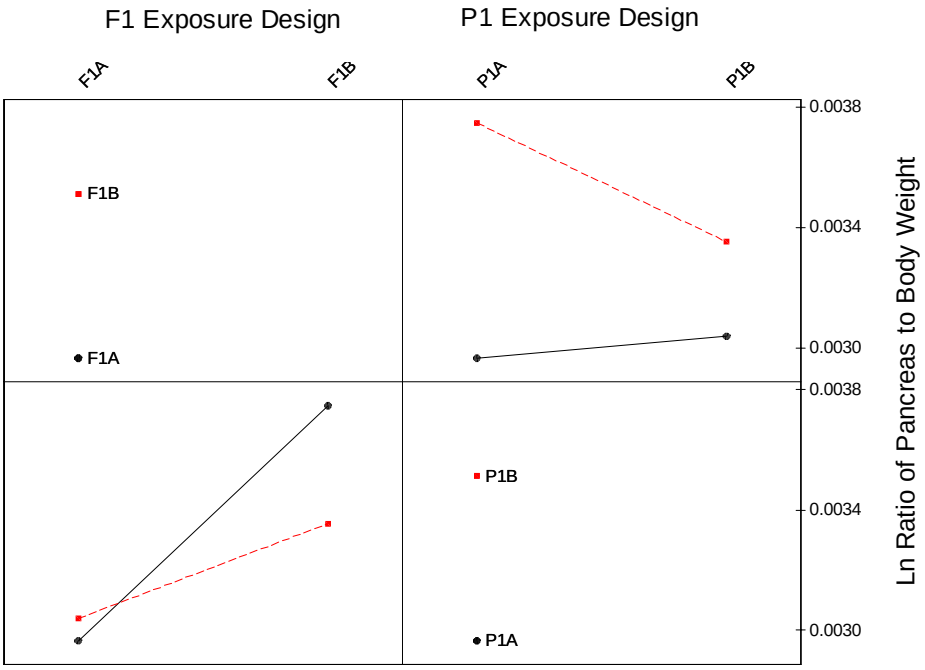


Figure 5.4-7. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the natural log-transformed pancreas-to-body weight ratios for female F1 birds. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1b, parents exposed to E2 post-maturation.

Liver

The liver weights in female birds of the F1 generation showed no significant effects of parental exposure scenario (p=0.69), dietary treatment with E2 (p=0.18), or F1 exposure design (p=0.61). Following normalization to body weight, a non concentration-linear effect of parental dietary concentration on liver-to-body-weight ratios was observed in female birds (p=0.07) (Figure 5.4-8).

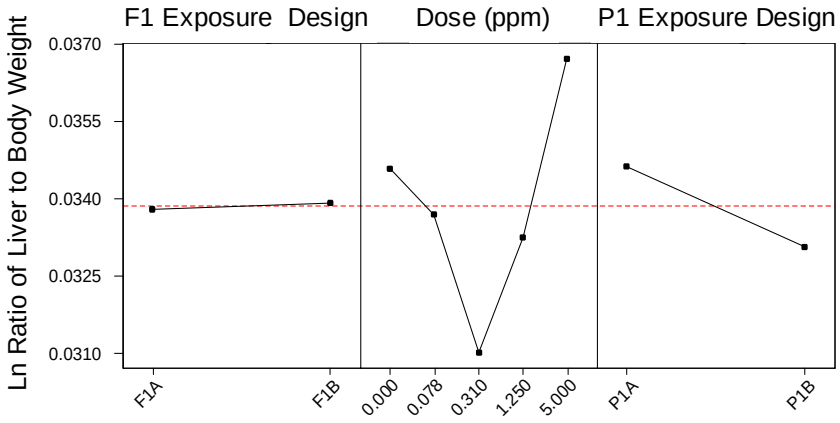


Figure 5.4-8. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed liver weight to body weight ratio of F1 females. General Linear Model analysis; nearly significant effect on parental dietary concentration was observed in female birds (p=0.07). F1a birds were fed the same dietary treatments as their parents.

Spleen

There appeared to be nearly significant effects of F1 treatment strategy ($p<0.09$) and P1 dietary concentration ($p<0.09$) on spleen weights of F1 hens (Figures 5.4-9 and 5.4-10). The F1 treatment and P1 dietary concentration effects were also observed when spleen weight was normalized to body weight ($p = 0.051$ and $p=0.12$, respectively) or brain weight ($p=0.11$ and 0.14, respectively). However, a nearly significant interaction between F1 and P1 designs was observed only when spleen weight was normalized to brain weight ($p=0.13$). As seen in Figure 5.4-11, F1a-P1A females had larger relative spleen weights than the F1a-P1B. P1 exposure scenario had no effect on absolute spleen weights or spleen-to-body weight ratios in F1 female quail ($p=0.96$ and $p=0.578$, respectively).

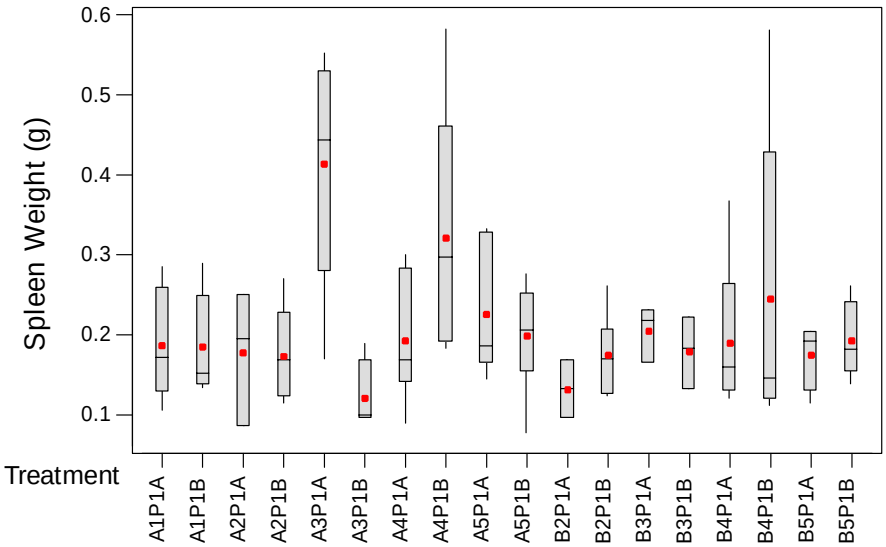


Figure 5.4-9. Box plots of gross spleen weight (g) of F1 hens by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

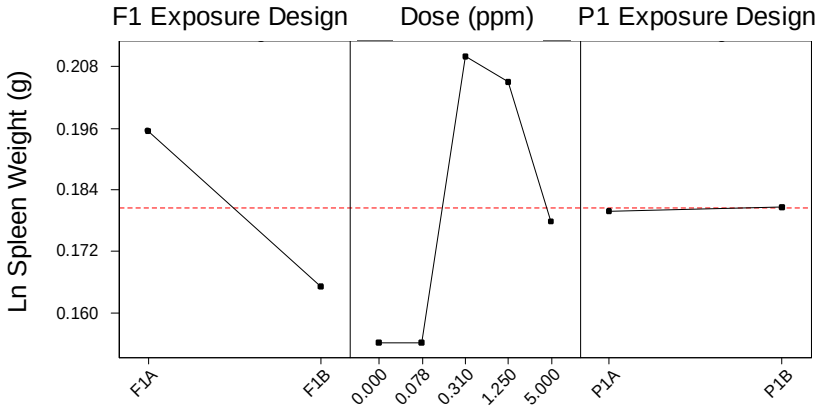


Figure 5.4-10. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed spleen weight of F1 females. General Linear Model analysis; F1 exposure strategies and P1 dietary concentrations to affected spleen weight, $p<0.09$. F1a birds were fed the same dietary treatments as their parents.

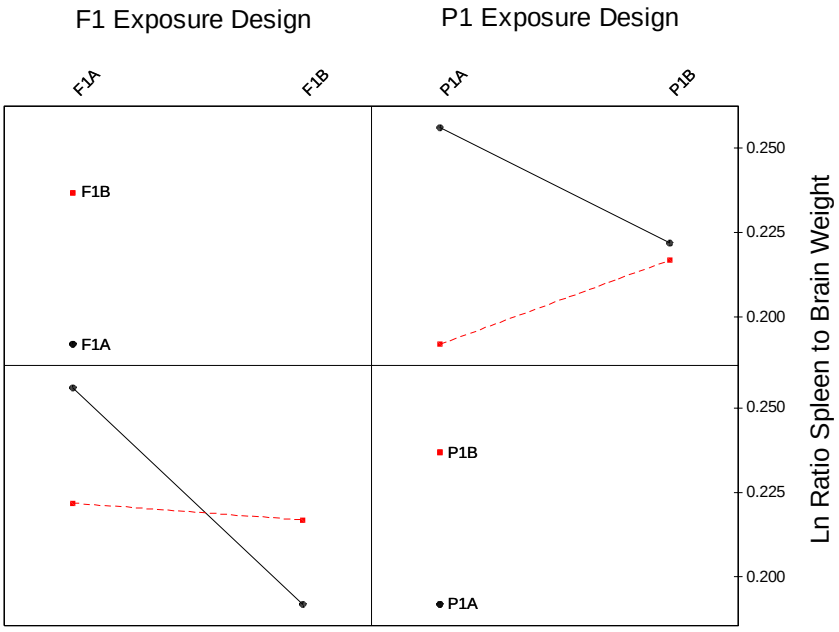


Figure 5.4-11. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the natural log-transformed spleen-to-brain weight ratios for female F1 birds. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1b, parents exposed to E2 post-maturation.

Ovary

Parental exposure scenario nearly significantly affected ovary weight of F1 offspring (p=0.097), with ovaries of hens from P1A exposure groups weighing less than those of hens from P1B groups. Nearly significantly smaller (p=0.063) ovarian weights in treated (F1a) hens was also observed (Figure 5.4-12). When normalized to body weight or to brain weight, only the tendency for smaller ovaries in F1a treated hens was still apparent (p=0.05 and p=0.07, respectively).

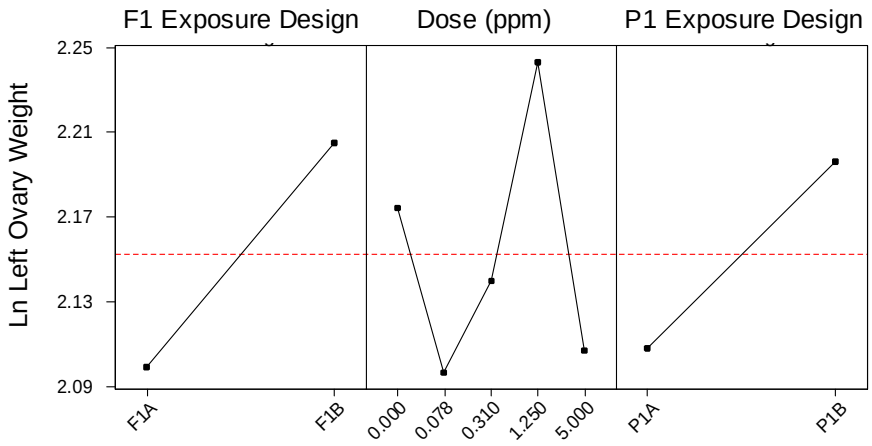


Figure 5.4-12. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed ovary weight (grams) of F1 females. General Linear Model analysis; nearly significant F1 and P1 exposure design effects on ovary weight, $p<0.10$. F1a birds were fed the same dietary treatments as their parents.

A nearly significant interaction ($p = 0.09$) between the F1 and P1 exposure designs was observed for the number of oocytes in the ovary that had initiated rapid growth (yellow oocytes greater than 4 mm in diameter). The number of active oocytes of F1a-P1A hens were increased in weight compared to F1b-P1A birds. However, when F1 exposure strategies were analyzed separately, a significant P1 design effect on oocyte number was not observed in either the treated or untreated F1 hens ($p = 0.25$ and $p=0.55$ respectively). Differences in oocyte numbers across dietary treatments were not significant ($p = 0.72$). Figure 5.4-13 summarizes the distribution of the oocyte number per ovary in the F1 hens.

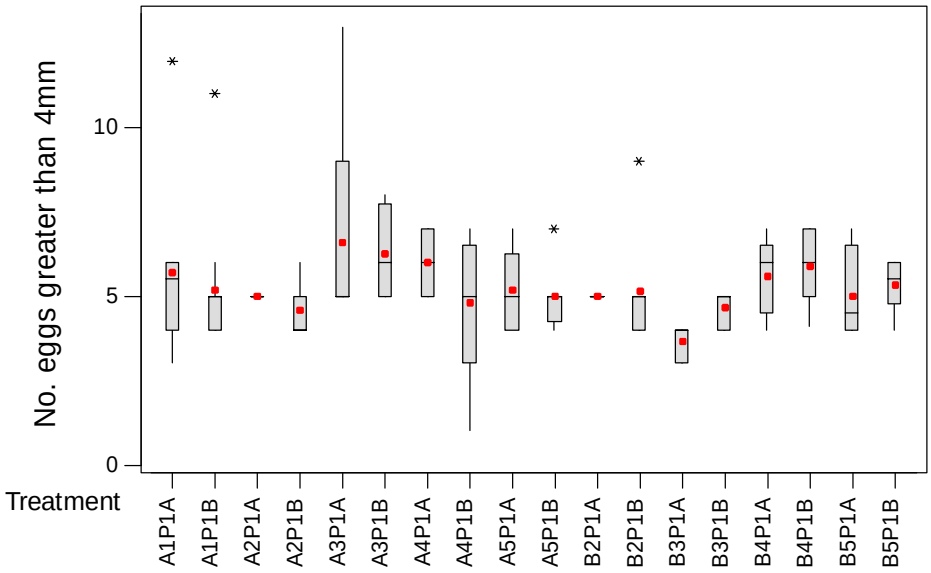


Figure 5.4-13. Box plots of the number of oocytes that have initiated rapid growth in the ovary of F1 hens by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

Oviduct

Left oviduct weight of F1 females had a significant interaction between the F1 and P1 exposure designs (p=0.025). The effect of parental dietary treatment was not significant (p=0.18). When each F1 generation was analyzed separately, the treated F1 female offspring of parents exposed for 13 weeks to E2 (F1a-P1A females) had a nearly significantly greater oviduct weight (p = 0.13) than the treated offspring of parents exposed for only 5 weeks (F1a-P1B females); however, the overall mean oviduct weight difference between the F1a-P1A and the F1a-P1B populations was modest (10%) (Table 5.4-1). Oviducts of untreated (F1b) females from both the P1A and P1B parents were not significantly different (p = 0.55). When the oviduct weight was normalized to body weight (Figure 5.4-14) or brain weight, the interaction between the P1 and F1 exposure designs was still significant (p = 0.04) (Figure 5.4-15). Analyzing the exposure designs separately, the oviduct weight normalized to body weight of treated F1 females was found to be significantly affected by P1 exposure scenario (p = 0.001), whereas the normalized oviduct weight of untreated F1 hens was not affected by P1 exposure strategy (p = 0.46). For oviduct weight normalized to brain weight, the P1 design effect on the F1a oviduct was reduced to marginal significance (p = 0.13).

Table 5.4-1. Left oviduct gross weight (g) and ratio of oviduct to body weight of F1 females.

Exposure Strategy	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Gross Weight									
F1a-P1A	30	10.4	9.99	1.88	5.74	14.0	9.34	11.4	18%
F1a-P1B	35	9.36	9.54	1.54	5.00	11.5	8.56	10.6	16%
F1b-P1A	14	10.0	9.93	1.05	8.49	11.7	9.17	10.9	10%
F1b-P1B	24	10.6	10.9	1.57	7.46	13.1	8.93	12.0	15%
Normalized Weight									
F1a-P1A	30	0.0382	0.0376	0.0053	0.0292	0.0490	0.0349	0.0417	14%
F1a-P1B	35	0.0335	0.0346	0.0049	0.0210	0.0435	0.0304	0.0373	15%
F1b-P1A	14	0.0377	0.0375	0.0037	0.0333	0.0453	0.0346	0.0393	10%
F1b-P1B	24	0.0369	0.0370	0.0046	0.0290	0.0456	0.0329	0.0401	12%

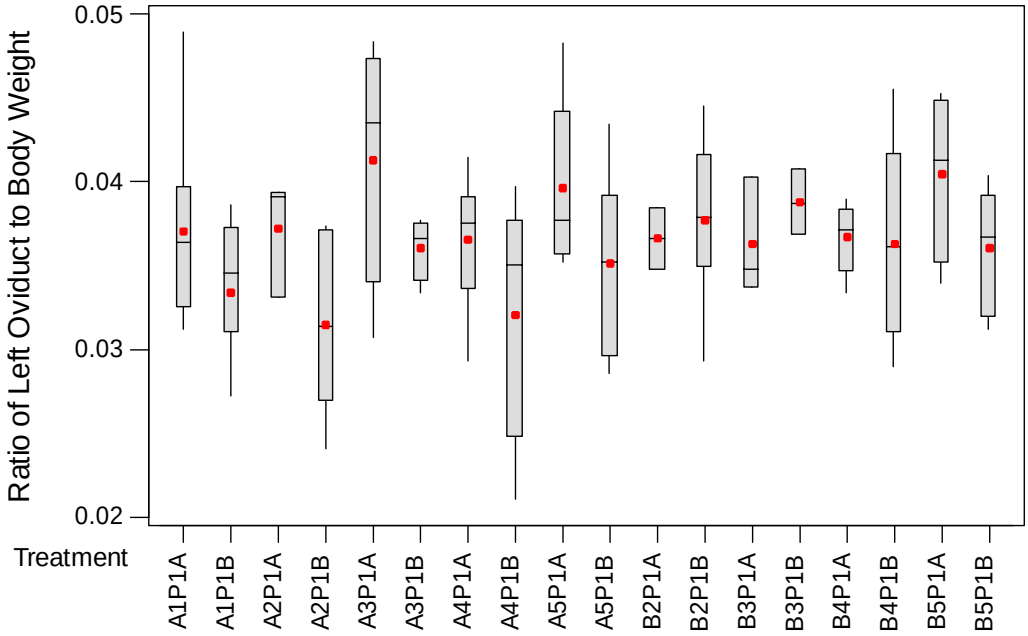


Figure 5.4-14. Box plots of the oviduct-to-body weight ratio of F1 hens by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

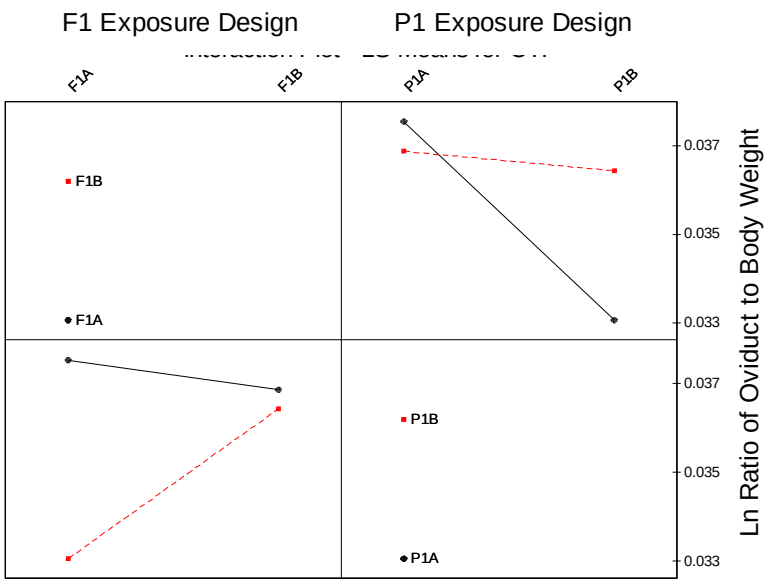


Figure 5.4-15. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the oviduct-to-body weight ratio for female F1 birds. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1b, parents exposed to E2 post-maturation.

Brain

Brain weights of F1 hens showed no significant effects of F1 or P1 exposure design (p=0.89 and p=0.47, respectively), or parental dietary treatment with E2 (p=0.31). However, after normalization to body weight, a nearly significant (p=0.06) effect of parental exposure scenario on relative brain weight was observed, but the mean difference in relative brain weight between the two scenarios was less than 5%. Brain-to-body weight ratio was unaffected by F1 exposure design or P1 dietary treatment (p>0.52) (Figure 5.4-16).

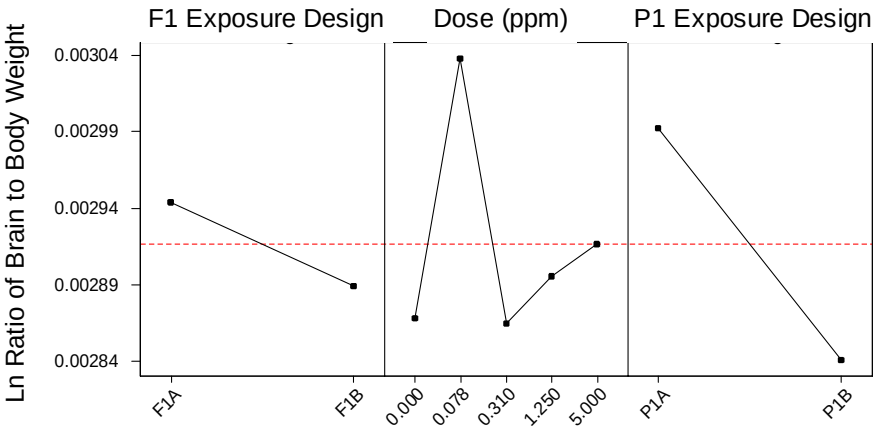


Figure 5.4-16. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed brain-to-body weight ratio of F1 females. General Linear Model analysis; nearly significant P1 exposure designs effects on relative brain weight, $p<0.06$. F1a birds were fed the same dietary treatments as their parents.

Adrenal Gland

Absolute and normalized weights of adrenal glands of F1 female quail were unaffected by F1 and P1 exposure designs or parental dietary treatment with E2 ($p>0.25$).

5.4.2 Males

Thyroid

No significant effects of F1 or P1 exposure design on gross thyroid weight ($p>0.47$) or thyroid weight normalized to brain ($p>0.39$) or body weight ($p>0.45$) of males of the F1 generation were found (Figure 5.4-17). However, there were significant differences in thyroid weight ($p<0.03$) and thyroid weight normalized to body ($p=0.023$) or brain ($p=0.014$) weight as a result of parental dietary treatment. Absolute and relative thyroid weight of male offspring exposed to 1.25 ppm *in ovo* was significantly increased ($p=0.004$, Kruskal-Wallis test) over control weights regardless of the duration of the P1 exposure scenario, but no increases were observed in groups exposed to either higher or lower concentrations. The distribution of thyroid weights in F1 male quail by dietary treatment within the F1 and P1 exposure designs is shown in Figure 5.4-18.

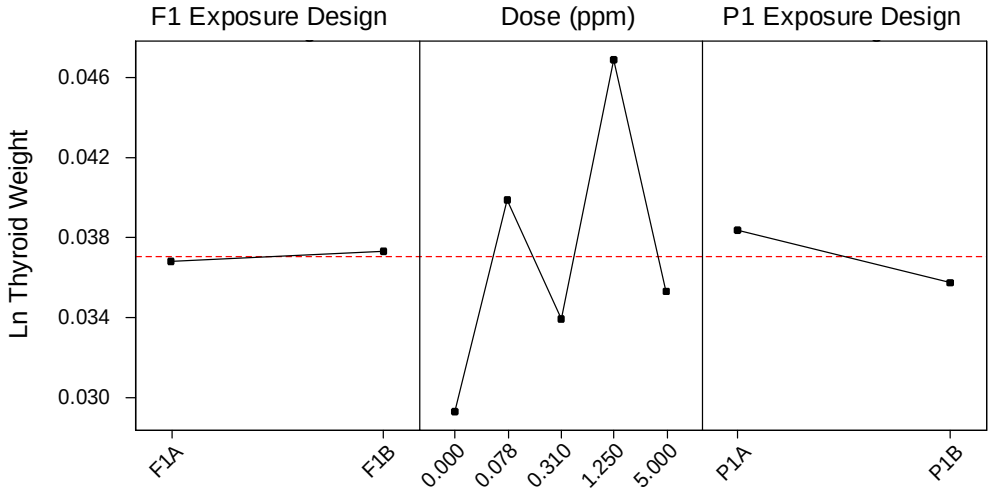


Figure 5.4-17. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed thyroid weights (g) of F1 males. General Linear Model analysis; significant differences between parental dietary treatments, p<0.03.

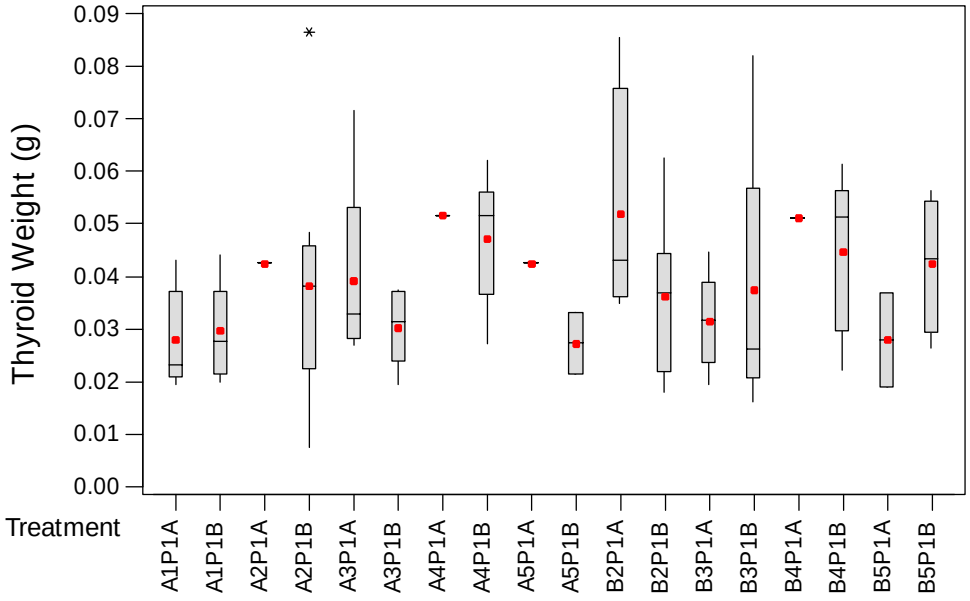


Figure 5.4-18. Box plots of thyroid weight (g) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Significant differences (p=0.004) in thyroid weights between the control and 1.25 ppm dietary treatment groups.

Liver

An interaction between the F1 and P1 exposure designs significantly affected absolute liver weights (p=0.005) and liver-to-brain weight ratios (p<0.01) of male F1 quail (Figure 5.4-19). No significant P1 dietary treatment effects on the gross or relative weight of the liver (p>0.36 and p>0.26, respectively) were detected. When the F1 exposure strategies were analyzed separately, it was found that livers of treated (F1a) males were affected by P1 exposure design, but livers of the untreated (F1b) males were not (p = 0.01 and 0.36, respectively). Similarly, after normalization of the liver weight to brain weight, relative liver weight of the F1a birds had a significant P1 design effect (p < 0.01) but the F1b males did not (p > 0.26). Both the gross liver weights and the liver-to-brain weight ratios were increased in F1a males of P1A parents compared to those whose parents were exposed to the steroid for a shorter period of time (P1B) (Figure 5.4-19). The interaction was reduced to nearly significant when the body weight normalization was used (p=0.09). Figure 5.4-20 shows the distribution of the liver-to-brain weight ratios by dietary treatment within F1 and P1 exposure designs.

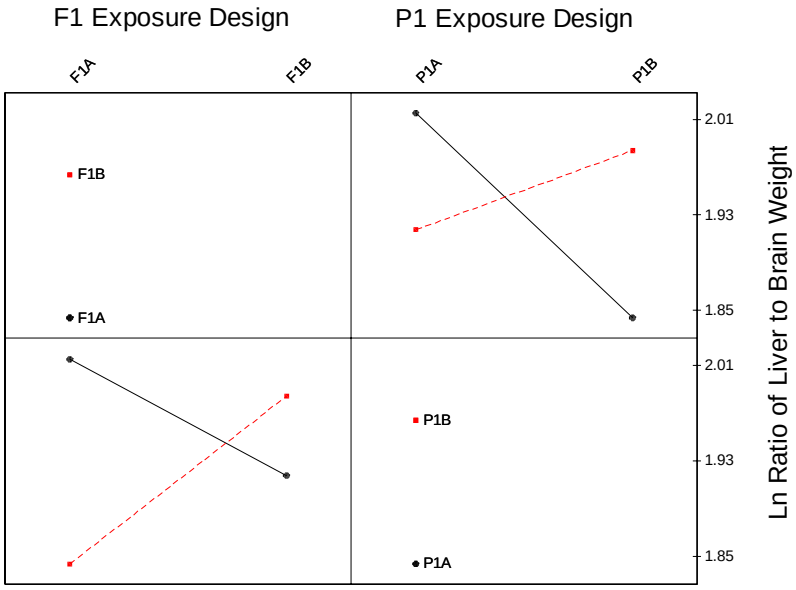


Figure 5.4-19. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed liver-to-brain weight ratios in F1 males. General Linear Model analysis; significant p<0.01.

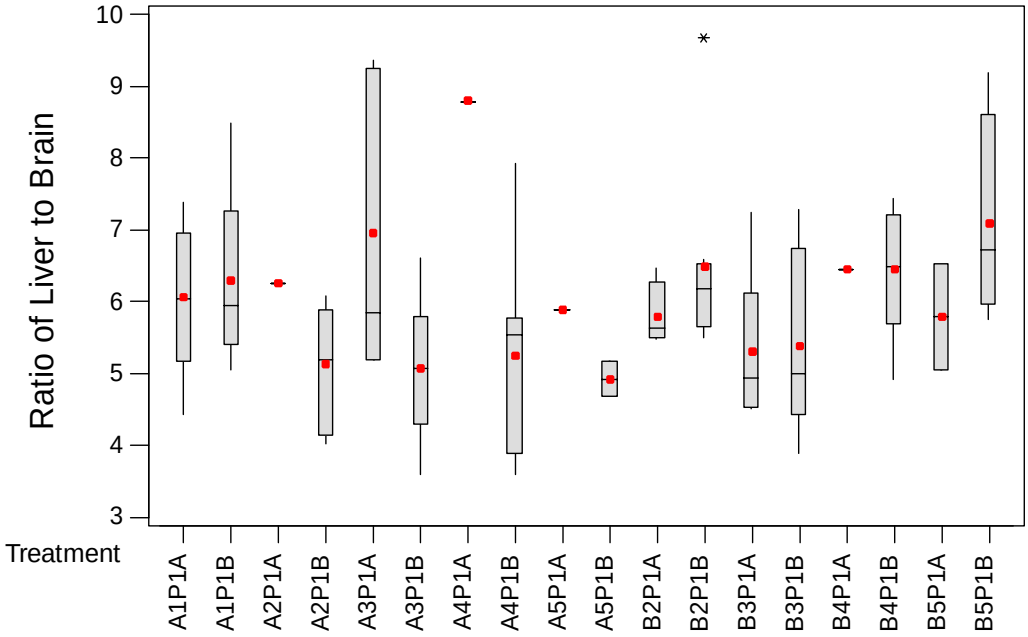


Figure 5.4-20. Box plots of liver-to-brain weight ratios of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Significant difference between the F1 and P1 dosing exposure effects (p=0.01).

Pancreas

Similar to liver weight in males from the F1 generation, a significant interaction between the F1 and P1 exposure designs affected pancreatic weight and the pancreas-to-brain weight ratio of F1 males ($p<0.03$ and $p=0.02$, respectively), but these weights were unaffected by parental dietary treatment ($p=0.16$ and $p=0.48$, respectively). Figure 5.4-21 shows the interaction of the F1 and P1 designs and Figure 5.4-22 shows the distribution of the pancreatic weights by dietary treatment within the F1 and P1 exposure scenarios. When the F1 exposure scenarios were analyzed separately, gross pancreas weight and pancreas weight normalized to brain weight of treated F1 males were significantly increased by P1 design ($p=0.04$ and $p=0.03$, respectively), but were unaffected by P1 design in untreated F1 males ($p=0.99$ and $p=0.80$, respectively). (Figure 5.4-23).

Normalization of pancreas weight to body weight resulted in a nearly significant parental dietary treatment effect ($p=0.07$) and of F1*P1 interaction effects on the relative pancreas weight ($p=0.14$). However, when the F1 exposure designs were analyzed separately, neither the pancreas-to-body weight ratios of the F1a males or the F1b males were significantly affected by P1 exposure scenarios ($p > 0.28$).

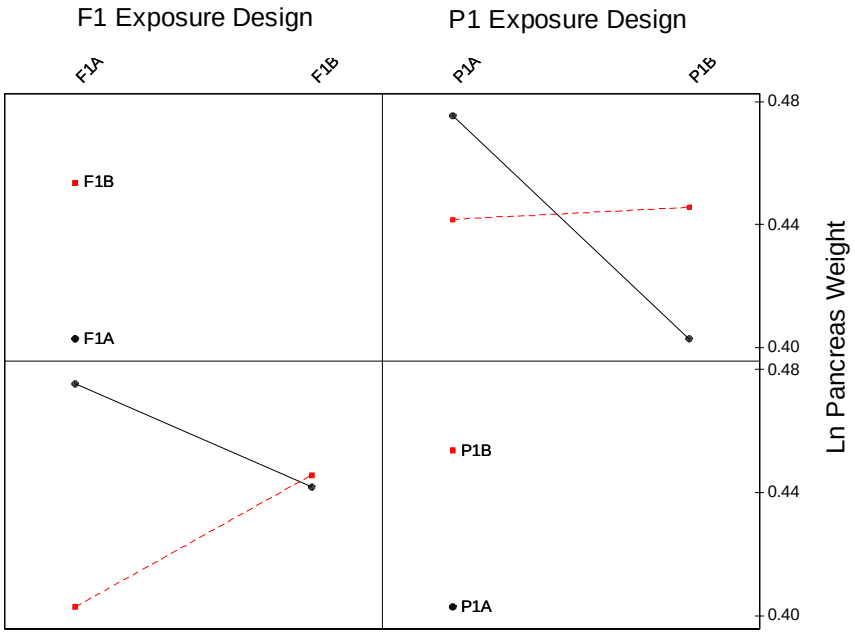


Figure 5.4-21. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on the natural log-transformed pancreas weight in F1 males. General Linear Model analysis; significant interaction, p<0.03.

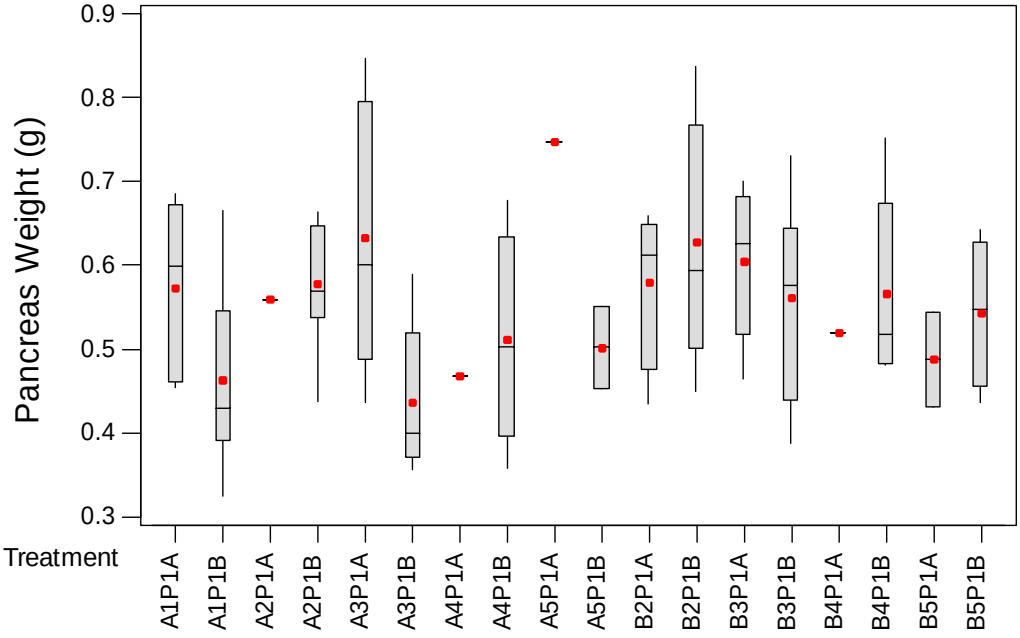


Figure 5.4-22. Box plots of pancreas weight (g) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

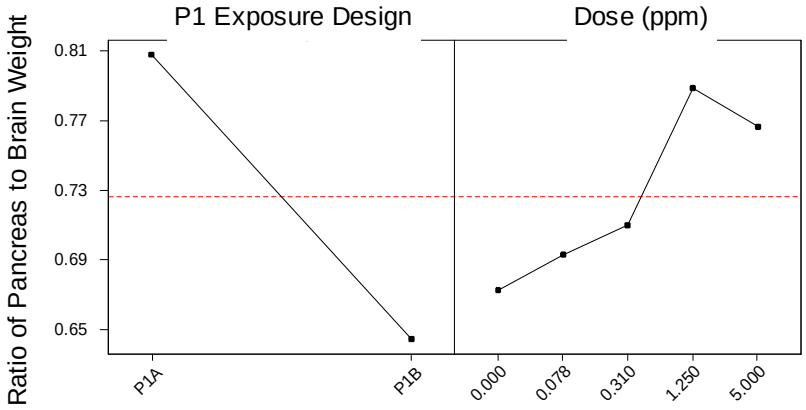


Figure 5.4-23. Effects of parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) and parental dietary treatment with E2 on the pancreas-to-brain weight ratio of treated (F1a) male offspring. General Linear Model analysis; significant difference between parental exposure scenarios, p=0.03.

Spleen

Spleen weights in males were nearly significantly different between treated (F1a) and untreated (F1b) males (p=0.072) and between parental dietary concentrations (p=0.069). Spleen weights tended to be higher in the 1.25 ppm exposure group than the controls (p=0.14). The effect of P1 exposure scenario on spleen weight in F1 males was not significant (p>0.20). However, when spleen weights in male F1 birds were normalized to body weight, there was a nearly significant difference (p=0.149) between spleen-to-body weight ratios in F1 males from P1A parents and those from P1B parents. F1 exposure design (p=0.06) and parental dietary treatment effects (p=0.11) were nearly significant as they were for the gross spleen weights. Normalizing spleen weights to brain weight also showed the nearly significant effects of F1 design (p=0.14) and parental dietary treatment (p=0.053), but not the differences between the parental exposure scenarios (p>0.20). Figures 5.4-24 and 5.4-25 show results for spleen weights.

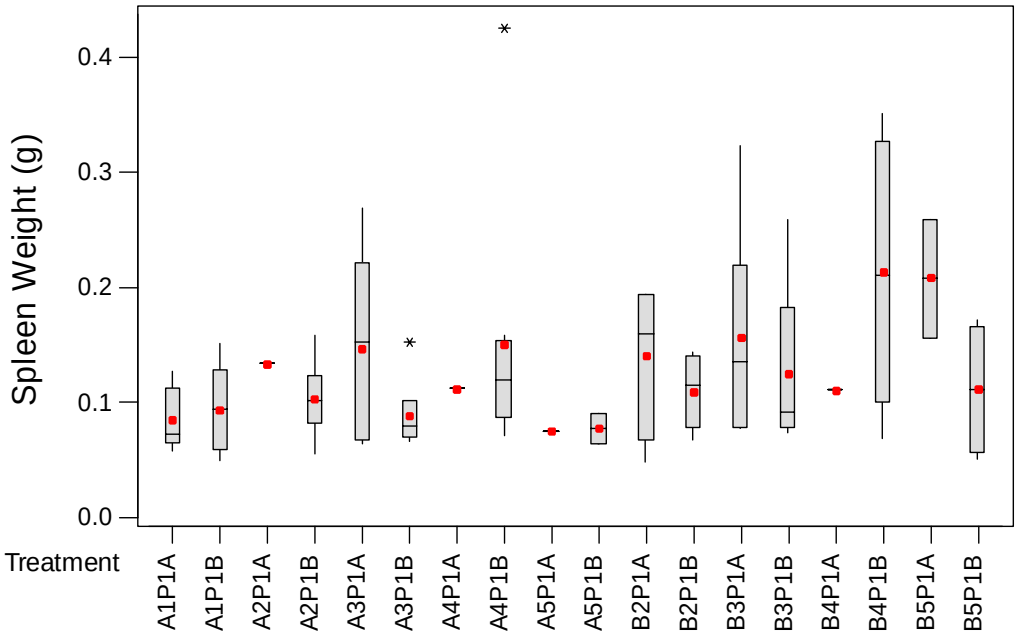


Figure 5.4-24. Box plots of spleen weight (g) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

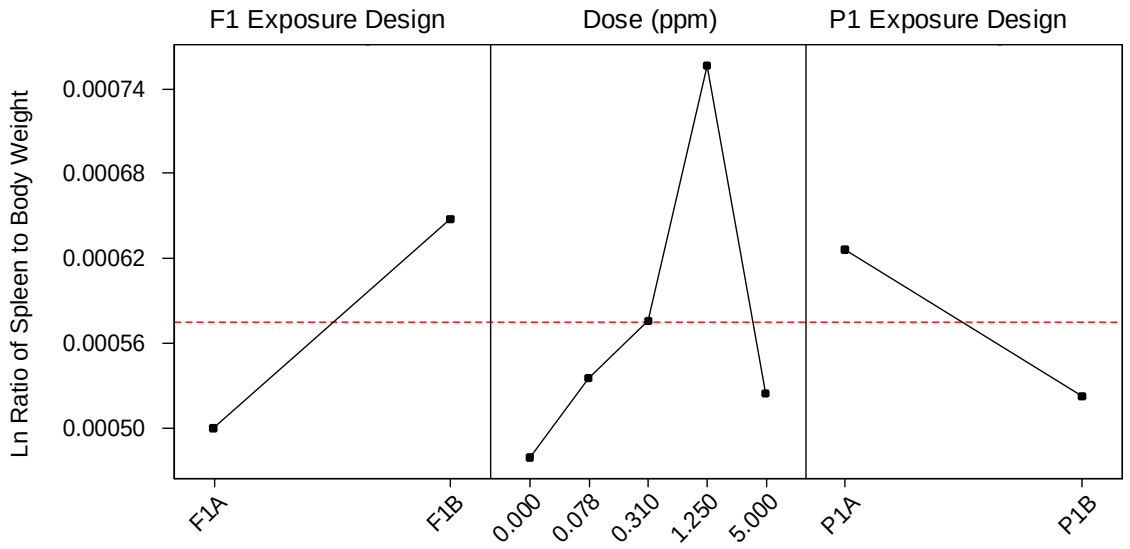


Figure 5.4-25. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed spleen-to-body weight ratios of F1 males. General Linear Model analysis; nearly significant differences between F1 treatment scenarios, p=0.06, parental dietary treatments, p=0.11, and P1 exposure design, p>0.15.

Left Testis

A nearly significant interaction between the F1 and P1 exposure designs affecting the absolute weight of the left testis (p=0.096) and the testis-to-brain weight ratio (p=0.065) was observed, but no significant parental dietary treatment effect was found (p>0.77) (Figure 5.4-26). When the F1 exposure scenarios were analyzed separately, the absolute and relative left testicular weights of the treated (F1a) males were significantly affected by the P1 design (p=0.05 and p=0.03, respectively), whereas the gross and relative weights of the testis of untreated (F1b) males were not significantly changed (p>0.22). F1a-P1A males had increased absolute and relative testicular weights (Figures 5.4-26 and 5.4-27). No interaction between P1 and F1 exposure scenarios was detected when the left testis weights were normalized to body weight ($p \geq 0.29$).

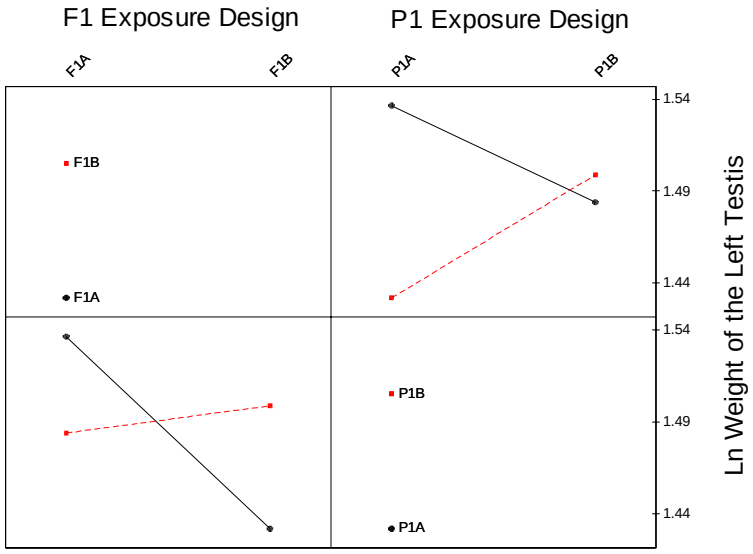


Figure 5.4-26. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the natural log-transformed weight of the left testis in F1 males. General Linear Model analysis; p=0.096.

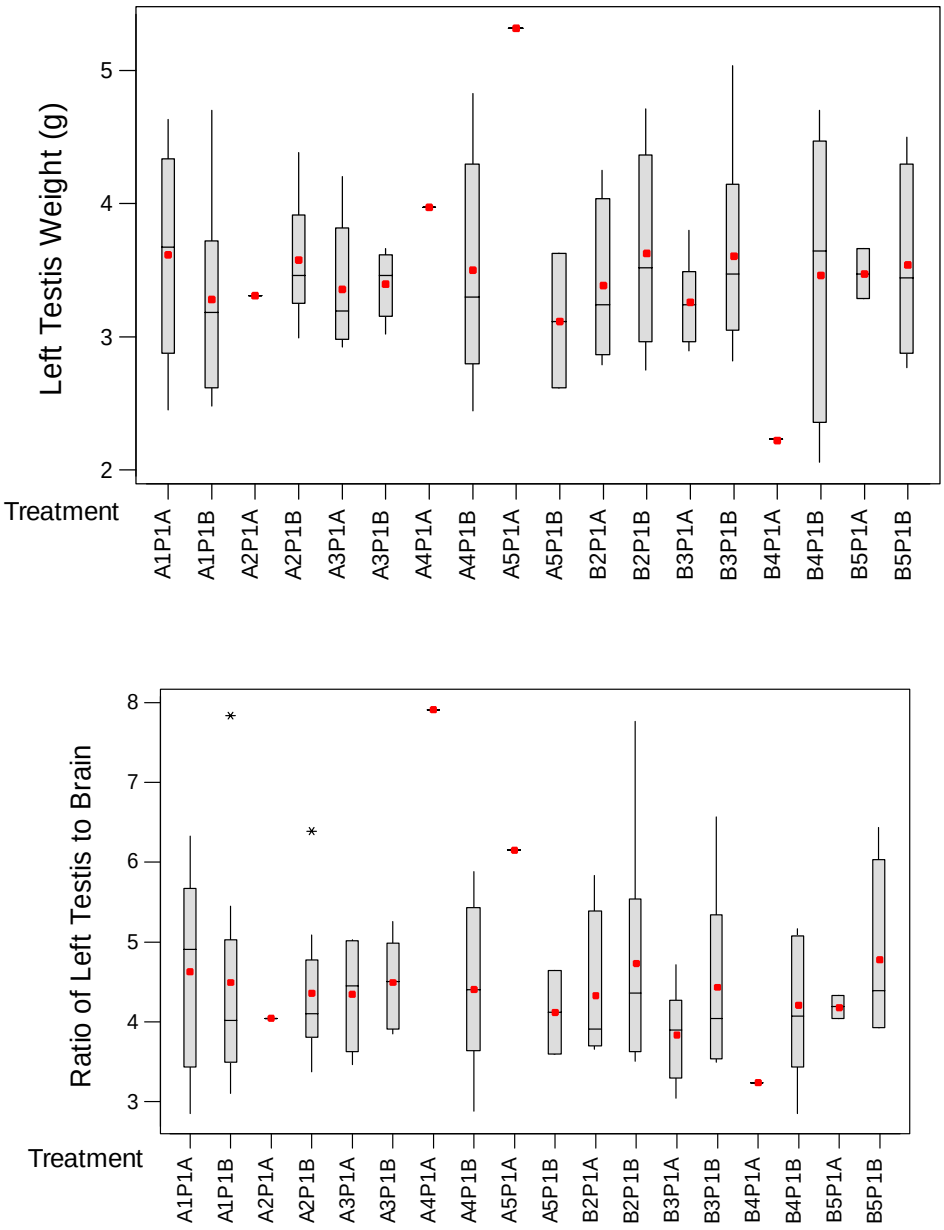


Figure 5.4-27. Box plots of left testis weight in grams (above) and left testis weight normalized to brain weight (below) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

Right Testis

Dietary treatments, F1 exposure strategies, and P1 exposure scenarios did not significantly affect the absolute weight of the right testis ($p>0.23$). Normalization of the right testis weight to body weight showed that parental dietary treatment ($p=0.105$) and parental exposure scenario ($p=0.084$) nearly significantly affected this index of testis weight (Figure 5.4-28). The dietary treatment effects were non dose-linear. When the right testicular weights were normalized to brain weight, no significant effects were observed. F1 exposure design had no effect on either index of right testicular weight in F1 male quail ($p\geq0.41$). The distribution of the relative weights of the right testis by dietary treatment within the F1 and P1 exposure regimes is shown in Figure 5.4-29.

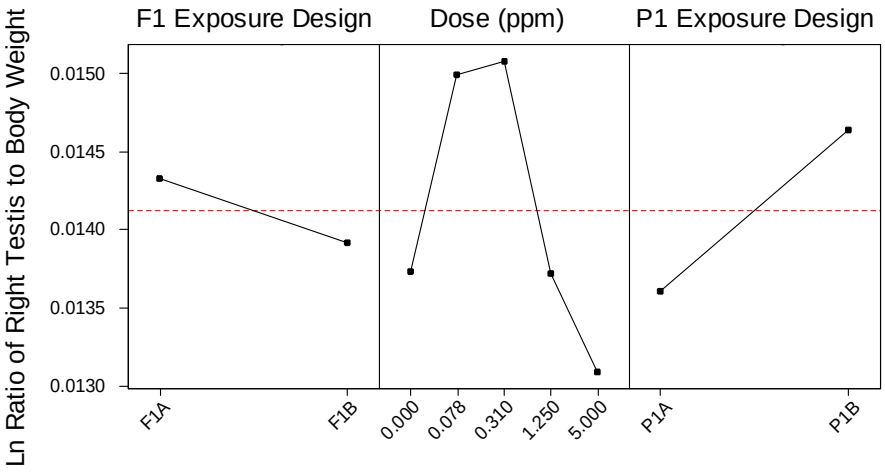


Figure 5.4-28. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed right testis-to-body weight ratios of F1 males. General Linear Model analysis; nearly signficant differences between P1 exposure scenarios, p=0.084, parental dietary treatments, p=0.105.

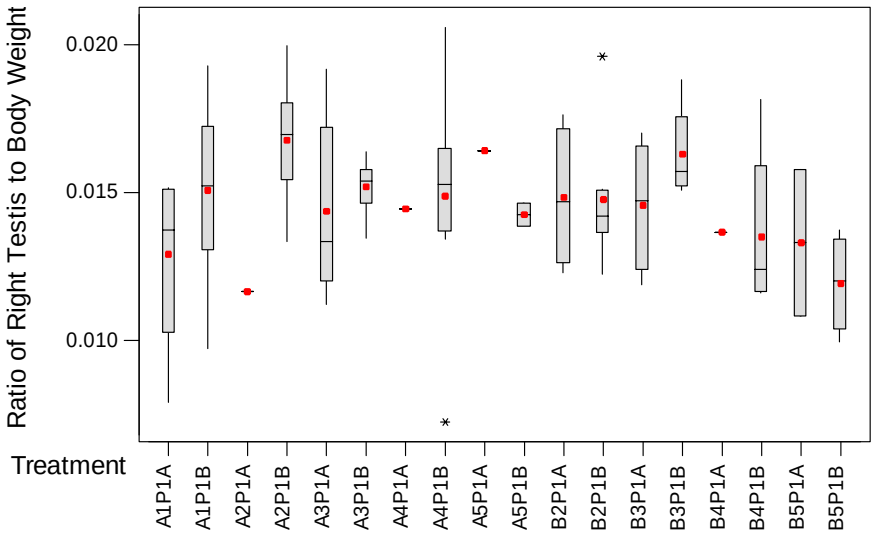


Figure 5.4-29. Box plots of ratio of the right testis-to-body weight of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

Testicular Asymmetry

A nearly significant interaction between the F1 and P1 exposure designs to affect the ratio of the left to right testis weight (p=0.12) was found in males of the F1 generation (Figure 5.4-30). Parental dietary treatment effects were not significant (p = 0.51). When the F1 exposure designs were analyzed separately, neither the treated (F1a) nor untreated (F1b) males were significantly affected (p>0.25) by the parental exposure scenarios (Figure 5.4-31).

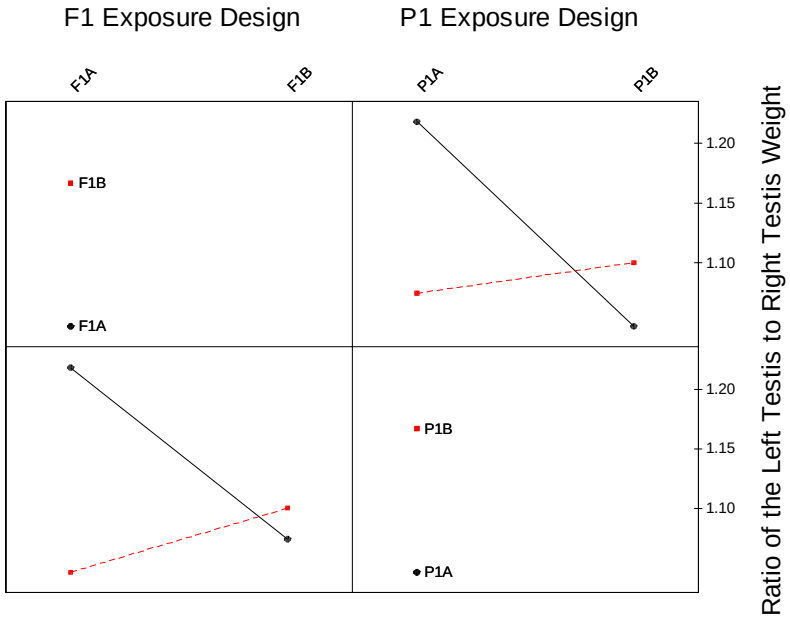


Figure 5.4-30. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on testicular asymmetry, left testis-to-right testis weight ratio in F1 males. General Linear Model analysis; p=0.12.

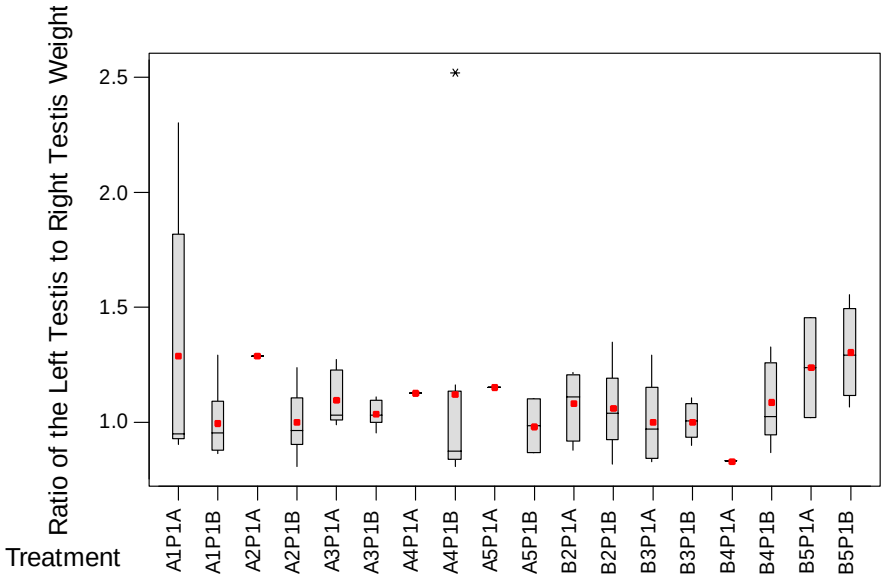


Figure 5.4-31. Box plots of the ratio of the left testis-to-right testis weight of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). (* = extreme value.)

Cloacal Gland

The effect of P1 exposure design on cloacal gland weight of their offspring (F1) was not significant ($p = 0.972$). However, the cloacal gland weight was nearly significantly smaller in treated F1 males ($p=0.139$) compared to untreated males (Figure 5.4-32). A nearly significant parental dietary treatment effect on cloacal gland weight ($p=0.140$) was also detected. F1 males fed the 5 ppm E2 diet tended ($p=0.145$) to have smaller cloacal gland weights than males fed any of the other dietary concentrations of E2 (Figure 5.4-32).

When cloacal gland weight was normalized to body weight, the dietary treatment effect was no longer significant ($p=0.163$) and a nearly significant ($p=0.13$) interaction between the F1 and P1 exposure designs affecting the relative weight of the gland was detected (Figure 5.4-33). Treated (F1a) male offspring of P1A birds appeared to have smaller cloacal gland weights than untreated offspring of P1A birds. Mean cloacal weights of the F1 males were unaffected by the P1B exposure scenario (Figure 5.4-33).

However, analyzing the two F1 exposure designs separately showed that the normalized cloacal gland weights of the untreated (F1b) males were significantly affected ($p=0.017$) and the treated (F1a) males tended to be affected ($p=0.12$) by an interaction between parental exposure scenario and parental dietary concentration (Figure 5.4-34). Offspring of P1B and P1A birds that had been fed 5 ppm E2 had offspring with much smaller cloacal glands. However, the controls of both the treated and untreated male offspring of the P1A birds also had greatly reduced cloacal gland relative weights, diminishing the ability to compare the effects of the two P1 exposure scenarios on cloacal gland weight in their offspring. When the responses of the treated groups are considered, then it appears that *in ovo* exposure of the F1 generation and not direct consumption of E2 affects cloacal gland weight of the F1 generation. This is in agreement with the observation that dietary exposure did not affect cloacal gland weight in the P1 generation (see above). Distribution of cloacal gland weights and ratios of cloacal gland weight-to-body weight by dietary treatment within F1 and P1 exposure designs are shown in Figure 5.4-35.

No significant differences in F1 exposure design, dietary treatment, or P1 exposure scenario were found after normalizing cloacal gland weight to brain weight ($p>0.16$).

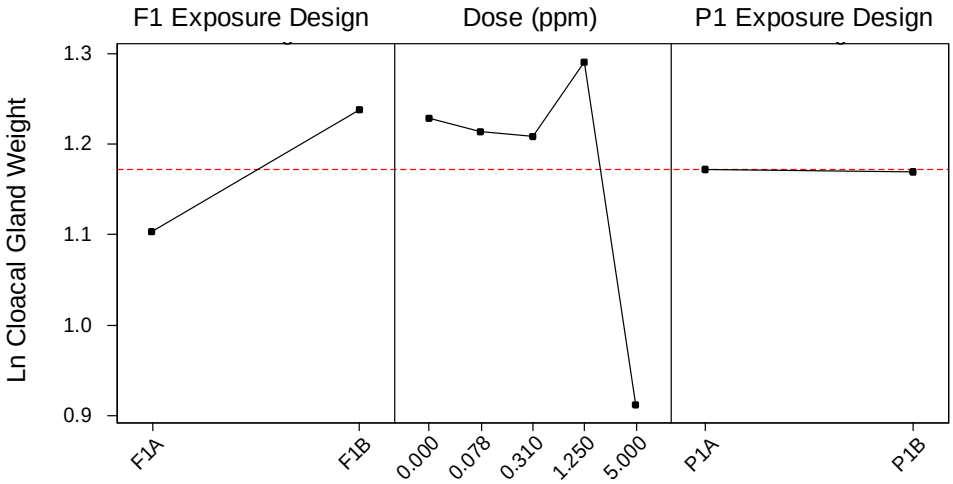


Figure 5.4-32. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed cloacal gland weights of F1 males. (General Linear Model analysis; nearly significant differences between F1 exposure scenarios, p=0.139, parental dietary treatments, p=0.140) Nearly significant effect of 5 ppm treatment on cloacal gland weights, p=0.145, Kruskal-Wallis test.

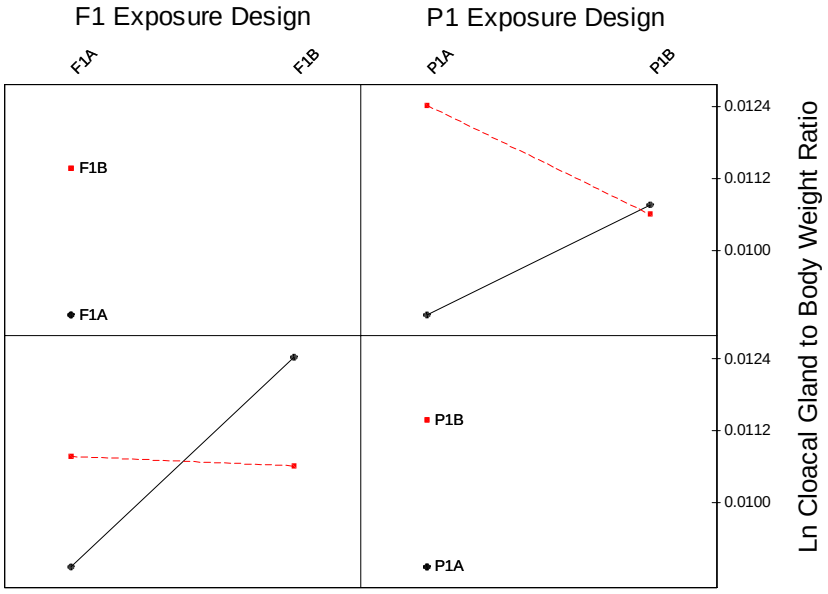


Figure 5.4-33. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed cloacal gland-to-body weight ratio in F1 males. (General Linear Model analysis; p=0.13)

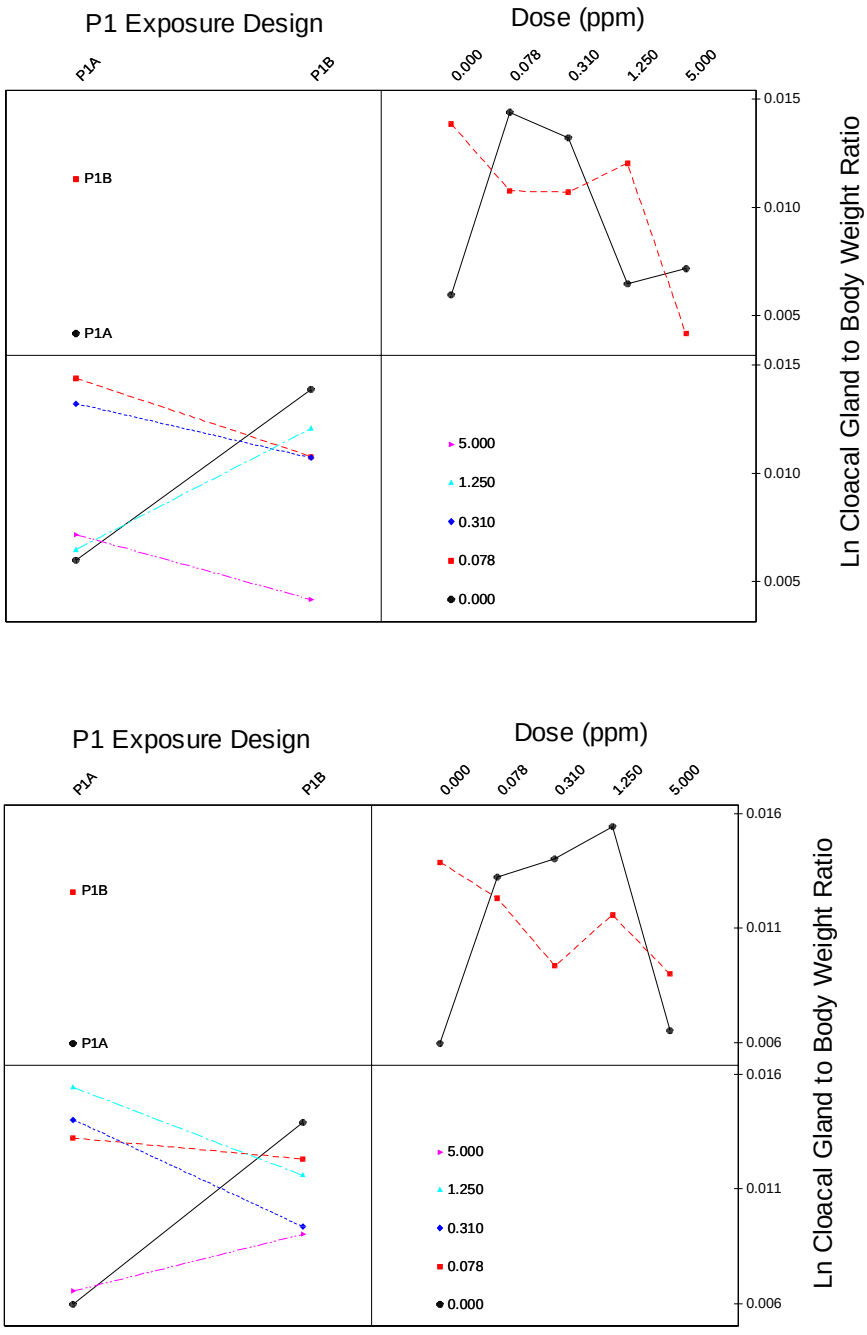


Figure 5.4-34. Interaction of P1 exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) and P1 dietary concentration on natural log-transformed cloacal gland-to-body weight ratio of F1a (above) and F1b (below) males. (General Linear Model analysis; nearly significant effects in F1a birds, p=0.12 and significant effects in F1b birds, p=0.017.)

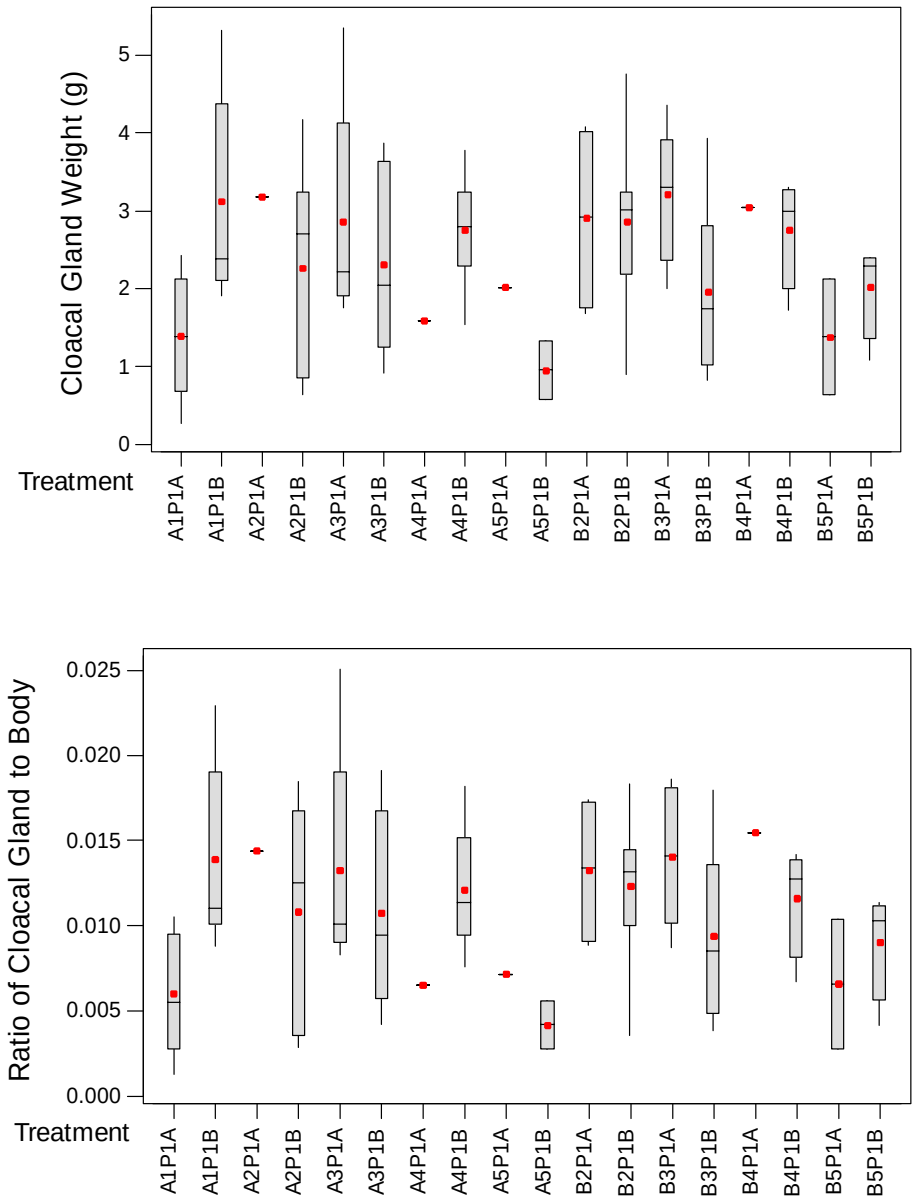


Figure 5.4-35. Box plots of cloacal gland weight in grams (above) and cloacal gland weight normalized to body weight (below) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

Brain

No significant effects of dietary treatment, F1 treatment strategy, or P1 exposure design on brain weight were found ($p \geq 0.44$). However, the ratio of the brain to body weight had a nearly significant P1*F1 interaction ($p < 0.10$). Parental dietary treatment effect remained non-significant ($p > 0.20$) after normalization of brain weight to body weight. When the F1 exposure designs were analyzed separately, a nearly significant effect of P1 exposure scenario on the

normalized brain weight of treated (F1a) males (p = 0.07) was detected; however, no P1 effect was detected in untreated (F1b) males (p > 0.32). Figures 5.4-36 and 5.4-37 show results for brain weights.

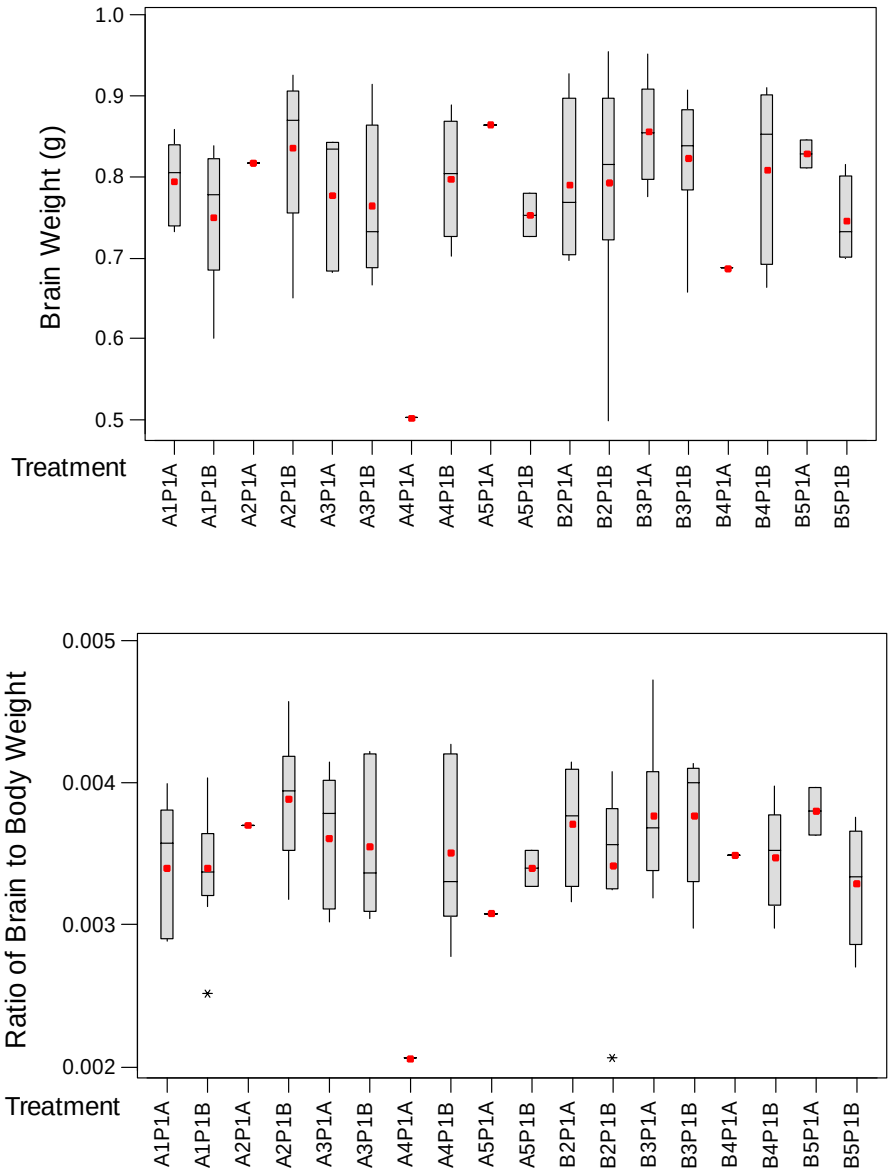


Figure 5.4-36. Box plots of brain weight in grams (above) and brain weight normalized to body weight (below) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). (* = extreme value.) Means are indicated by solid circles.

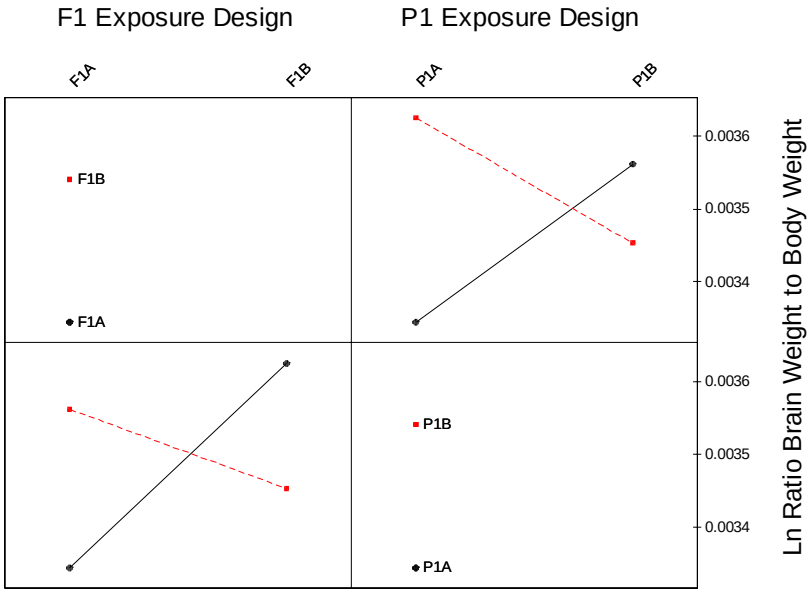


Figure 5.4-37. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on brain weight-to-body weight ratio in F1 males. General Linear Model analysis; p<0.10.

Adrenal Gland

Absolute and normalized weights of adrenal glands of F1 male quail were unaffected by F1 and P1 exposure designs or parental dietary treatment with E2 (p>0.23).

5.5 Histology (F1)

Females

Ovary

Ovarian changes (increased follicles, degenerating follicles) associated with reproductive function were observed in most groups (Table 5.5-1). There appeared to be a decrease in the percentage of hens with degenerating follicles in the treated groups (40% to 67%) of the F1a-P1A population compared to the percentage in the shared control group of the P1A offspring (80%). The proportion of these hens that had increased follicle numbers was also somewhat increased in the E2-treated groups (80% to 100%) compared to the controls (60%). However, these differences were not consistent with exposure concentration. No difference in the incidence rate for either follicular change was apparent in the F1b-P1A groups compared to the shared control group of the P1A offspring. Similarly, no treatment-related changes were observed in the tests groups of the P1B offspring (Table 5.5-1). The severity of the ovarian changes was minimal to slight in all test groups, with the exception of the F1a-P1A E2-treated groups that had incidents of moderate level increases in follicle number in all but the control and 5 ppm treatment group (Table 5.5-2).

Oviduct

Hyperplasia and hypertrophy of the epithelial cells of the oviduct were evident in nearly all hens, indicating active reproductive function (Table 5.5-1). The severity of the observed epithelial hyperplasia/hypertrophy in the oviducts of F1 females appeared to be somewhat greater in offspring of parents from the P1A exposure scenario than in those from the P1B scenario and from the F1a (treated) vs. F1b (untreated) populations (Table 5.5-2).

Table 5.5-1. Incidence of histological changes in ovaries and oviducts of Japanese quail by dietary treatment of F1 female offspring of parents exposed prior (P1A) or post (P1B) puberty and exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Ovary Degenerating Follicles	Ovary Increased Follicles	Oviduct Epithelial Hyperplasia/ Hypertrophy
F1a-P1A				
0	10	8	6	10
0.078	3	2	3	3
0.31	5	2	4	5
1.25	6	3	6	6
5	6	4	6	6
F1b-P1A				
0.078	2	1	1	2
0.31	3	2	1	3
1.25	5	4	3	5
5	4	1	3	4
F1a-P1B				
0	11[a]	5	8	10
0.078	7	2	6	7
0.31	4	3	0	4
1.25	5	2	4	5
5	8	6	5	7
F1b-P1B				
0.078	7	3	2	7
0.31	3	3	1	3
1.25	8[b]	5	5	8
5	6	5	3	6

[a] One oviduct was not examined (10 were examined).
[b] There were 8 oviducts and 7 ovaries examined.

Table 5.5-2. Average severity[a] of histological changes in ovaries and oviducts of Japanese quail by dietary treatment of F1 female offspring of parents exposed prior (P1A) or post (P1B) puberty and exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Ovary Degenerating Follicles	Ovary Increased Follicles	Oviduct Epithelial Hyperplasia/ Hypertrophy
F1a-P1A				
0	10	1	1.5	1.8
0.078	3	1.5	2.0	2.3
0.31	5	1	2.3	2.2
1.25	6	1.7	2.3	3.5
5	6	1.5	1.2	1.8
F1b-P1A				
0.078	2	1	1	1.5
0.31	3	1.5	1	2.7
1.25	5	1	2	1.4
5	4	1	2	2.0
F1a-P1B				
0	11[b]	1	1.5	1.5
0.078	7	1	1.7	1.3
0.31	4	1.3	0	1.8
1.25	5	2	1.5	1.8
5	8	1.3	1.6	2.0
F1b-P1B				
0.078	7	1.3	1.5	1
0.31	3	1.3	1	1.3
1.25	8[c]	1.2	1	1.4
5	6	1	1.7	1.2

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.
[b] One oviduct was not examined (10 were examined).
[c] There were 8 oviducts and 7 ovaries examined.

Adrenal Gland

For the adrenal gland, only adrenals from the 5 ppm E2 treatment were compared histologically to adrenals from the 0 ppm exposure concentration. Diffuse hypertrophy of the adrenal gland was observed with greater incidence in treated (F1a) females compared to untreated (F1b) hens (Table 5.5-3). Parental exposure scenario appeared to have no effect on the incidence of adrenal lesions. The severity of the hypertrophy may be slightly greater in P1A birds treated with 5 ppm E2.

Table 5.5-3. Incidence and severity of diffuse hypertrophy in adrenal glands of Japanese quail by dietary treatment of F1 female offspring of parents exposed prior (P1A) or post (P1B) puberty and exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Incidence of Diffuse Hypertrophy	Proportion Affected	Average Severity [a] of Diffuse Hypertrophy
F1a-P1A				
0	10	4	0.4	1
5	6	4	0.7	1.8
F1b-P1A				
5	4	1	0.3	2
F1a-P1B				
0	11	6	0.5	1.5
5	8	5	0.6	1.4
F1b-P1B				
5	6	0	0	0

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.

Liver

Only the 5 ppm E2 treatment was compared histologically to the 0 ppm parental exposure concentration for the livers of the F1 generation. Focal mineralization of the liver was not found in the shared controls of the P1A or P1B offspring, but was prevalent in treated hens. From 75% to 100% of the 5 ppm E2 treated hens from the F1b-P1A, F1a-P1B, and F1b-P1B populations had minimal to slight mineralization of hepatic tissue. No incidence of mineralization was found in the high dietary concentration group of the F1a-P1A population (Table 5.5-4). A high incidence rate (50% to 67%) of centrilobular vacuolation was observed in the offspring of P1B parents whether they were treated with 0 ppm or 5 ppm E2. Livers of F1a-P1A hens consuming 5 ppm E2 had 10 times the incident rate of vacuolation as that of the shared controls of the offspring of the P1A population. However, untreated offspring (F1b) of parents that consumed 5 ppm E2 had a lower incident rate (0.25) of the lesion (Table 5.5-4).

Table 5.5-4. Incidence and severity of focal mineralization and centrilobular vacuolation in livers of Japanese quail by dietary treatment of F1 female offspring of parents exposed prior (P1A) or post (P1B) puberty and exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Incidence of Focal Mineralization	Average Severity	Incidence of Centrilobular Vacuolation	Average Severity [a]
F1a-P1A					
0	10	0	0	1	2
5	6	0	0	6	3
F1b-P1A					
5	4	3	2	1	3
F1a-P1B					
0	11	0	0	6	2
5	8	6	1.7	4	2.5
F1b-P1B					
5	6	6	1.5	4	2.8

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.

No treatment-related effects in the thyroid or brain tissue of female quail from the F1 generation were found (Appendix J).

Males

Testes

Incidence of seminiferous tubule degeneration in the testis of treated males in the F1 generations was lower than observed in the P1 generation. Only untreated F1 male offspring of P1B parents showed a slight increase in the incidence rate of tubule degeneration compared to controls (Table 5.5-5). However, there was no clear dose response in the incidence or severity of the lesion in the F1b-P1B males.

Epididymis

As seen with the testis lesion, the incidence of hypospermia in the epididymis of treated males in the F1 generations was lower than observed in the P1 generation. The incidence of hypospermia was greater in male offspring of P1B birds, particularly those that did not receive dietary exposure to E2 (F1b-P1B) (Table 5.5-5). Severity of the lesion did not appear to be related to dietary concentration of E2 (F1a) or parental exposure to the steroid.

Table 5.5-5. Incidence of histological changes in testes and epididymis by dietary treatment of F1 male offspring of parents exposed prior (P1A) or post (P1B) puberty exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Testis Degeneration Seminiferous Tubules	Average Severity of Lesion	N	Epididymis Hypospermia	Average Severity[a] of Lesion
F1a-P1A						
0	5	1	1	4	1	4
0.078	1	0	0	1	0	0
0.31	5	0	0	5	1	4
1.25	1	0	0	1	0	0
5	1	0	0	1	0	0
F1b-P1A						
0.078	4	0	0	3	2	4
0.31	6	1	1	5	0	0
1.25	1	0	0	1	0	0
5	2	0	0	1	0	0
F1a-P1B						
0	9	1	2	7	0	0
0.078	9	0	0	8	1	5
0.31	6	0	0	5	1	4
1.25	8	2	2	6	1	4
5	2	0	0	2	1	4
F1b-P1B						
0.078	8	2	1	8	3	2.3
0.31	8	2	3	6	3	3.7
1.25	5	1	2	5	2	3
5	4	2	2	4	4	4

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.

Cloacal Gland

Hypertrophy of the submucosal glands of the cloacal gland occurred almost exclusively in the untreated male offspring of parents that were exposed to E2 prior to puberty. Although the total incidence number was small for each treatment group of the F1b-P1A population, the percentage of affected males within each group was high (50% to 100%) because of the small number of males in some groups (Table 5.5-6). Dilatation of the lumens of the submucosal glands occurred largely in the offspring of P1B parents, but no clear dose-response was observed. Severity of both lesions were minimal or slight (Table 5.5-6).

Table 5.5-6. Incidence of histological changes of the cloacal gland by dietary treatment of F1 male offspring of parents exposed prior (P1A) or post (P1B) puberty exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Hypertrophy of the Submucosal Glands	Average Severity of Lesion	Dilatation of Lumens of Submucosal Glands	Average Severity[a] of Lesion
F1a-P1A					
0	5	0	0	0	0
0.078	1	0	0	0	0
0.31	5	0	0	0	0
1.25	1	0	0	0	0
5	1	0	0	0	0
F1b-P1A					
0.078	4	0	0	3	1.7
0.31	6	3	1.7	0	0
1.25	1	1	1	0	0
5	2	1	1	0	0
F1a-P1B					
0	9	0	0	0	0
0.078	9	0	0	2	1.5
0.31	6	0	0	4	1.3
1.25	8	0	0	4	1.3
5	2	0	0	1	1
F1b-P1B					
0.078	8	1	1	4	1.8
0.31	6	0	0	2	1.5
1.25	4	0	0	0	0
5	4	0	0	1	1

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.

Adrenal Glands

Minimal to slight diffuse hypertrophy was present in the adrenal glands of F1b males of parents that were fed 5 ppm E2 under either the P1A or P1B exposure scenario (Table 5.5-7). The change was not observed in control birds or F1a males consuming the 5 ppm E2 diet (F1a).

Table 5.5-7. Incidence and severity of diffuse hypertrophy in adrenal glands of Japanese quail by dietary treatment of F1 male offspring of parents exposed prior (P1A) or post (P1B) puberty and exposed to dietary E2 (F1a) or receiving no additional E2 exposure above *in ovo* (F1b).

Treatment (ppm diet)	N	Incidence of Diffuse Hypertrophy	Proportion Affected	Average Severity [a] of Diffuse Hypertrophy
F1a-P1A				
0	5	0	0	0
5	1	0	0	0
F1b-P1A				
5	2	2	1	2
F1a-P1B				
0	9	0	0	0
5	2	0	0	0
F1b-P1B				
5	4	3	0.8	1.3

[a] Severity was graded on a scale of 1 through 5, with 1 indicating minimal change over baseline, and 5 indicating severe/high change.

No treatment-related effects in the thyroid or brain tissue of male quail from the F1 generation were found (Appendix J).

5.6 Sexual Maturation (F1)

Males

A significant interaction ($p<0.01$) between the F1 and P1 exposure designs affected the maturation of F1 males (Figure 5.6-1). Of the offspring of the P1A birds, the treated (F1a) males matured sooner than the untreated (F1b) males, whereas the untreated male offspring matured sooner than the treated male offspring of the P1B birds. The greatest difference in average age to maturity was seen between the F1b-P1A males and the F1b-P1B males (5 days). Male maturation was also significantly affected by P1 dietary treatment ($p<0.01$), but the response was not concentration-linear (Figure 5.6-2). The age at maturation (first production of foam exudates in the cloacal gland) of males by parental dietary concentration within each exposure design combination is shown in Table 5.6-1.

Table 5.6-1. Age (d) to first production of foam exudate of the cloacal gland in F1 males.

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
F1a-P1A-0 ppm	5	37	37	2	35	39	36	39	4%
F1a-P1B-0 ppm	7	36	35	3	33	43	33	36	10%
F1a-P1A-0.078 ppm	1	41	41	NC	41	41	NC	NC	NC
F1a-P1B-0.078 ppm	9	35	34	2	33	37	33	36	4%
F1a-P1A-0.31 ppm	3	35	34	2	34	37	34	37	5%
F1a-P1B-0.31 ppm	4	34	34	2	33	36	33	36	4%
F1a-P1A-1.25 ppm	1	36	36	NC	36	36	NC	NC	NC
F1a-P1B-1.25 ppm	5	38	39	2	35	40	37	40	5%
F1a-P1A-5 ppm	1	39	39	NC	39	39	NC	NC	NC
F1a-P1B-5 ppm	2	36	36	0	36	36	NC	NC	0%
F1b-P1A-0.078 ppm	4	39	40	2	36	41	37	41	6%
F1b-P1B-0.078 ppm	6	34	33	2	32	38	32	35	7%
F1b-P1A-0.31 ppm	6	36	36	2	34	39	35	38	5%
F1b-P1B-0.31 ppm	3	33	33	1	32	34	32	34	3%
F1b-P1A-1.25 ppm	1	44	44	NC	44	44	NC	NC	NC
F1b-P1B-1.25 ppm	5	35	36	2	32	37	33	37	7%
F1b-P1A-5 ppm	2	40	40	1	39	41	NC	NC	4%
F1b-P1B-5 ppm	4	35	35	2	33	38	33	38	6%

NC Not calculable because n is too small.

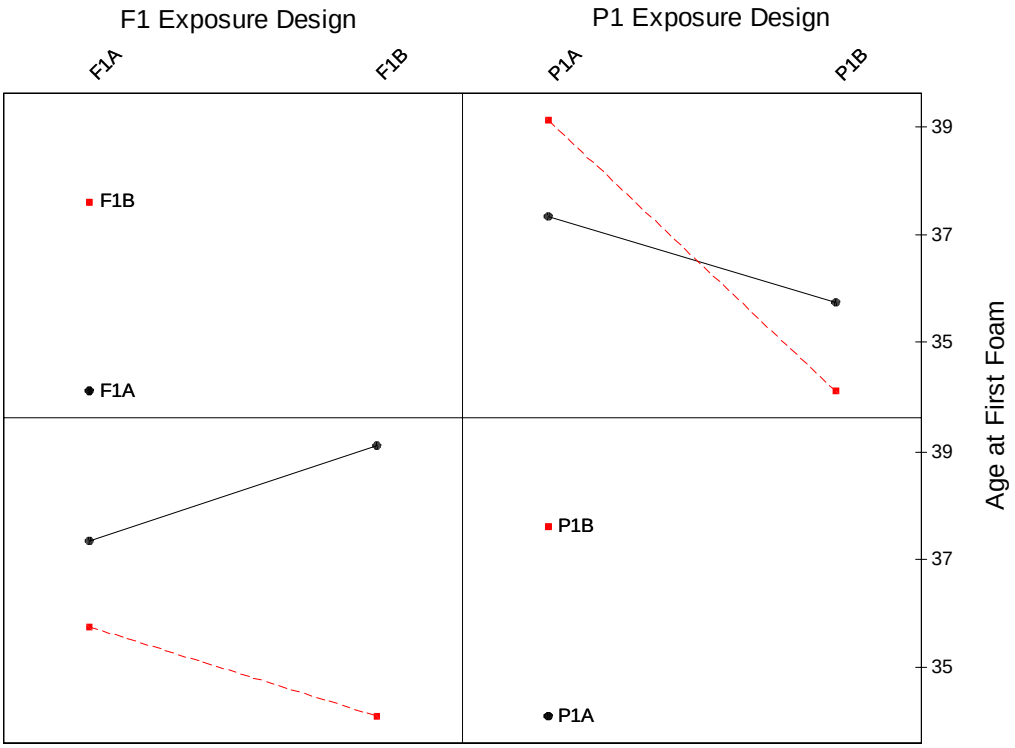


Figure 5.6-1. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on maturation age (days) of F1 males.

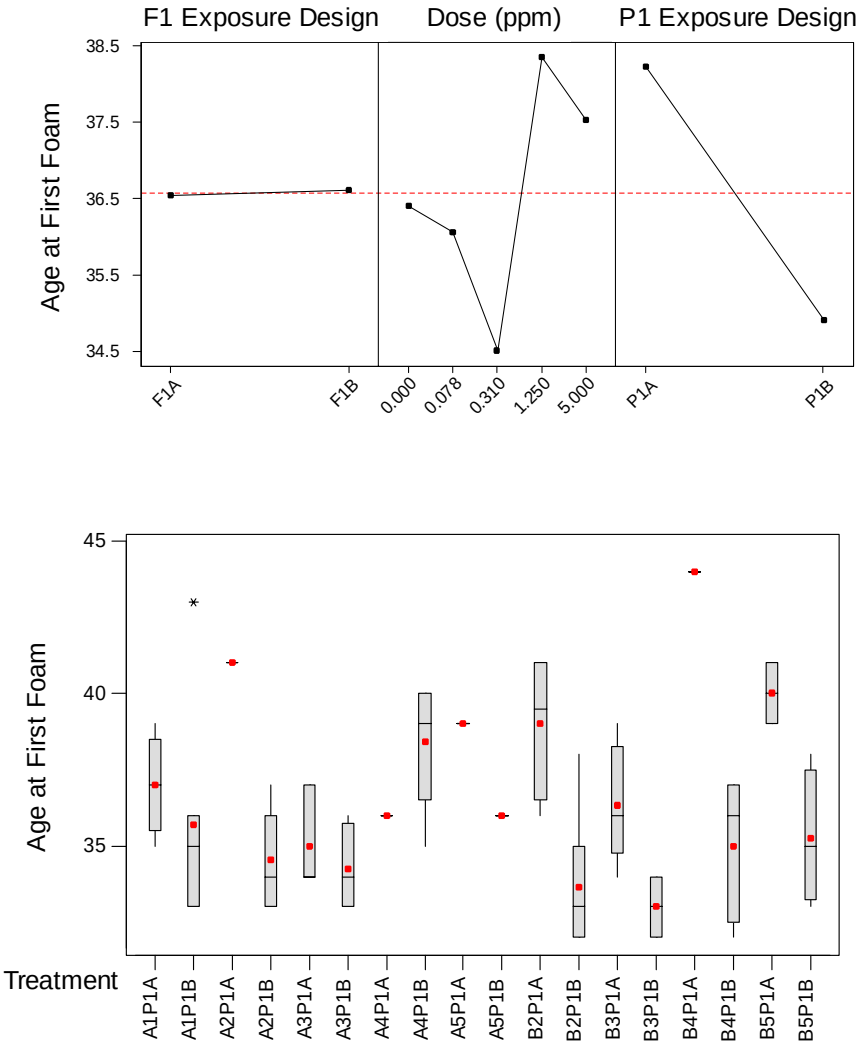


Figure 5.6-2. General Linear Model analysis (above) and box plots (below) of the effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on maturation age (days) of F1 males. Significant differences across dietary concentrations, $p<0.01$. Means are indicated by solid circles.

Females

Treated (F1a) females tended ($p = 0.065$) to mature (begin egg laying) later than untreated F1 females when extra birds were included in the analysis. When the extra birds are removed from the analysis, the nearly significant effect was no longer apparent ($p>0.27$) (Figures 5.6-3 and 5.6-4). However, there is a significant dietary concentration effect observed with or without the inclusion of the extra females in the statistical analysis ($p<0.01$ or $p<0.02$). The concentration effect was not linear (Figures 5.6-3 and 5.6-4). The age at maturation (first egg) of females by parental dietary concentration within each exposure design combination is shown in Table 5.6-2.

Table 5.6-2. Female F1 age to first egg (d), all F1 females.

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
F1a-P1A-0 ppm	10	41	41	3	37	45	38	44	8%
F1a-P1B-0 ppm	12	40	39	3	35	47	37	41	8%
F1a-P1A-0.078 ppm	3	43	42	3	40	46	40	46	7%
F1a-P1B-0.078 ppm	7	39	39	1	36	40	39	40	4%
F1a-P1A-0.31 ppm	5	46	46	4	41	51	43	49	8%
F1a-P1B-0.31 ppm	4	43	44	5	36	47	38	47	12%
F1a-P1A-1.25 ppm	6	40	41	3	36	44	38	42	7%
F1a-P1B-1.25 ppm	5	47	46	6	41	53	42	53	12%
F1a-P1A-5 ppm	6	43	44	3	39	46	39	45	7%
F1a-P1B-5 ppm	8	41	42	4	36	49	38	44	10%
F1b-P1A-0.078 ppm	2	41	41	7	36	46	NC	NC	17%
F1b-P1B-0.078 ppm	7	38	38	3	33	42	36	41	8%
F1b-P1A-0.31 ppm	3	42	43	2	39	43	39	43	6%
F1b-P1B-0.31 ppm	3	38	40	4	33	41	33	41	11%
F1b-P1A-1.25 ppm	5	41	39	3	39	47	39	44	9%
F1b-P1B-1.25 ppm	8	44	43	4	39	50	40	48	10%
F1b-P1A-5 ppm	4	42	42	2	39	44	40	44	5%
F1b-P1B-5 ppm	6	40	39	5	35	46	37	46	11%

NC Not calculable because n is too small.

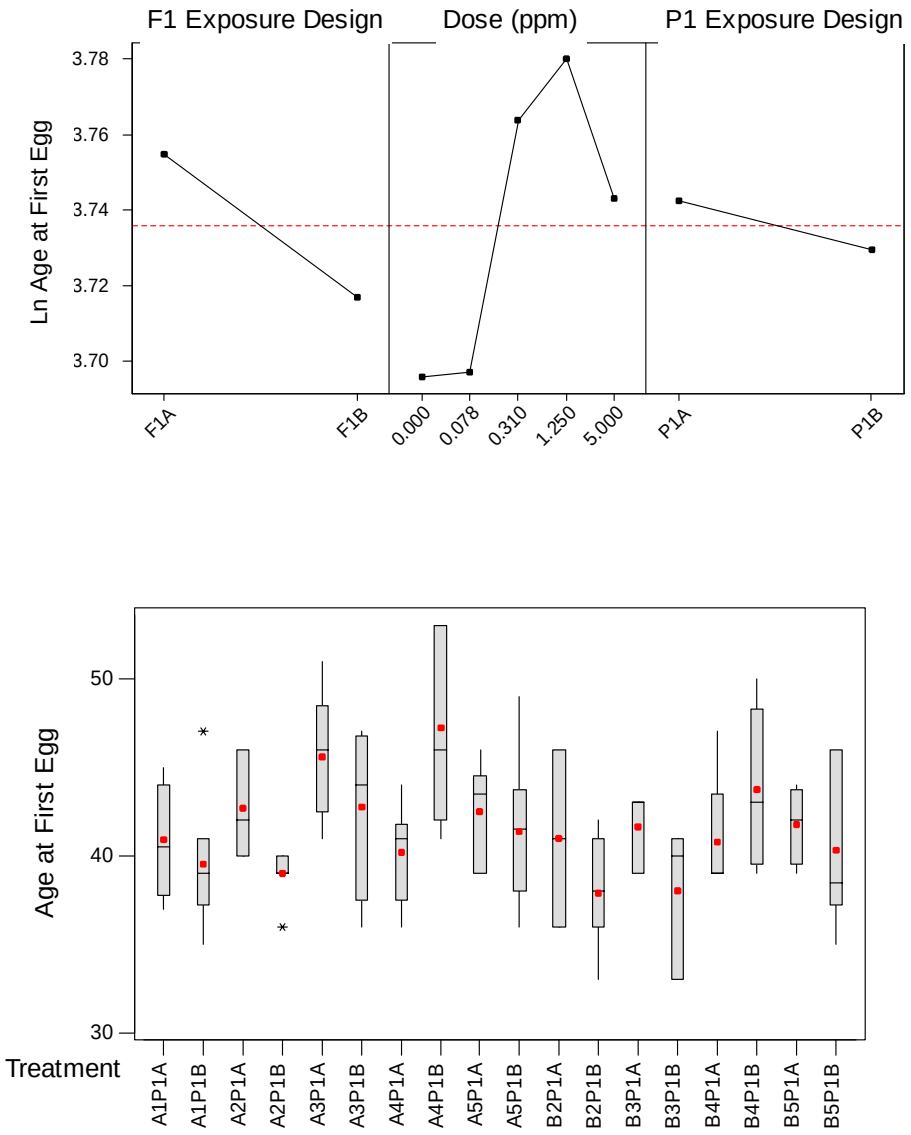


Figure 5.6-3. Natural log transformed General Linear Model analysis (above) and boxplots (below) of the effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on maturation age (days) of F1 females. Significant differences between F1 treatment scenarios, $p<0.07$ and dietary concentrations, $p<0.01$. Data include extra females. Means are indicated by solid circles.

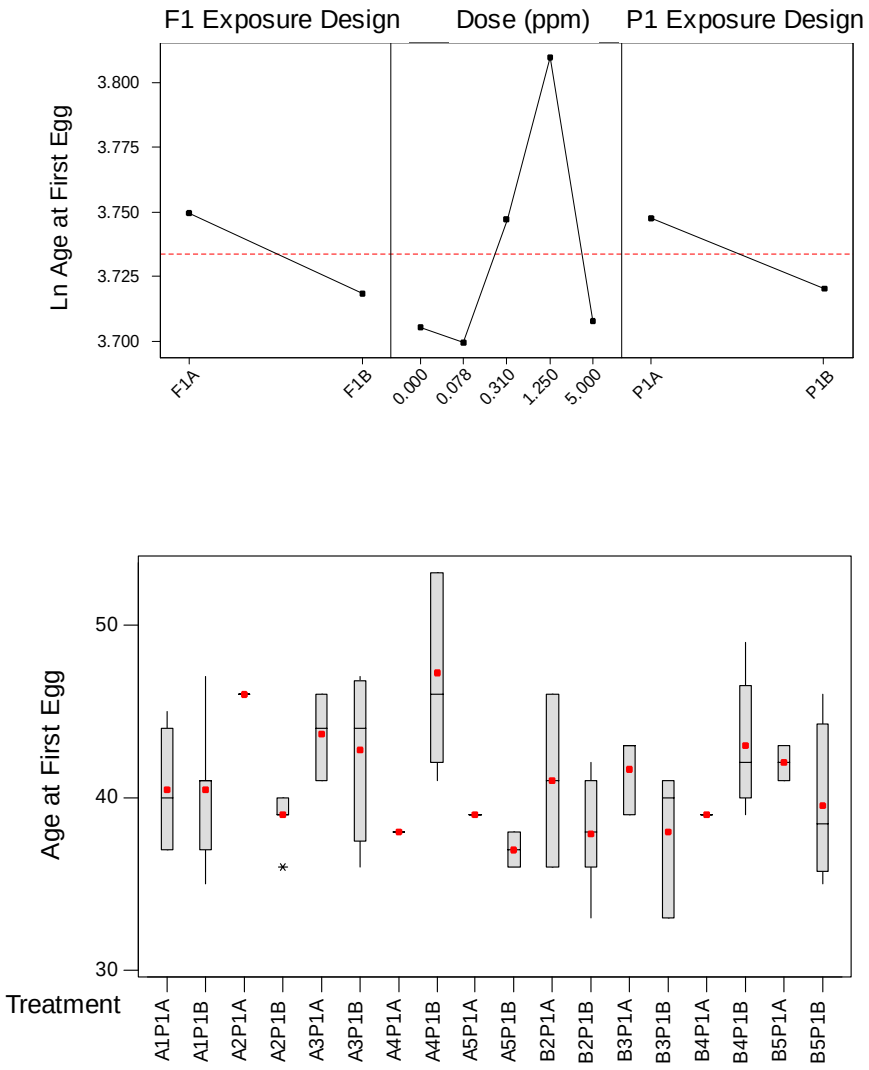


Figure 5.6-4. Natural log transformed General Linear Model analysis (above) and boxplots (below) of the effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on maturation age (days) of F1 females. No significant differences between F1 treatment scenarios, $p>0.27$ and parental dietary concentrations, $p<0.02$. Data do not include extra females.

5.7 Plumage Dimorphism (F1)

Males

As seen in the P1 generation, dietary treatment with E2 resulted in increased incidence of males with phenotypic female or mixed-gender plumage (plumage with both male and female characteristics). However, unlike mixed-gender plumage of feminized males in the P1 generation, the female-type spots of some of the F1 males with mixed plumage were reddish in color rather than the phenotypic brown. Spots on feathers of F1 males with phenotypic female plumage were the typical brown of female-type plumage (Figure 5.7-1). The incidence of feminized plumage in male F1 birds is shown in Figure 5.7-2.

Figure 5.7-1. Normal plumage dimorphism in male (left) and female (right) Japanese quail (above). Phenotypic female plumage of a F1 male (left) treated with E2 from hatch and receiving an *in ovo* dose from P1B parents (below). Plumage phenotype of the female was unchanged by E2 treatment.

The proportion of males out of all F1 males with feminized plumage was significantly greater in treated males (39%) than untreated males (14%) ($p < 0.001$) and significantly greater for the 5 ppm dose than for the control ($p < 0.03$) (Figure 5.7-3). In addition, when all F1 males were considered together, the length of the feminized plumage was highly significantly different for both the F1 exposure strategy and across dietary concentrations of E2 ($p < 0.001$). The degree of feminization (length of female-type plumage) also increased with increasing dietary concentration of E2 (Figure 5.7-4). P1 exposure scenario had no effect on either the proportion of males with non-male plumage characteristics or the length of the feminized feathers ($p>0.95$).

In those F1 males with non-male plumage, the length of the plumage was significantly longer in F1a than F1b males ($p < 0.04$) and significantly shorter in the 0.078 ppm parental dose treatment ($p < 0.03$); parental dosing strategy resulted in nearly significantly longer non-male plumage in male offspring of P1A birds (Figure 5.7-5).

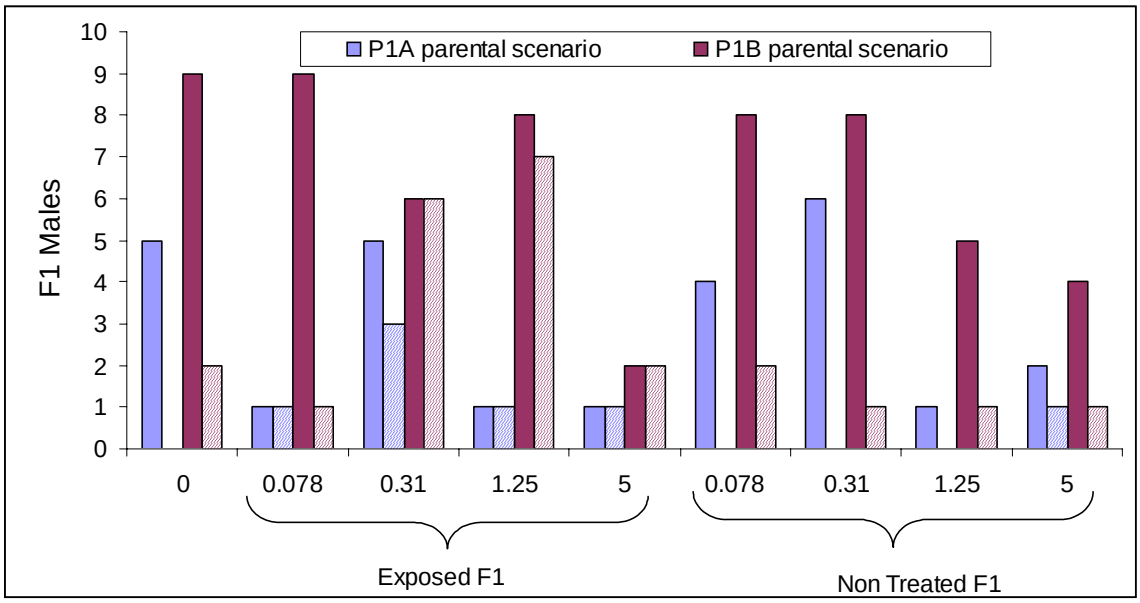


Figure 5.7-2. Total number of male offspring (solid bars) and number of males with feminized plumage (cross hatched) in each scenario. X-axis is feed concentration (ppm) of the parental group. The exposed F1a groups were administered feed with the same concentrations of E2 as the parental group. F1b groups were untreated.

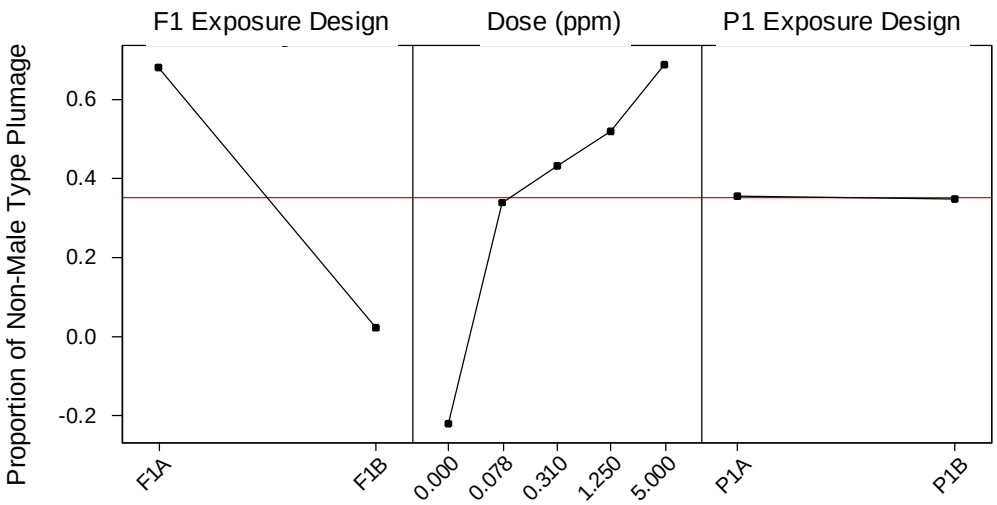


Figure 5.7-3. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the proportion of non-male type plumage (mixed or female phenotypic) in F1 males. General Linear Model analysis; highly significant difference between F1 exposure strategies ($p<0.001$) and significant differences across parental dietary treatments ($p<0.03$). F1a birds were fed the same dietary treatments as their parents.

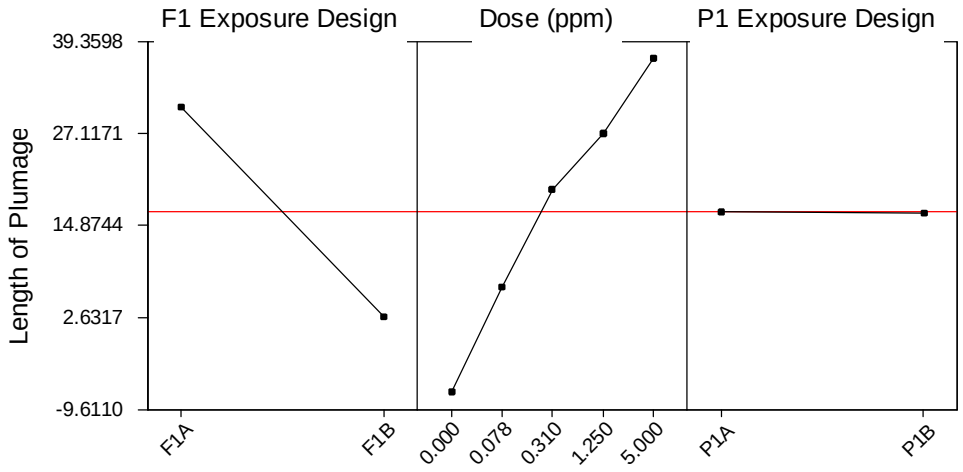


Figure 5.7-4. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the length (mm) of non-male type plumage (mixed or female phenotypic) in F1 males. General Linear Model analysis; highly significant differences ($p<0.001$) between F1 exposure strategies and across parental dietary treatments. F1a birds were fed the same dietary treatments as their parents.

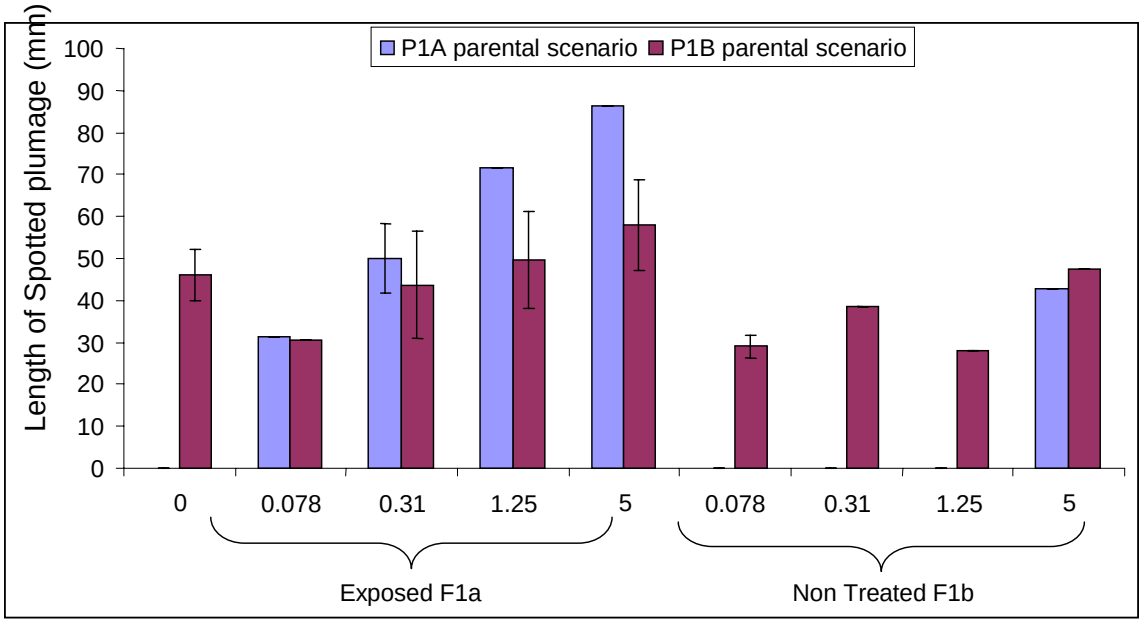


Figure 5.7-5. Length of spotted plumage in male offspring with feminized plumage. Values represent mean ± SD of N=1-7 (no error bars indicate n=1). X-axis is feed concentration (ppm) of the parental group. No data indicates no males with female plumage. The exposed F1a groups were administered feed with the same concentrations of E2 as the parental group. F1b groups were untreated.

Females

No effect of F1 exposure strategy, parental exposure scenario or dietary treatment on plumage phenotype was observed in female F1 birds. Only two F1 females had non-female type plumage. They were both from the untreated F1 exposure (F1b) whose parents were fed diets containing 1.25 ppm E2. (Table 5.7-1).

Table 5.7-1. Proportion of F1 females with female, male, or mixed-gender breast plumage characteristics.

Design	P1 Parental Generation	P1 Treatment Group (ppm)	N	Proportion of Females with Plumage Type			
				Female	Male	Mixed	Cinnamon-colored Spots
F1a	P1A	0	10	1	0	0	0
F1a	P1A	0.078	3	1	0	0	0
F1a	P1A	0.31	5	1	0	0	0
F1a	P1A	1.25	6	1	0	0	0
F1a	P1A	5	6	1	0	0	0
F1a	P1B	0	11	1	0	0	0
F1a	P1B	0.078	7	1	0	0	0
F1a	P1B	0.31	4	1	0	0	0
F1a	P1B	1.25	5	1	0	0	0
F1a	P1B	5	8	1	0	0	0
F1b	P1A	0.078	2	1	0	0	0
F1b	P1A	0.31	3	1	0	0	0
F1b	P1A	1.25	5	0.8	0	0.2	0
F1b	P1A	5	4	1	0	0	0
F1b	P1B	0.078	7	1	0	0	0
F1b	P1B	0.31	3	1	0	0	0
F1b	P1B	1.25	8	0.875	0	0.125	0
F1b	P1B	5	6	1	0	0	0

The length of female phenotypic breast plumage in females of the F1 generation was significantly different between the F1a and F1b exposure strategies (Figures 5.7-6 and 5.7-7), with plumage length significantly less in the treated hens than in untreated birds ($p<0.01$). Neither dietary concentration ($p=0.15$) or parental exposure design ($p=0.52$) had a significant effect on plumage length (Figure 5.7-7).

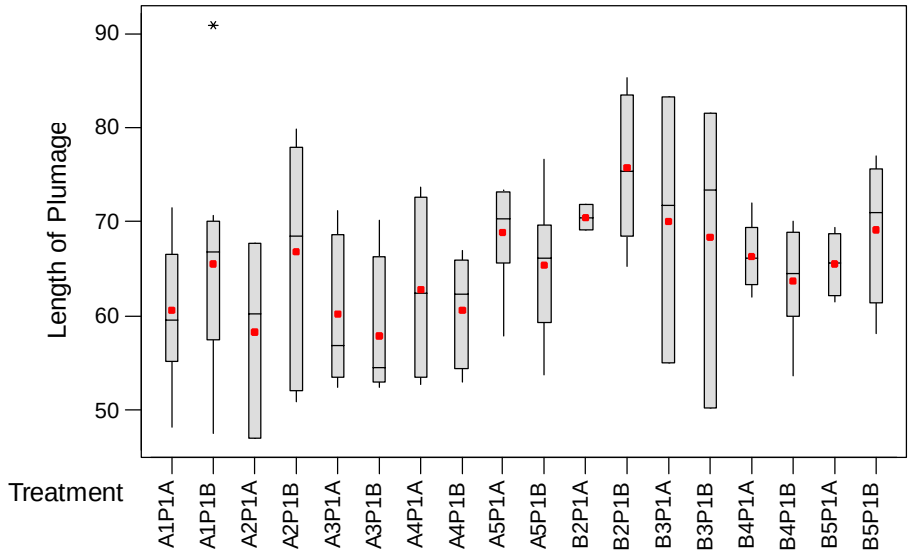


Figure 5.7-6. Box plots of the length (mm) of the spotted phenotypic female breast plumage in F1 females by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentrations of E2 (1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles. (* = extreme value)

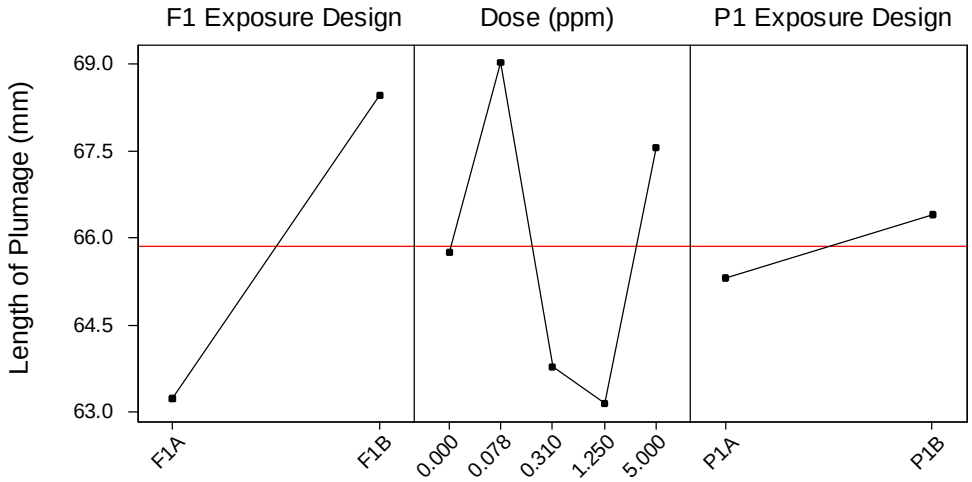


Figure 5.7-7. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the length (mm) of female type plumage in F1 females. General Linear Model analysis; highly significant differences ($p<0.01$) between F1 exposure strategies. F1a birds were fed the same dietary treatments as their parents.

5.8 Cloacal Gland Size (F1)

Cloacal gland measurements were obtained on the same calendar day for all F1 males. No significant differences in cloacal gland surface area were detected between F1 or P1 exposure designs in F1 males at 27, 34, 41, or 51 days of age. However, a significant interaction between F1 exposure strategy and the P1 exposure scenario (p=0.012) affected the cloacal gland surface area at necropsy. Of the offspring of the P1A parents, the treated males (F1a) had greater cloacal gland areas than the untreated males (F1b). In contrast, cloacal gland surface area was not different between the F1a and F1b males of P1B parents (Figure 5.8-1). The mean effect of the parental dietary treatment was not significant (p=0.82).

Because the mean cloacal gland area appeared to differ between the offspring in the P1A and P1B control groups (Figure 5.8-2), possibly contributing to the observed difference in response between the two designs, a best fit model was applied to the natural-log transformed surface areas of the each of the control groups, and the fits of the data from the two parental exposure scenarios were compared. No significant difference between the sigmoidal growth curves for the two control groups was detected (p>0.05; R^2=0.87) (Figure 5.8-3).

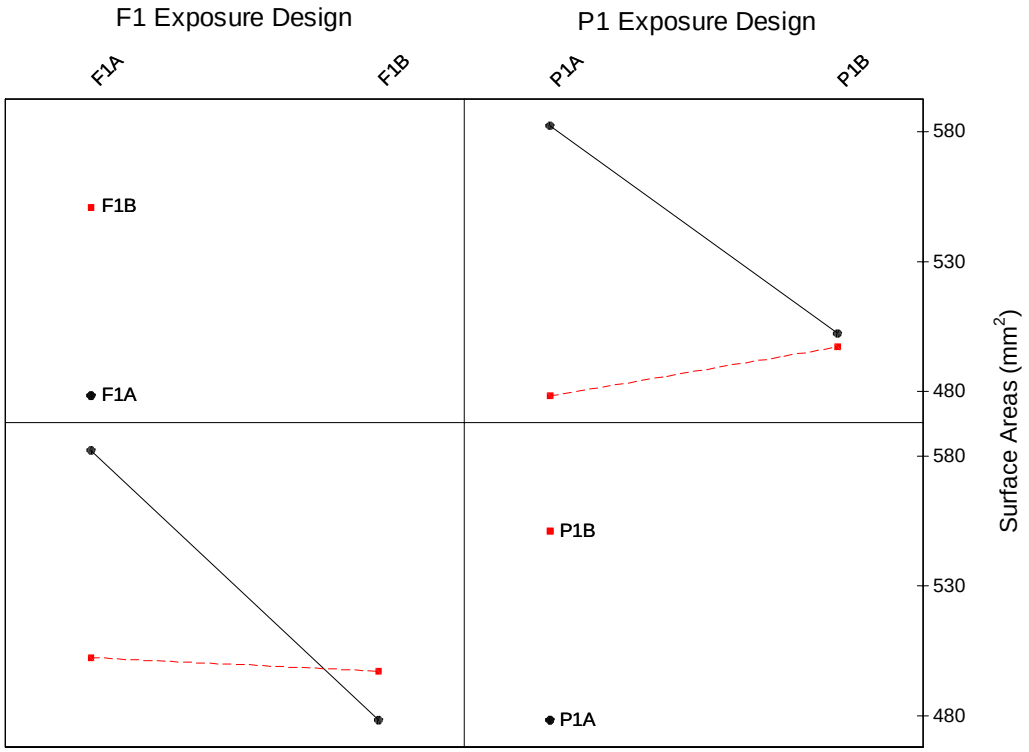


Figure 5.8-1. Interaction of F1 exposure strategy (F1a, treated; F1b, untreated) and parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on surface areas (mm^2) of the cloacal gland in F1 males. (General Linear Model analysis; p=0.13)

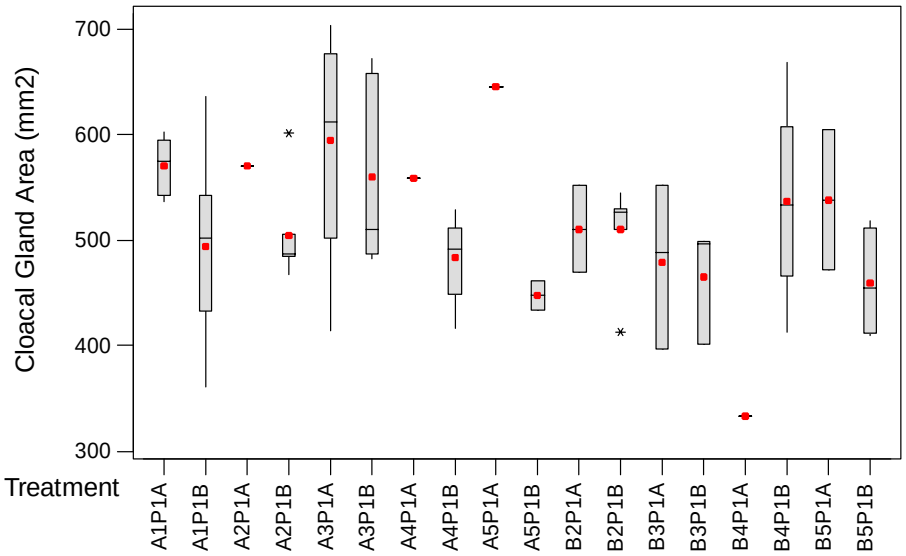


Figure 5.8-2. Box plots of cloacal gland surface area (mm^2) of F1 males by parental exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), parental dietary concentration of E2 (1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

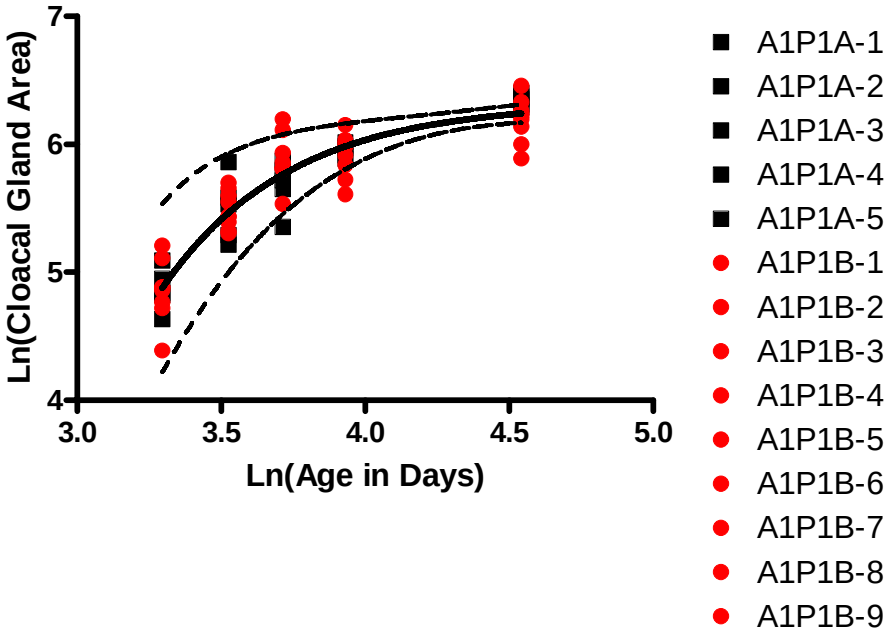


Figure 5.8-3. Best fit model applied to the natural-log transformed surface areas of the control F1 male offspring of parents from the P1A exposure scenario and the control F1 male offspring from the P1B exposure scenario. No significant difference between the sigmoidal dose-response curves for the two control groups was detected ($p>0.05$; $R^2=0.87$).

5.9 Reproductive Parameters (F1)

5.9.1 Egg Production

Eggs produced from offspring of the P1 parental dosing strategies were collected for 5 weeks after the onset of laying to measure test endpoints. Because of the lack of males in some groups and the difficulty in identifying the gender of some birds and therefore assigning appropriate pairs, unmated ("extra") F1 birds produced within groups were maintained under the same E2 dietary treatments as their mated cohorts. Eggs collected from these birds were included in the egg production data analysis to increase the experimental number (N), as some groups had small numbers of mated pairs. The results of the statistical analyses with and without the extra eggs are reported below.

5.9.2 Total Eggs Produced

When the eggs from extra, non-paired birds were included, total egg production was not significantly different between F1 exposure strategies, P1 dietary treatments, or between P1 exposure scenarios ($p > 0.19$). The slope for cumulative number of eggs was not significantly different between F1 exposure designs ($p = 0.47$), P1 dietary treatments ($p = 0.21$) or the P1A and P1B exposure scenarios ($p = 0.16$). The average cumulative number of eggs laid over time (days of age) by dietary treatment of E2 in hens exposed under the F1a and F1b exposure strategies are shown in Figures 5.9-1 and 5.9-2, respectively.

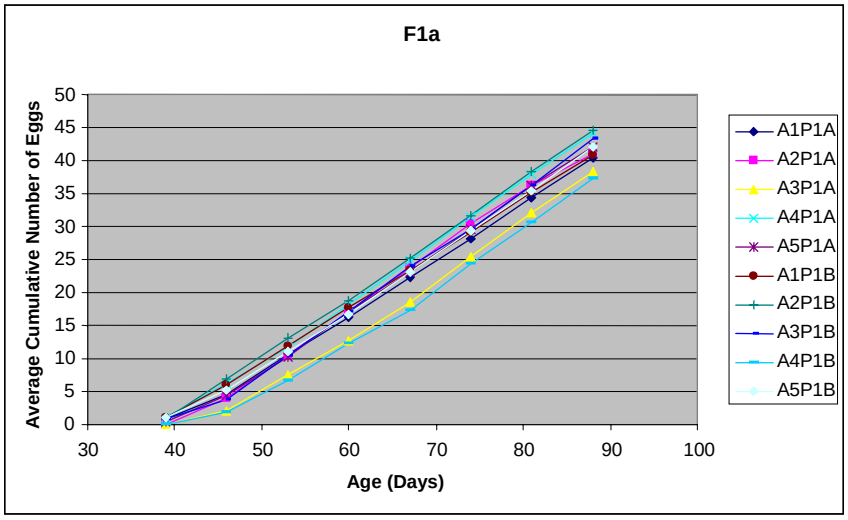


Figure 5.9-1. Average cumulative number of eggs over days of age by dietary treatment in F1 hens fed E2-treated feed from hatch. F1a hens were fed the same diets as their parents.

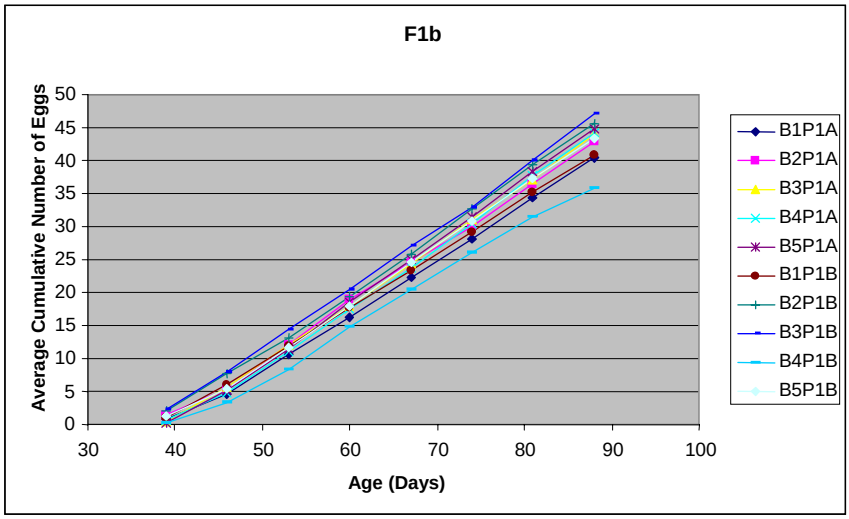


Figure 5.9-2. Average cumulative number of eggs over days of age by dietary treatment of parent in untreated (F1b) hens.

The total eggs laid per maximum number of eggs laid was not significantly different between F1 dosing strategies, P1 treatment dose, or between P1 dosing strategies ($p>0.48$). When the eggs from extra birds were not included in the calculation, and therefore there were fewer birds under consideration (N ranged from 1 to 7 birds/group), there was a statistically significant, but not concentration-linear, effect of treatment concentration on the total number of eggs laid ($p=0.044$). The total number of eggs laid by F1 hens exposed *in ovo* (i.e. from P1 dietary exposure) to 1.25 ppm E2 diet was lower ($p=0.049$) than for all other dietary treatments (Figure 5.9-3).

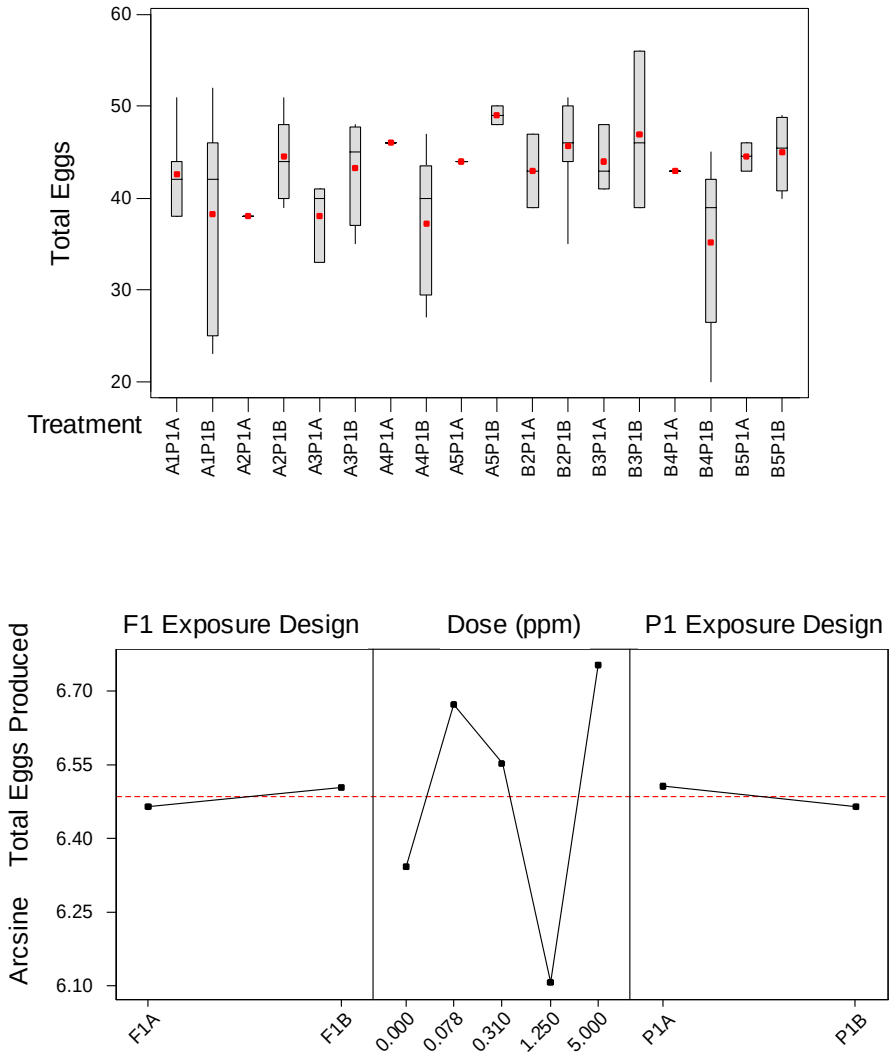


Figure 5.9-3. Box plots (above) of the total egg production per hen by dietary treatment within F1 exposure strategy (F1a, dietary treatment with E2; F1b, untreated) and parental exposure scenario (P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders). Main effects of the General Linear Model analysis of the arcsine-transformed[4] total number of eggs produced per hen (below). The effect of P1 dietary treatment was significant (p=0.044). Data do not include extra eggs from unmated hens.

[4] In the arcsine transformation, the square root of the proportion is converted to its arcsine

5.9.3 Viability of Eggs

Because extra hens were unmated, no extra eggs were used for viability comparisons. The number of viable eggs per number of eggs set per hen was not significantly different between F1 exposure strategies, P1 dietary treatments, or between P1 exposure scenarios on either day 8 ($p>0.20$) or day 15 ($p>0.15$) of incubation. Egg viability is summarized in Table 5.9-1.

When the proportions of eggs viable on day 8 per number of eggs set per hen were regressed against the age of the hens, no significant differences were found ($p>0.16$). However, for eggs that were viable on Day 15, nearly significant ($p=0.056$) F1 exposure design effect on the rate of production of viable eggs over time was detected. Greater positive slopes were observed for eggs laid by hens consuming E2 treated diet (F1a) compared to untreated F1b hens (Figure 5.9-4).

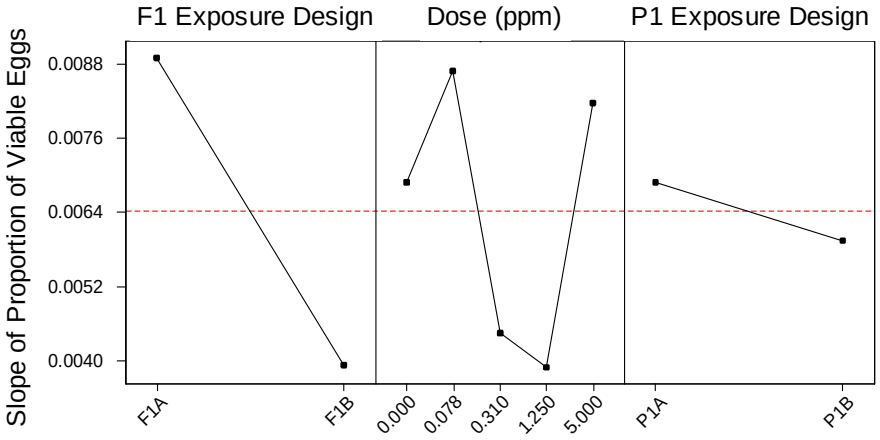


Figure 5.9-4. Main effects of the General Linear Model analysis of the slope of the proportion of Day 15 viable eggs per total eggs produced per F1 hen regressed against age of the hen. The effect of F1 exposure strategy was a ($p=0.056$). Data do not include extra eggs from unmated hens. (F1a, dietary treatment with E2; F1b, untreated; P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders).

5.9.4 Hatchlings Produced

Neither the proportional number of eggs that hatched per number of eggs set, nor the hatchling number per number of eggs viable at day 8 differed significantly between F1 exposure strategies, P1 dietary treatments, or between P1 exposure scenarios ($p>0.37$). However, the proportion of hatchlings out of the maximum number of eggs set had a significant but nonlinear treatment concentration effect ($p=0.02$). The exposure design of the F1 or P1 generation had no effect on the proportion of hatchlings produced out of the maximum number of eggs set ($p>0.42$). (Figure 5.9-5). Hatchling production by treatment is summarized in Table 5.9-1.

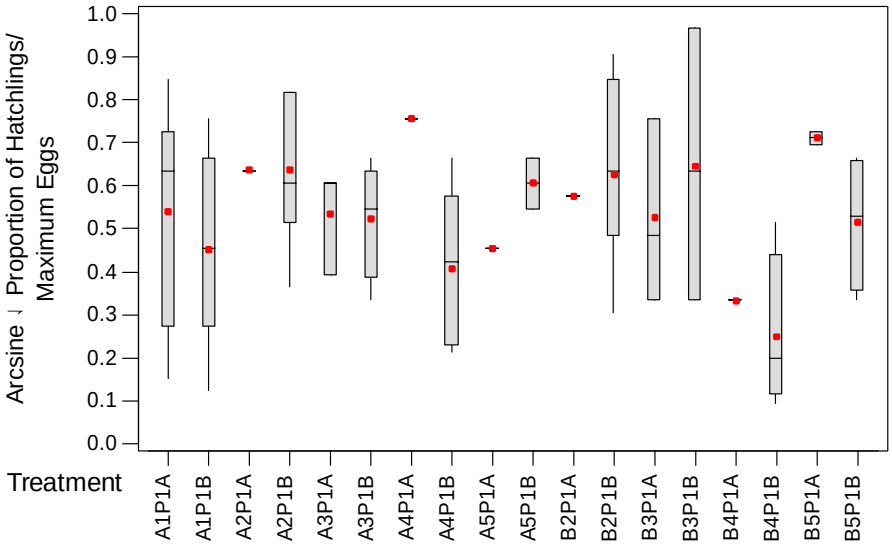
Table 5.9-1. F1 egg viability and hatch.

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Proportion Hatched out of Total Set									
F1a-P1A-0 ppm	7	0.77	0.88	0.20	0.39	0.92	0.63	0.88	25%
F1a-P1B-0 ppm	7	0.70	0.75	0.15	0.50	0.86	0.54	0.85	22%
F1a-P1A-0.078 ppm	1	0.91	0.91	NC	0.91	0.91	NC	NC	NC
F1a-P1B-0.078 ppm	7	0.81	0.82	0.09	0.63	0.90	0.79	0.87	11%
F1a-P1A-0.31 ppm	3	0.84	0.83	0.03	0.81	0.87	0.81	0.87	3%
F1a-P1B-0.31 ppm	4	0.70	0.69	0.12	0.56	0.85	0.59	0.81	17%
F1a-P1A-1.25 ppm	1	0.93	0.93	NC	0.93	0.93	NC	NC	NC
F1a-P1B-1.25 ppm	5	0.82	0.85	0.14	0.64	1.00	0.69	0.94	17%
F1a-P1A-5 ppm	1	0.58	0.58	NC	0.58	0.58	NC	NC	NC
F1a-P1B-5 ppm	2	0.70	0.70	0.08	0.64	0.76	NC	NC	12%
F1b-P1A-0.078 ppm	2	0.79	0.79	0.16	0.68	0.91	NC	NC	20%
F1b-P1B-0.078 ppm	7	0.78	0.76	0.13	0.59	0.97	0.70	0.88	16%
F1b-P1A-0.31 ppm	3	0.65	0.59	0.28	0.41	0.96	0.41	0.96	43%
F1b-P1B-0.31 ppm	3	0.79	0.88	0.24	0.52	0.97	0.52	0.97	30%
F1b-P1A-1.25 ppm	1	0.46	0.46	NC	0.46	0.46	NC	NC	NC
F1b-P1B-1.25 ppm	5	0.71	0.77	0.41	0.00	1.00	0.38	1.00	58%
F1b-P1A-5 ppm	2	0.86	0.86	0.05	0.82	0.89	NC	NC	6%
F1b-P1B-5 ppm	4	0.72	0.73	0.15	0.55	0.88	0.57	0.86	21%
Proportion of Total Set that were Viable at 8 Days									
F1a-P1A-0 ppm	7	0.84	0.88	0.12	0.64	0.96	0.71	0.95	14%
F1a-P1B-0 ppm	7	0.82	0.86	0.14	0.60	1.00	0.68	0.90	17%
F1a-P1A-0.078 ppm	1	1.00	1.00	*	1.00	1.00	*	*	*
F1a-P1B-0.078 ppm	7	0.89	0.87	0.07	0.80	1.00	0.84	0.94	7%
F1a-P1A-0.31 ppm	3	0.88	0.91	0.06	0.81	0.91	0.81	0.91	6%
F1a-P1B-0.31 ppm	4	0.81	0.81	0.07	0.72	0.88	0.74	0.87	9%
F1a-P1A-1.25 ppm	1	0.96	0.96	*	0.96	0.96	*	*	*
F1a-P1B-1.25 ppm	5	0.85	0.85	0.12	0.67	1.00	0.75	0.94	14%
F1a-P1A-5 ppm	1	0.75	0.75	*	0.75	0.75	*	*	*
F1a-P1B-5 ppm	2	0.83	0.83	0.12	0.75	0.92	*	*	14%
F1b-P1A-0.078 ppm	2	0.88	0.88	0.03	0.86	0.90	*	*	3%
F1b-P1B-0.078 ppm	7	0.85	0.83	0.09	0.75	1.00	0.76	0.92	11%
F1b-P1A-0.31 ppm	3	0.89	0.92	0.12	0.76	1.00	0.76	1.00	14%
F1b-P1B-0.31 ppm	3	0.87	0.91	0.16	0.69	1.00	0.69	1.00	19%
F1b-P1A-1.25 ppm	1	0.58	0.58	*	0.58	0.58	*	*	*
F1b-P1B-1.25 ppm	4	0.92	0.97	0.12	0.75	1.00	0.80	1.00	13%
F1b-P1A-5 ppm	2	0.92	0.92	0.00	0.92	0.92	*	*	0%
F1b-P1B-5 ppm	4	0.81	0.81	0.10	0.69	0.91	0.71	0.90	12%

Table 5.9-1. F1 egg viability and hatch (continued).

Treatment	N	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Proportion Hatched out of Maximum Set									
F1a-P1A-0 ppm	7	0.54	0.64	0.25	0.15	0.85	0.27	0.73	47%
F1a-P1B-0 ppm	7	0.45	0.46	0.22	0.12	0.76	0.27	0.67	48%
F1a-P1A-0.078 ppm	1	0.64	0.64	NC	0.64	0.64	NC	NC	NC
F1a-P1B-0.078 ppm	7	0.63	0.61	0.18	0.36	0.82	0.52	0.82	28%
F1a-P1A-0.31 ppm	3	0.54	0.61	0.12	0.39	0.61	0.39	0.61	23%
F1a-P1B-0.31 ppm	4	0.52	0.55	0.14	0.33	0.67	0.39	0.64	27%
F1a-P1A-1.25 ppm	1	0.76	0.76	NC	0.76	0.76	NC	NC	NC
F1a-P1B-1.25 ppm	5	0.41	0.42	0.19	0.21	0.67	0.23	0.58	46%
F1a-P1A-5 ppm	1	0.46	0.46	NC	0.46	0.46	NC	NC	NC
F1a-P1B-5 ppm	2	0.61	0.61	0.09	0.55	0.67	NC	NC	14%
F1b-P1A-0.078 ppm	2	0.58	0.58	0.00	0.58	0.58	NC	NC	0%
F1b-P1B-0.078 ppm	7	0.63	0.64	0.22	0.30	0.91	0.49	0.85	35%
F1b-P1A-0.31 ppm	3	0.53	0.49	0.22	0.33	0.76	0.33	0.76	41%
F1b-P1B-0.31 ppm	3	0.65	0.65	0.32	0.33	0.97	0.33	0.97	49%
F1b-P1A-1.25 ppm	1	0.33	0.33	NC	0.33	0.33	NC	NC	NC
F1b-P1B-1.25 ppm	4	0.25	0.20	0.18	0.09	0.52	0.11	0.44	74%
F1b-P1A-5 ppm	2	0.71	0.71	0.02	0.70	0.73	NC	NC	3%
F1b-P1B-5 ppm	4	0.52	0.53	0.16	0.33	0.67	0.36	0.66	31%

Regressing the proportion of hatchlings produced out of the maximum number of eggs set against age of hen showed that dietary treatment of the F1 females resulted in increased rate of hatchling production over time (p=0.015) compared to the production slopes of untreated (F1b) hens (Figure 5.9-6).



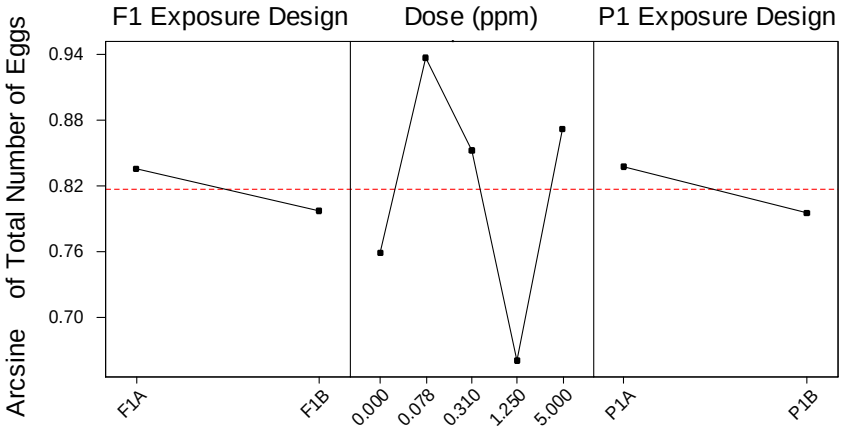


Figure 5.9-5. **Box plots (above) of the arcsine square root-transformed proportion of the number of hatchlings per maximum number of eggs set by dietary treatment within F1 exposure strategy (F1a, dietary treatment with E2; F1b, untreated) and parental exposure scenario (P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders).** Main effects of the General Linear Model analysis of the arcsine transformed total number of eggs produced per hen (below). The effect of P1 dietary treatment was significant (p=0.02). Data do not include extra eggs from unmated hens.

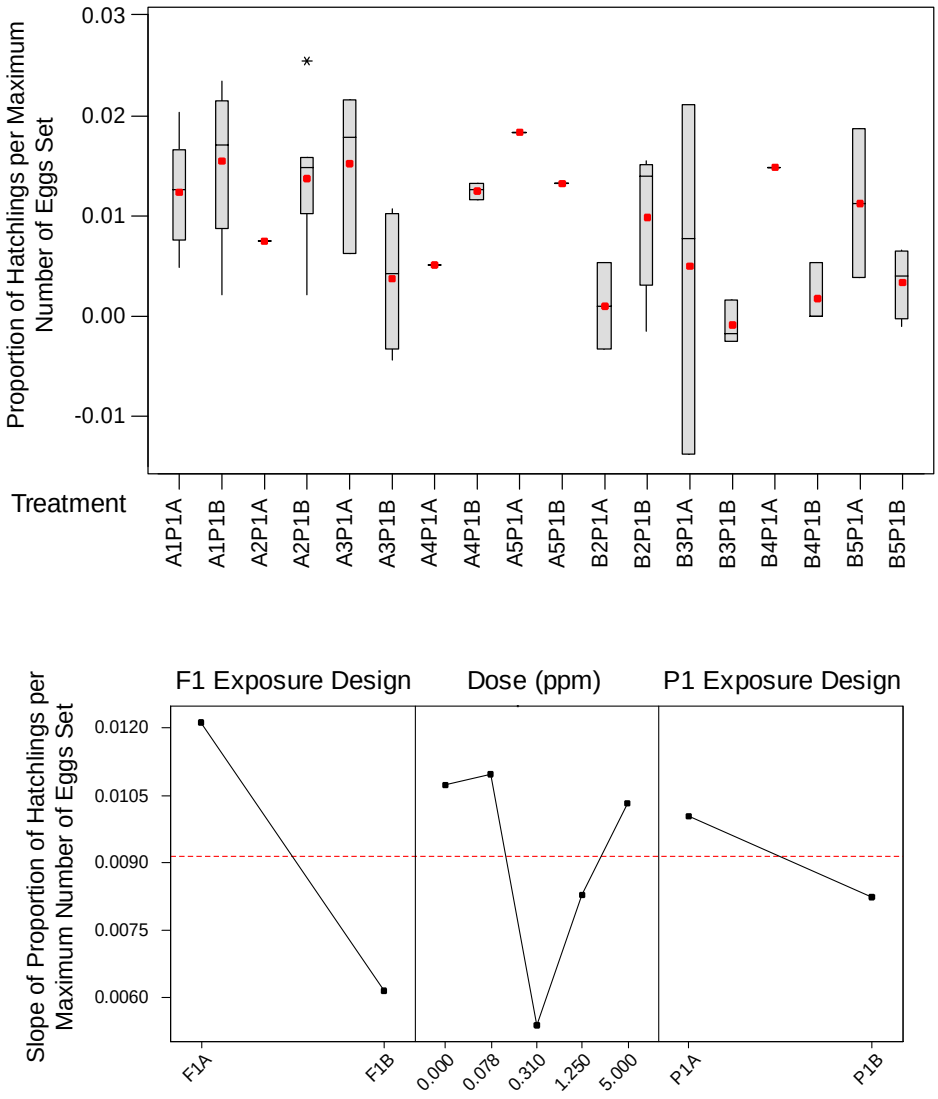


Figure 5.9-6. **Box plots (above) of the proportion of the number of hatchlings per maximum number of eggs set by dietary treatment within F1 exposure strategy (F1a, dietary treatment with E2; F1b, untreated) and parental exposure scenario (P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders).** Main effects of the General Linear Model analysis of the slope of the proportion of hatchlings produced per maximum eggs set (below). The effect of F1 dietary treatment was significant (p=0.015). Data do not include extra eggs from unmated hens.

5.10 Eggshell Quality (F1)

As described in Section 5.9, Reproductive Parameters, eggs were collected from unmated F1 birds maintained under the same E2 dietary treatments as their mated cohorts. Eggs collected from these birds were included in the eggshell quality data analysis to increase the experimental number (N), as some groups had small numbers of mated pairs. The results of the statistical analyses with and without the extra eggs are reported below.

5.10.1 Proportion of Cracked Eggs

Without including the extra eggs, the number of cracked eggs per total number of eggs laid by F1 females were significantly affected (p=0.001) by dietary treatment of the parents of the F1 generation, but the effect was non concentration-linear. A nearly significantly (p=0.06) higher proportion of cracked eggs laid by offspring of P1B parents was also observed (Figure 5.10-1). When the eggs from extra, non-paired birds were included, the number cracked/number laid eggs showed a significant interaction between the F1 and the P1 exposure strategies (p = 0.02) and had a significant but nonlinear P1 dietary treatment concentration effect (p = 0.001) (Figures 5.10-2 and 5.10-3). Eggs of the F1a hens had about the same proportion of cracked eggs whether they were offspring of P1A or P1B parents; however, the untreated F1 offspring of P1B parents had a greater proportion of cracked eggs than untreated offspring of the P1A parents (Figures 5.10-2 and 5.10-4). Figure 5.10-4 shows the distribution of the proportion of cracked eggs of total eggs produced per hen by dietary treatment within F1 exposure strategies and P1 exposure scenario.

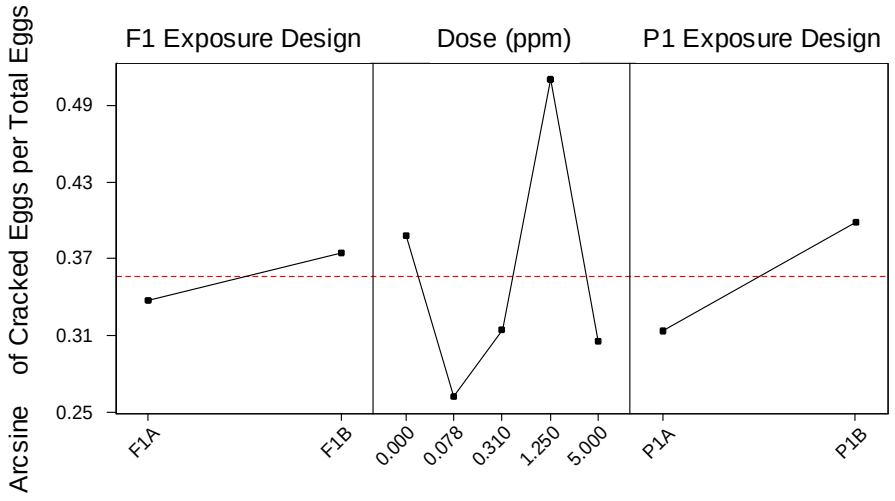


Figure 5.10-1. Main effects of the General Linear Model analysis of the arcsine square root transformed proportion of cracked eggs per total eggs produced per F1 hens. The effect of P1 dietary treatment was significant (p=0.001). Data do not include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed for 13 weeks from 3 weeks of age; P1B, parental population exposed for 5 weeks of egg laying.

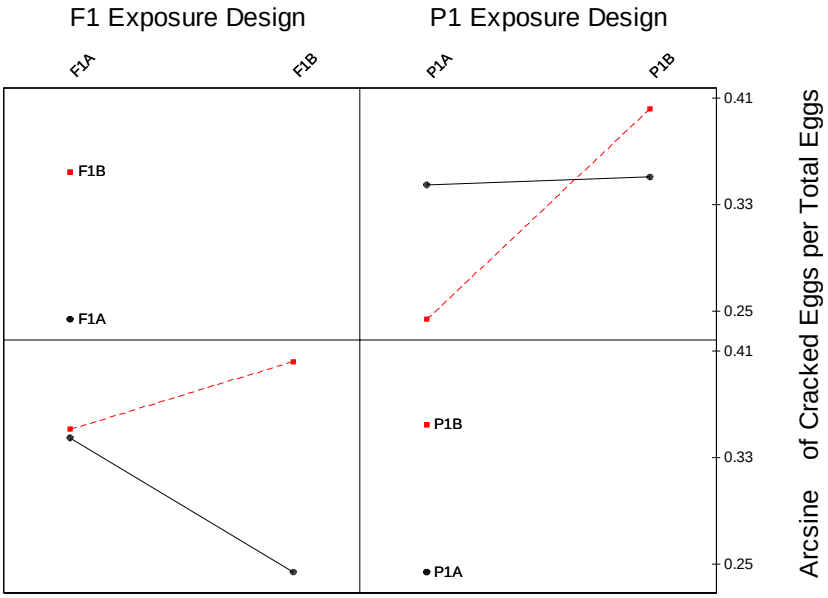


Figure 5.10-2. **Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.02) the arscine square root transformed proportion cracked eggs per total eggs produced per F1 hen.** Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

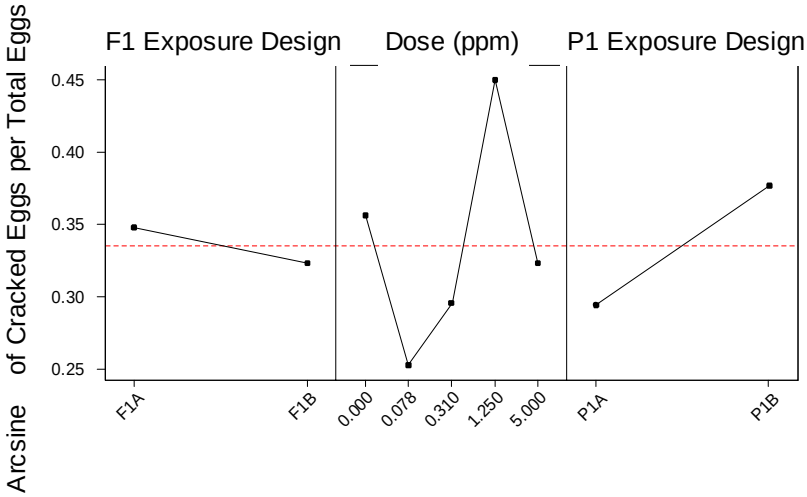


Figure 5.10-3. **Main effects of the General Linear Model analysis of the arcsine square root transformed proportion of cracked eggs per total eggs produced per F1 hen.** The effect of P1 dietary treatment was significant (p=0.001). Data include extra eggs from unmated hens. (F1a, dietary treatment with E2; F1b, untreated; P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders).

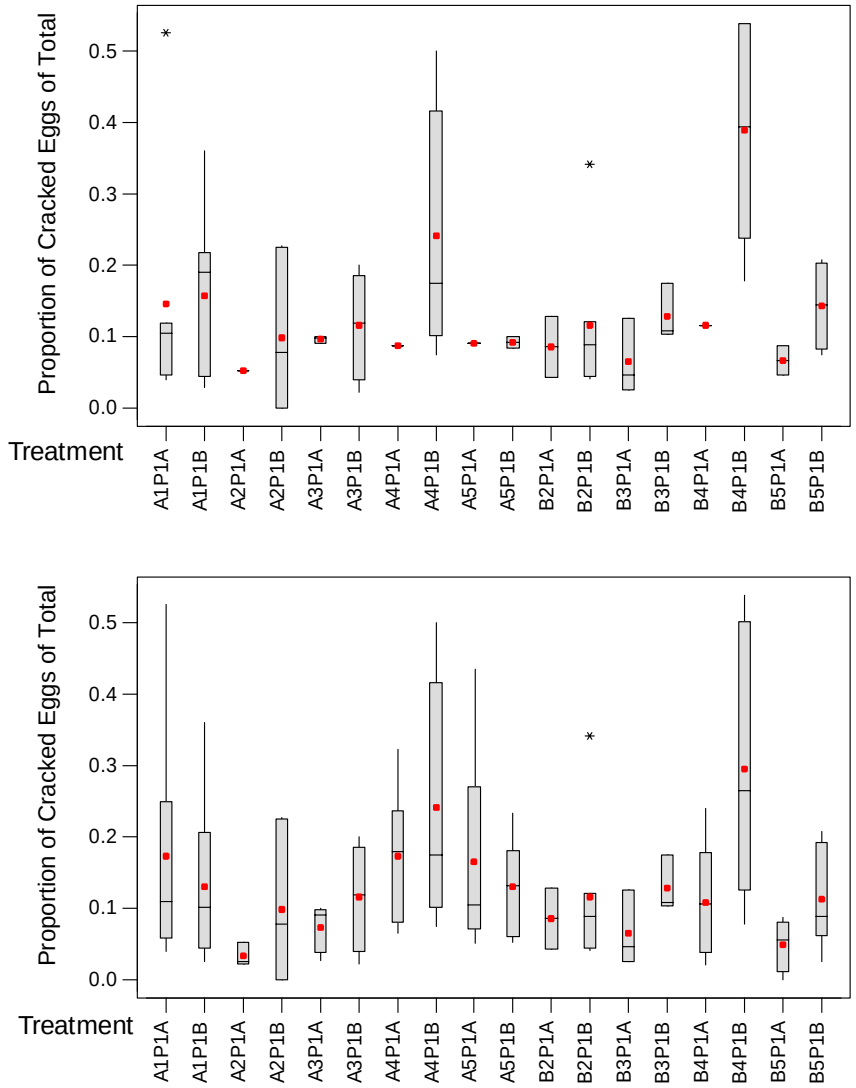


Figure 5.10-4. Box plots of the proportion of cracked eggs of total eggs laid per hen by dietary treatment within F1 exposure strategy (F1a, dietary treatment with E2; F1b, untreated) and parental exposure scenario (P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders). Data above do not include extra eggs from unmated hens, data below do include the extra eggs.

When the proportions of cracked eggs were regressed against the age of the hens, the slopes became significantly (p=0.046) more negative with age for treated (F1a) hens compared to untreated hens indicating a decrease in the proportion of cracked eggs with dietary exposure to E2 (Figure 5.10-5). P1 exposure scenario and dietary concentration of E2 did not have a significant effect on the proportion of cracked eggs produced over time (p>0.64).

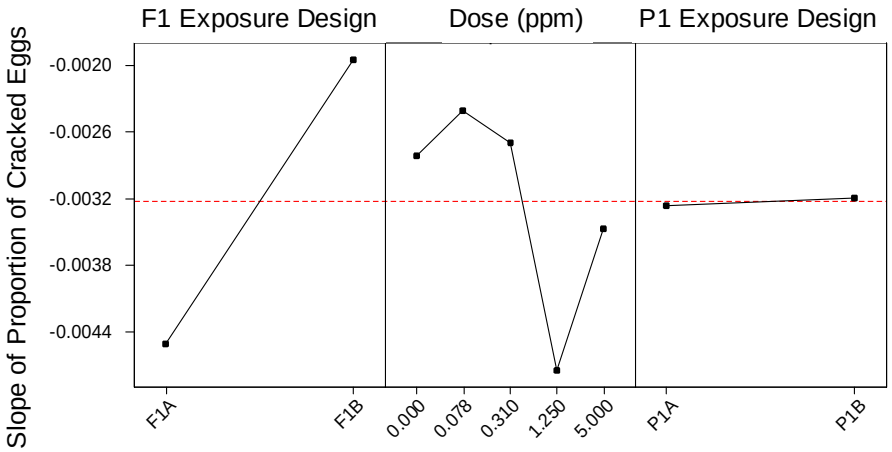


Figure 5.10-5. Main effects of the General Linear Model analysis of the slope of the proportion of cracked eggs per total eggs produced per F1 hen regressed against age of the hen. The effect of F1 exposure strategy was significant (p=0.046). Data include extra eggs from unmated hens. (F1a, dietary treatment with E2; F1b, untreated; P1A, dietary treatment from 3 weeks of age through egg laying; P1B, dietary treatment with E2 as proven breeders).

5.10.2 Shell Thickness

Mean shell thickness of eggs laid by the F1 adults was nearly significantly affected by F1 exposure strategy (p=0.10). Eggs laid by F1a birds (exposed from hatch to E2) had thicker shells than eggs laid by F1b birds (no dietary exposure to E2). Parental dietary treatment also appeared to have some effect on shell thickness (p=0.07), but the effect was limited to the 1.25 ppm E2 exposure (Figures 5.10-6 and 5.10-7). However, shell thickness over time changed only as a result of parental dietary treatments (p=0.06) and in a more dose-linear pattern (Figures 5.10-8 and 5.10-9). Most of the slopes of shell thickness regressed against time of groups consuming treated feed were negative compared to the positive slopes of the controls. The parental exposure scenario, P1A or P1B, had no effect on the shell thickness of the eggs laid by their offspring (p=0.66).

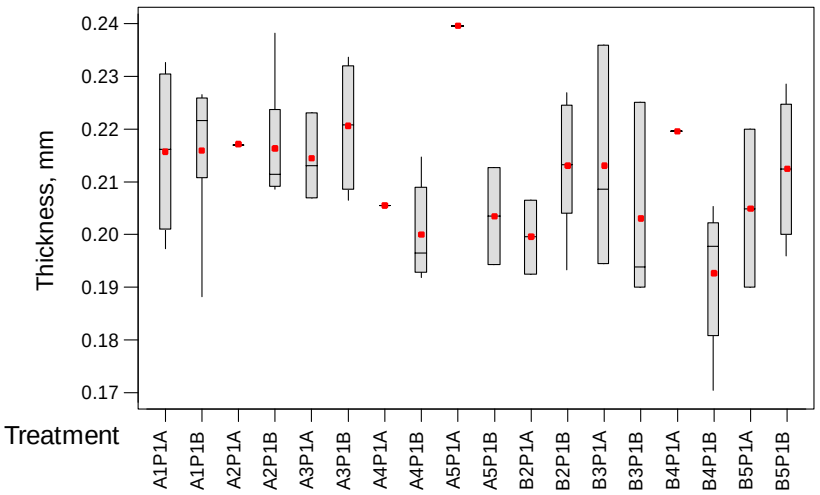


Figure 5.10-6. **Box plots of the shell thickness (mm) of eggs laid by F1 hens by F1 exposure strategy (a, treated from hatch with same diet of E2 as parents; b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and by parental dietary treatment group (1, control; 2, 0.078 ppm E2; 3, 0.31 ppm E2; 4, 1.25 ppm E2; 5, 5 ppm E2). Data do not include extra eggs from unmated hens.** Means are indicated by solid circles.

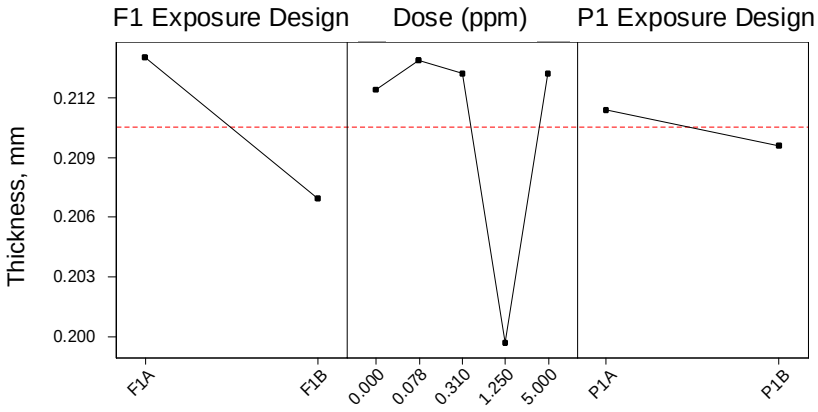


Figure 5.10-7. **General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on egg shell thickness (mm). Data do not include extra eggs from unmated hens.** Nearly significant F1 exposure strategy (p=0.10) and P1 dietary treatment (p=0.07) effects on shell thickness.

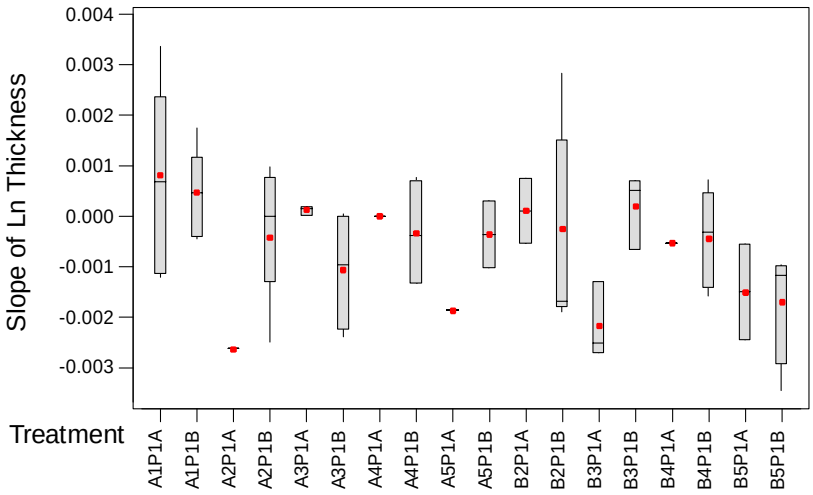


Figure 5.10-8. **Box plots of the natural log-transformed slopes of shell thickness over time of eggs laid by F1 hens by F1 exposure strategy (a, treated from hatch with same diet of E2 as parents; b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and by parental dietary treatment (1, control; 2, 0.078 ppm E2; 3, 0.31 ppm E2; 4, 1.25 ppm E2; 5, 5 ppm E2). Data do not include extra eggs from unmated hens.** Means are indicated by solid circles.

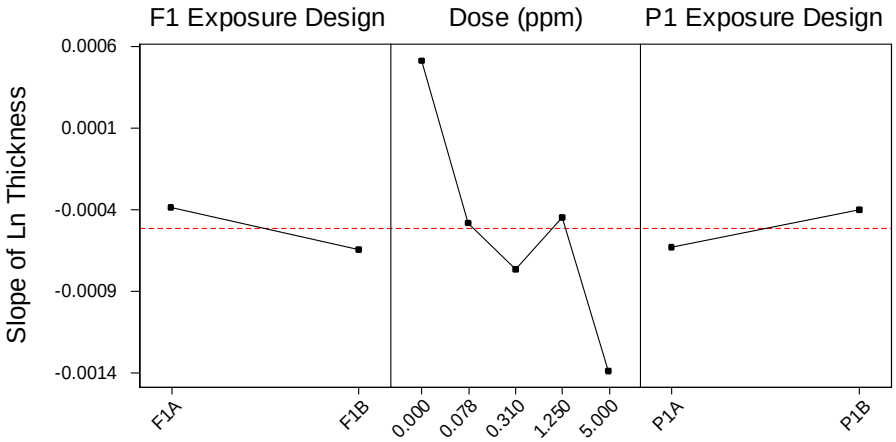


Figure 5.10-9. **General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on natural log-transformed slope of egg shell thickness over time. Data do not include extra eggs from unmated hens.** Nearly significant P1 dietary treatment (p=0.06) effect on shell thickness.

When the eggs from unmated birds were included in the analysis, and therefore there were more birds under consideration, only the nearly significant P1dietary treatment concentration effect on mean shell thickness was retained (p=0.131); the effect of the F1 exposure strategy was not detected (p=0.422) (Figure 5.10-10). In contrast, the change in shell thickness over time was no longer affected by parental dietary treatment (Figure 5.10-11), but was significantly affected (p=0.015) by an interaction between the F1 and P1 exposure designs (Figure 5.10-12) wherein treated offspring (F1a) of P1A parents laid eggs of greater thickness than untreated offspring (F1b) of the same P1 exposure scenario. P1B exposure had no effect on shell thickness eggs laid by the F1 generation.

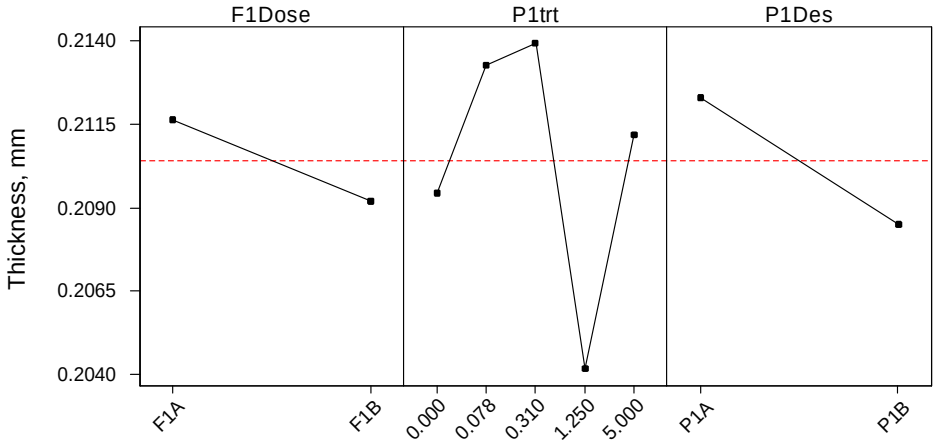


Figure 5.10-10. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on egg shell thickness (mm). Data include extra eggs from unmated hens. Nearly significant P1 dietary treatment (p=0.131) effect on shell thickness. Doses in ppm.

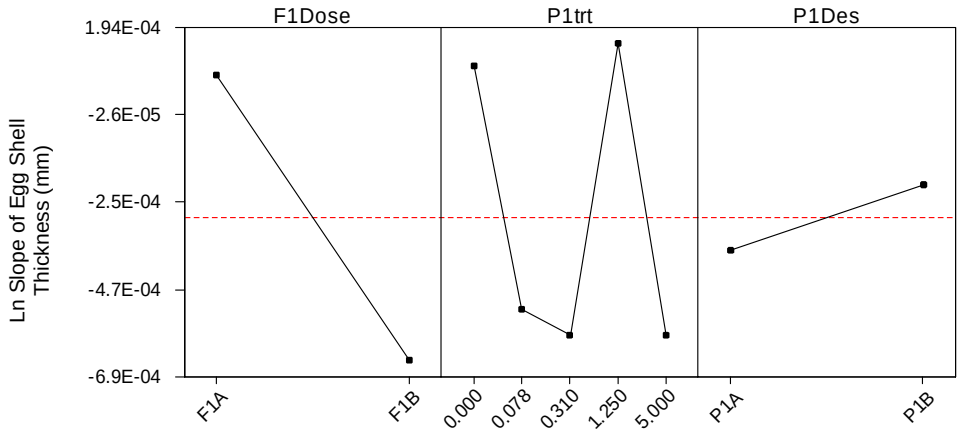


Figure 5.10-11. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on natural log-transformed slope of egg shell thickness. Data includes extra eggs from unmated hens. Dietary concentration effect no longer statistically significant. Doses in ppm.

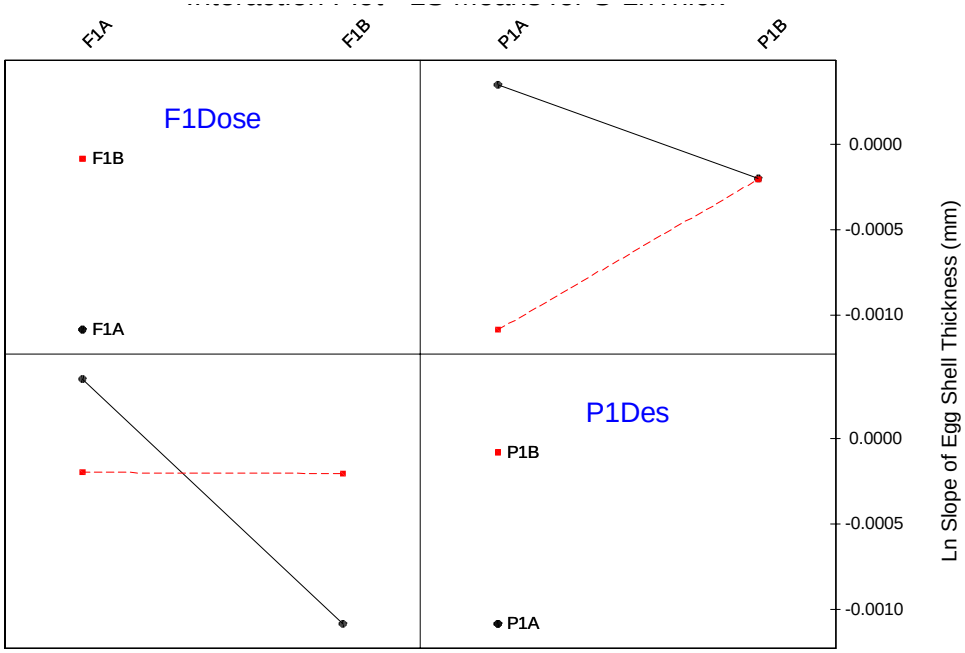


Figure 5.10-12. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.015) the natural log-transformed slope of shell thickness of eggs laid by F1 hens. Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders. Data include extra eggs from unmated hens.

5.10.3 Eggshell Strength

Maximum Load to Rupture

The breaking strength (maximum load to rupture) of F1 eggs was not significantly affected by the F1 or P1 exposure scenarios ($p>0.25$) when eggs from unmated hens were not included in the analysis. There were, however, significant differences in shell strength induced by parental dietary concentration ($p= 0.005$) and a nearly significant increase in breaking strength ($p=0.09$) over time in eggs laid by offspring of P1B parents (Figures 5.10-13 and 5.10-14). These parental dietary concentration effects were similar to those observed for shell thickness (Figures 5.10-7 and 5.10-9) and shell stiffness (Figure 5.10-18, presented below).

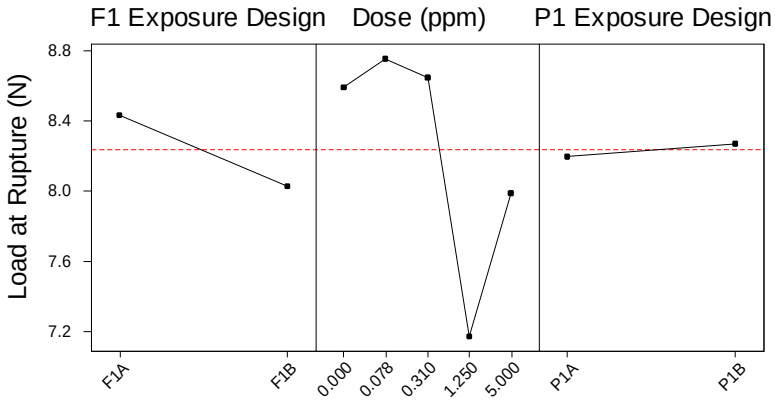


Figure 5.10-13. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on breaking strength in Newtons. Data do not include extra eggs from unmated hens. Significant effect ($p=0.005$) of parental dietary treatment on breaking strength of F1 eggs.

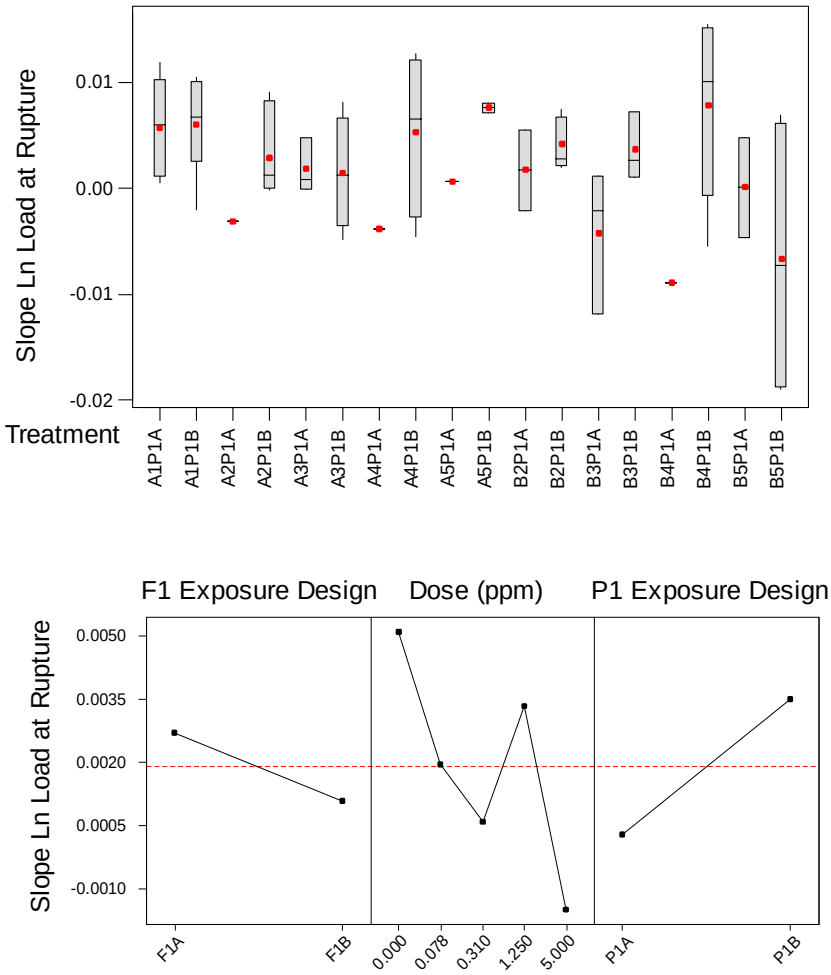


Figure 5.10-14. Box plots of the natural log-transformed slopes of the shell breaking strength (N/days of age, above) and General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on natural log-transformed slope of the shell breaking strength over time (below). Data do not include extra eggs from unmated hens. Nearly significant P1 exposure scenario effect on breaking strength over time (p=0.09). Means are indicated by solid circles.

In contrast to the analysis without the extra eggs, a parental dietary concentration effect was not detected when the number of eggs examined was increased (p=0.423). However, breaking strength of eggs laid by the F1 generation remained unaffected by F1 or P1 exposure scenarios (p>0.228) when the extra eggs from unmated hens were considered in the analysis. Breaking strength over time of F1 eggs was nearly significantly affected (p=0.102) by an interaction between the F1 and P1 exposure designs when the extra eggs were used (Figure 5.10-15). The eggs of untreated (F1b) hens of P1A parents diminished in strength over time compared to eggs of their F1b-P1B counterparts. Eggs from treated (F1a) hens were unaffected by P1 exposure design and the breaking strength over time of eggs from parents with P1B exposure scenario

history was unaffected by F1 exposure strategy (Figure 5.10-15). When the F1 generations were examined separately, shell strength over time from F1a birds were unaffected by P1 exposure scenario or by dietary concentration (Figure 5.10-16), whereas, eggs from F1b birds with P1B parents tended to increase in strength over time more than F1b birds with P1A parents (p=0.105) (Figure 5.10-17).

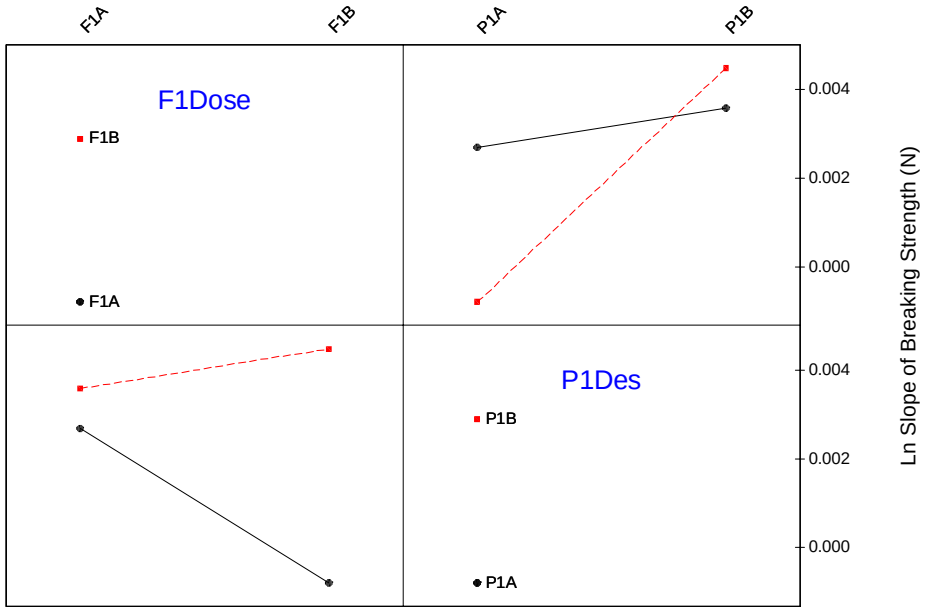


Figure 5.10-15. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.102) the natural log-transformed slope of breaking strength shells of eggs laid by F1 hens. Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

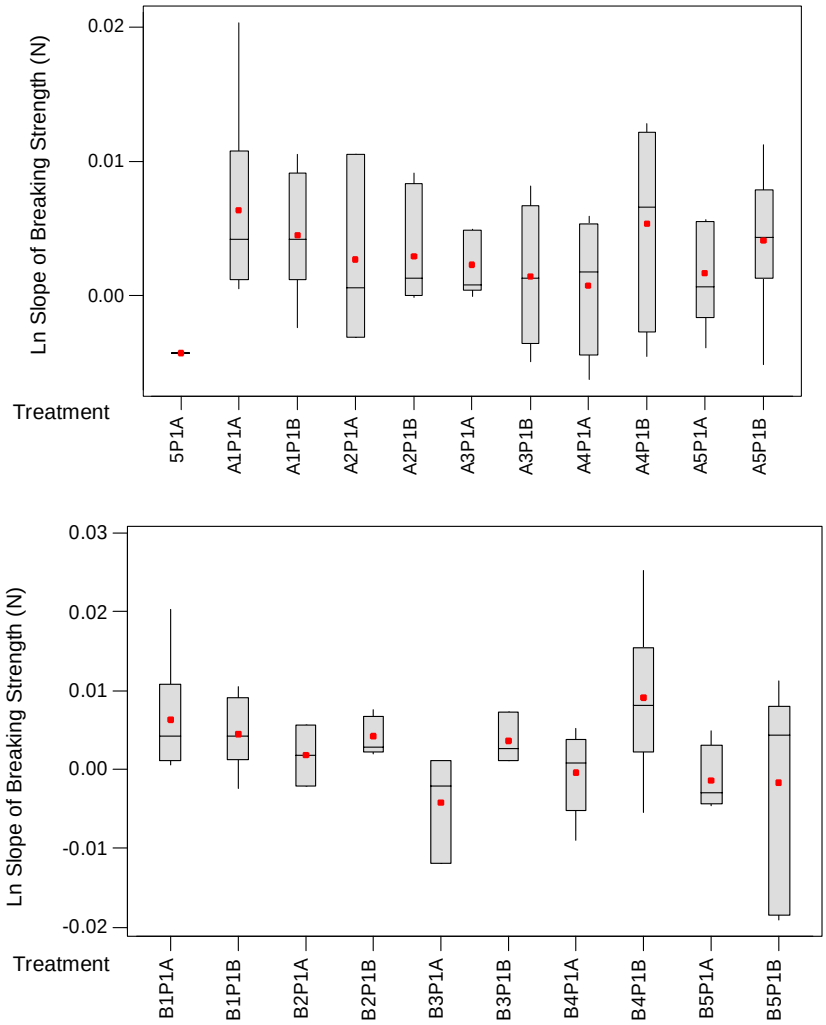


Figure 5.10-16. Box plots of the natural log-transformed slopes of shell breaking strength over time of eggs laid by F1a hens (above) and F1b hens (below). Data include extra eggs from unmated hens. F1a, treated from hatch with same diet of E2 as parents; F1b, untreated; P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying; 1, control; 2, 0.078 ppm E2; 3, 0.31 ppm E2; 4, 1.25 ppm E2; 5, 5 ppm E2). Means are indicated by solid circles.

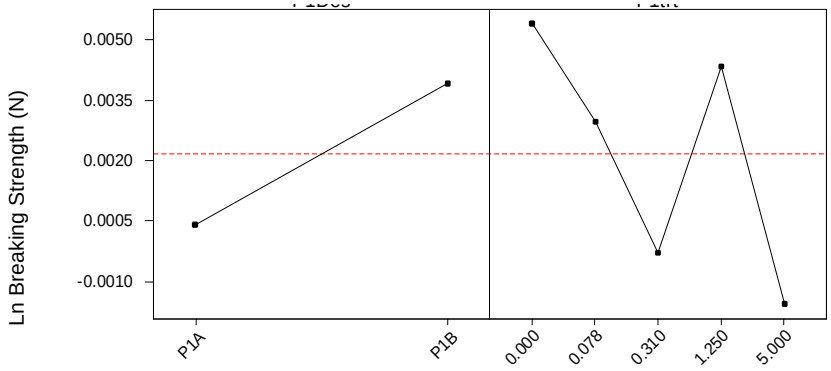


Figure 5.10-17. General Linear Model analysis of the effects of P1 exposure scenario on natural log-transformed breaking strength over time of shells of untreated (F1b) hens. Data include extra eggs from unmated females. (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying). Nearly significant P1 effect on breaking strength over time (p=0.105). Doses in ppm.

Shell Stiffness

Mean shell stiffness and shell stiffness over time was unaffected by F1 and P1 exposure designs (p=0.793). However, a nearly significant parental dietary treatment effect on mean shell stiffness (p=0.07) and shell stiffness over time (p=0.06) were detected. As seen in Figure 5.10-18, the effect was non-concentration linear and largely limited to the 1.25 ppm treatment group.

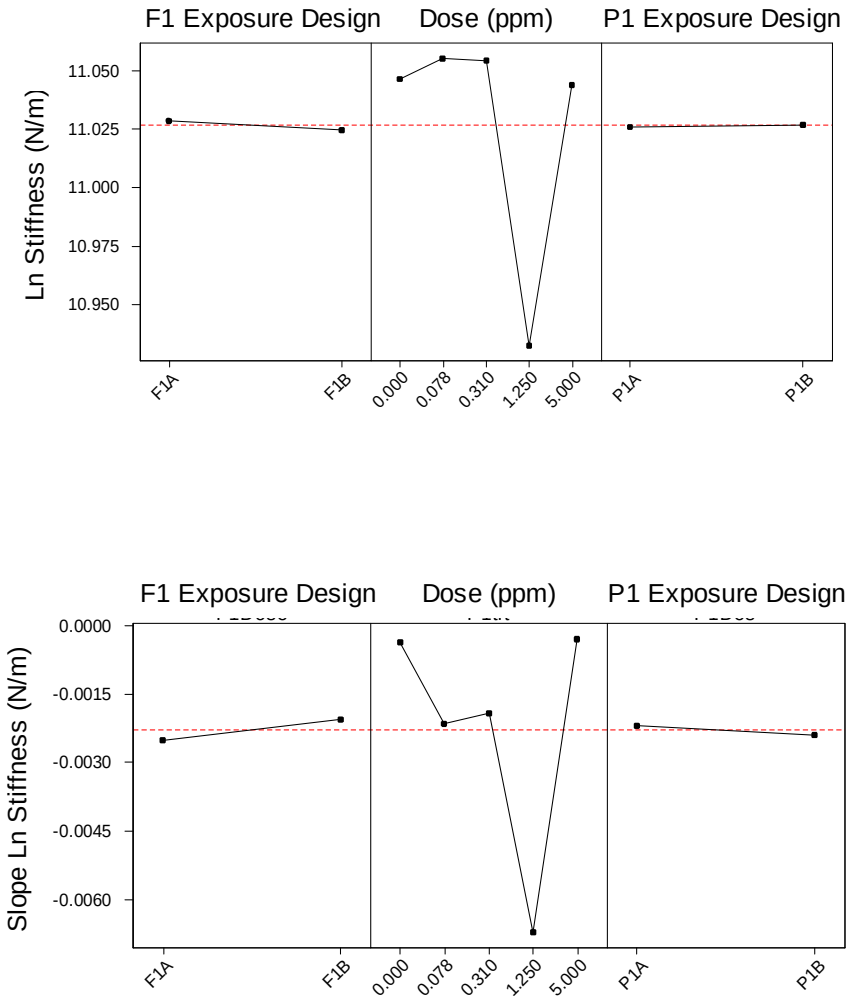


Figure 5.10-18. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on natural log-transformed shell stiffness (N/m, above) and natural log-transformed slope of shell stiffness over time (below). Data do not include extra eggs from unmated hens. Nearly significant parental dietary treatment effect on mean shell stiffness (p=0.07) and shell stiffness over time (p=0.06).

With extra eggs from unmated hens used in the analysis, shell stiffness was significantly affected by parental diet concentration (p=0.042), but the effects were not linear (Figure 5.10-19). No significant differences as a result of dietary concentration or F1 or P1 exposure designs were detected in eggshell stiffness over time (p>0.176).

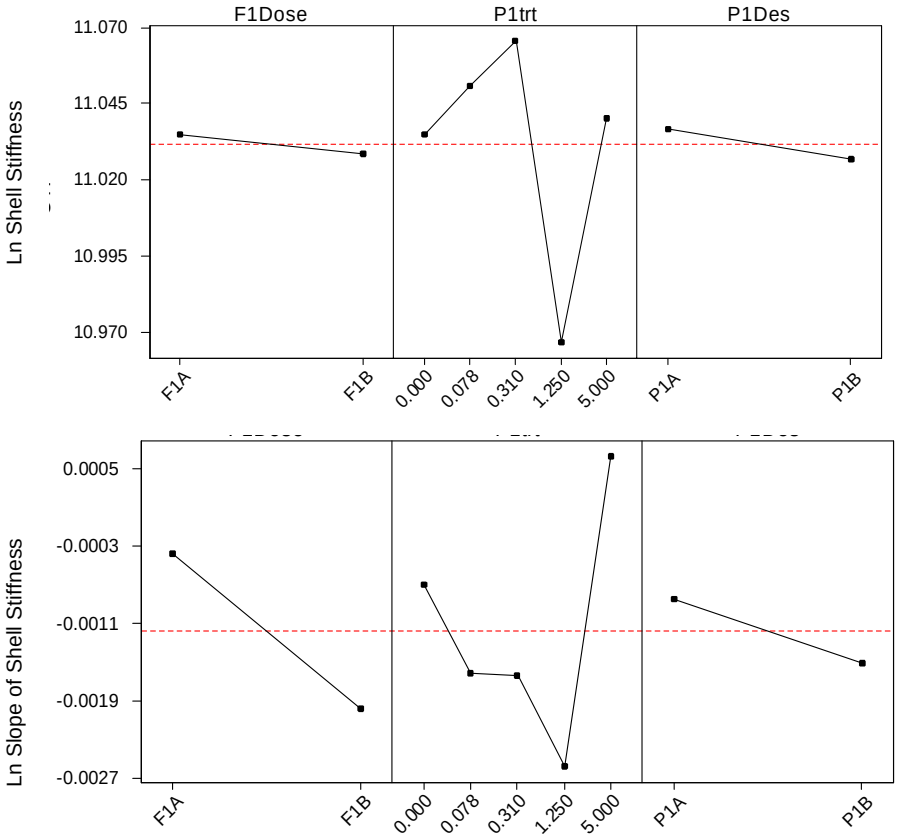


Figure 5.10-19. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on natural log-transformed shell stiffness (N/m, above) and natural log-transformed slope of shell stiffness over time (below). Data include extra eggs from unmated hens. Doses in ppm.

Coefficient of Variation of Shell Quality

The effect of the test variables (F1 exposure strategy, P1 exposure scenario and P1 dietary treatment) on the coefficient of variation (CV) were analyzed by General Linear Model analysis for each of the eggshell quality measures. For shell thickness, there were no significant differences in the CV between F1 or P1 exposure designs or P1 dietary treatments ($p>0.288$). However, variability of breaking strength was significantly affected by F1 exposure strategy ($p=0.04$) and was statistically smaller for eggs laid by F1a birds (Figure 5.10-20). A nearly significant effect ($p=0.07$) of P1 dietary concentration of E2 on CV of breaking strength was also observed, but it was non-linear in effect (Figure 5.10-20). An interaction ($p=0.115$) between F1 and P1 exposure design appeared to increase variability of shell stiffness of eggs laid by

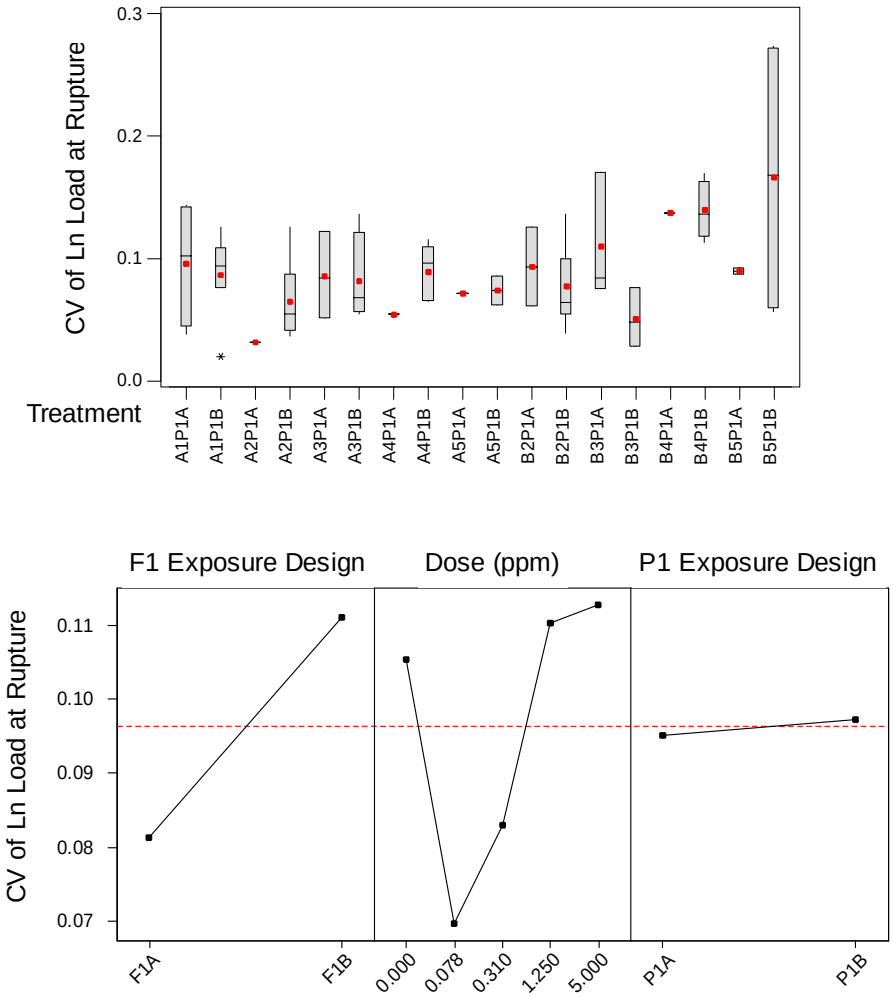


Figure 5.10-20. Box plots of coefficients of variation of the natural log-transformed breaking strength (above) and General Linear Model analysis (below) of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on Coefficient of Variation of the natural log-transformed breaking strength. Significant effect of F1 exposure strategy (p=0.04); nearly significant P1 dietary treatment (p=0.07) effect on the Coefficient of Variation of the breaking strength. Means are indicated by solid circles.

untreated (F1b) offspring of P1B parents. Variability of the F1a shell stiffness of F1a birds was unaffected by the P1A or P1B exposure period of their parents (Figure 5.10-21). There was also a nearly significant effect (p=0.102) of P1 dietary concentration of E2 on the CV of shell stiffness, with greater variability in shell stiffness observed in hens in the higher dietary treatment groups, particularly within the P1B exposure scenario (Figure 5.10-22).

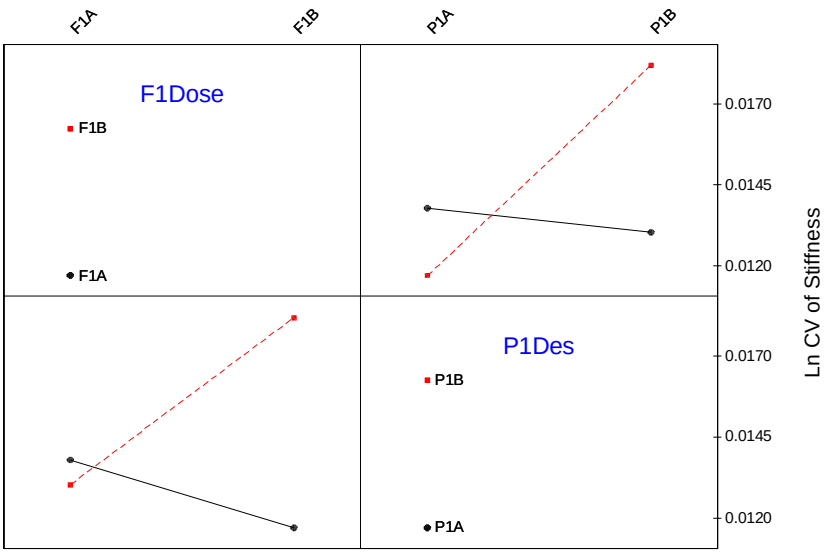


Figure 5.10-21. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.115) Coefficient of Variation of the natural log-transformed shell stiffness of eggs laid by F1 hens. Data do not include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

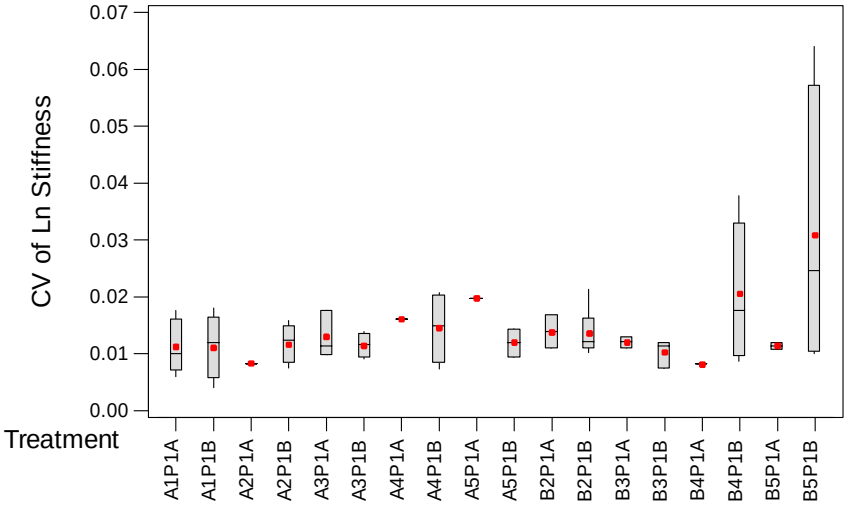


Figure 5.10-22. Box plots of coefficients of variation of the natural log-transformed shell stiffness. Exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group (1, 0 ppm E2; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm). Means are indicated by solid circles. Data do not include extra eggs from unmated hens.

When the extra eggs from unmated hens were considered, a nearly significant interaction of F1 and P1 exposure design on the variability of shell thickness was detected (p=0.095). The

interaction resulted in eggs from treated (F1a) hens of P1B parents having greater variability in shell thickness than those from untreated (F1b) hens. Variability in shell thickness of eggs laid by F1 birds was unaffected by *in ovo* exposures from P1A parents (Figure 5.10-23). A similar interaction (p=0.117) was detected between the F1 and P1 exposure designs resulting in increased variability of the breaking strength in eggs laid by F1b-P1B birds (Figure 5.10-24). For CV of shell stiffness, this interaction was significant (p=0.005) (Figure 5.10-25). The CV of this strength measure also showed a trend of increasing variability with increasing parental dietary concentration of E2 (p=0.093).

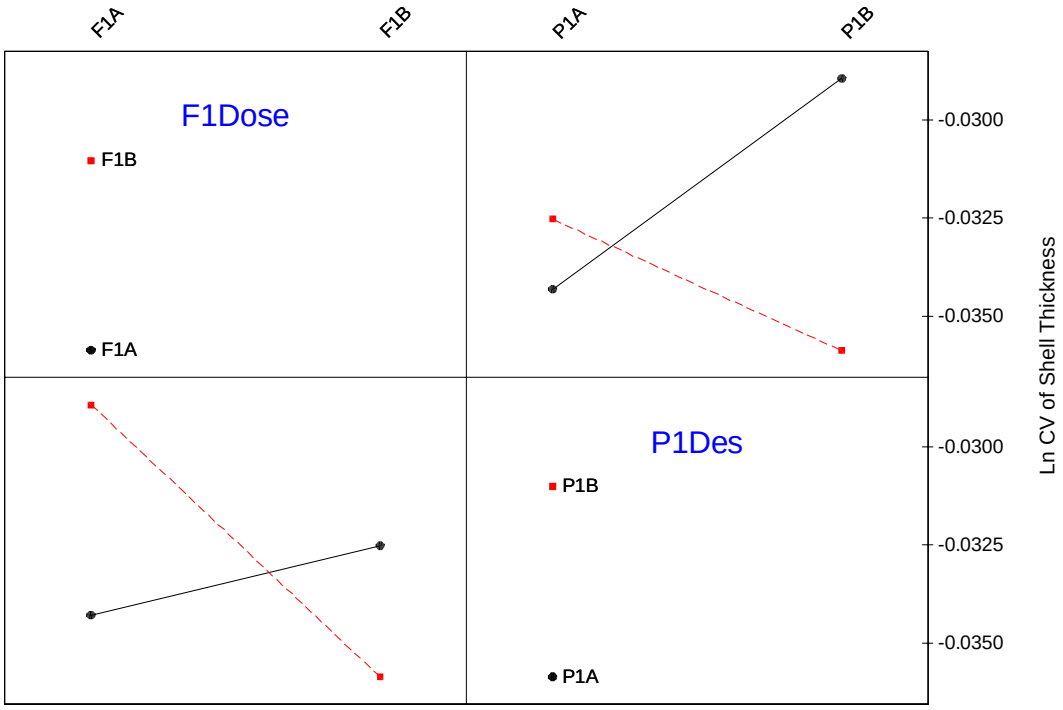


Figure 5.10-23. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.095) Coefficient of Variation of the natural log-transformed shell thickness of eggs laid by F1 hens. Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

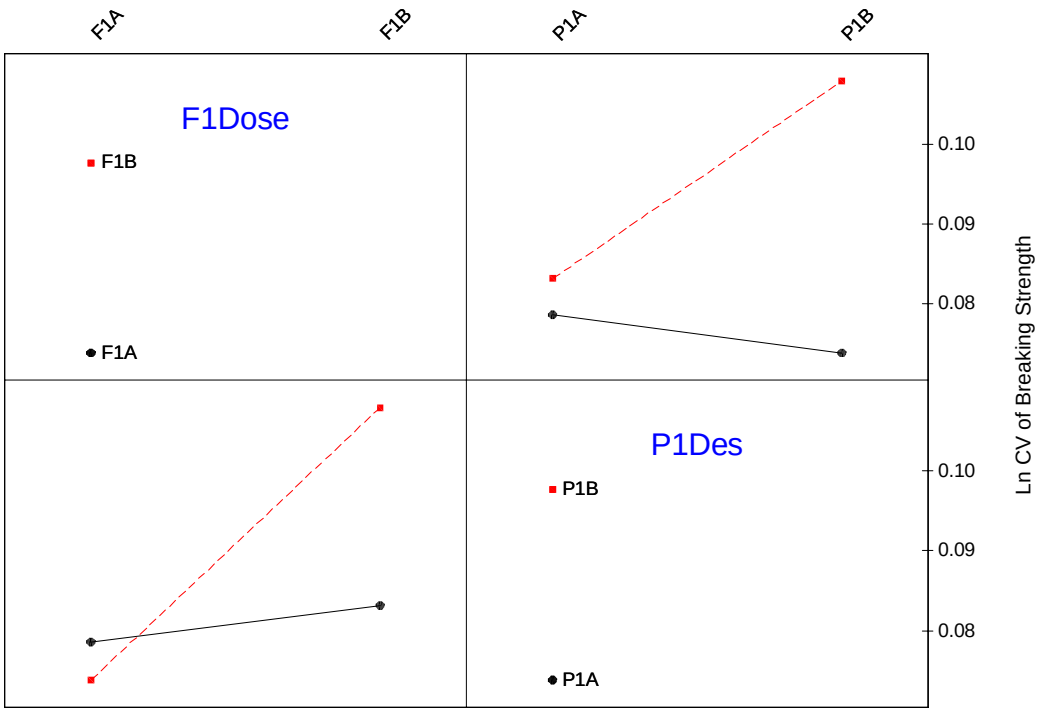


Figure 5.10-24. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.117) Coefficient of Variation of the natural log-transformed load to rupture of eggs laid by F1 hens. Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

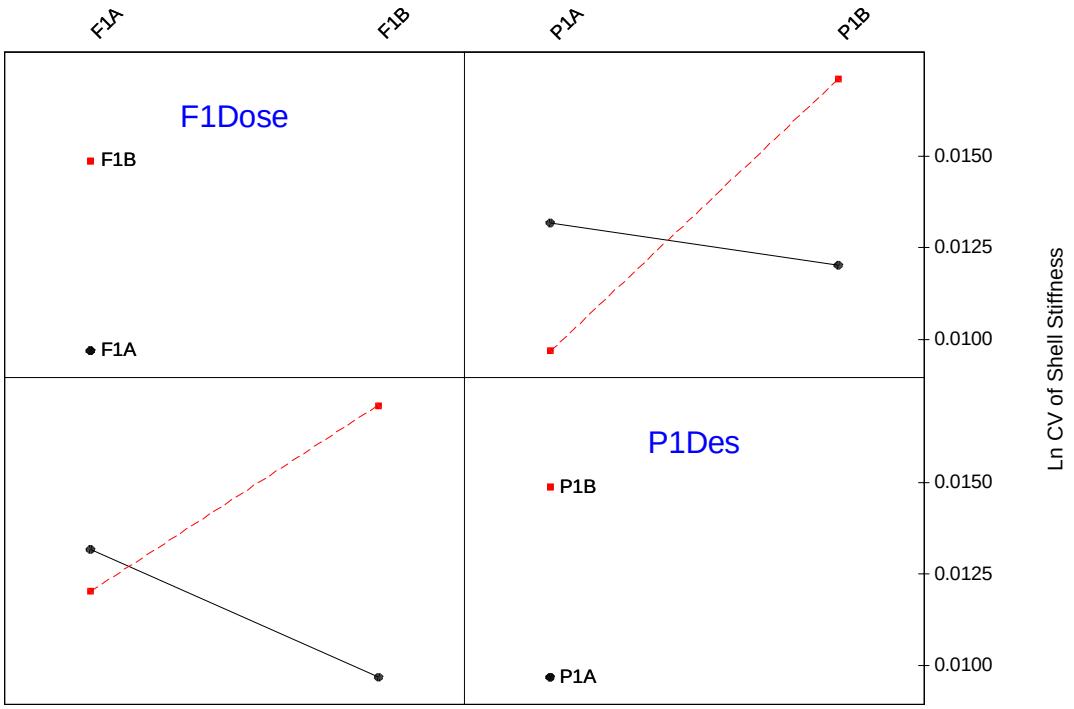


Figure 5.10-25. Interaction between the F1 treatment strategies and the P1 exposure scenarios affecting (p=0.005) Coefficient of Variation of the natural log-transformed shell stiffness of eggs laid by F1 hens. Data include extra eggs from unmated hens. F1a, fed E2 treated diet; F1b, fed untreated diet; P1A, parental population exposed from 3 weeks of age through egg laying; P1B, parental population exposed as proven breeders.

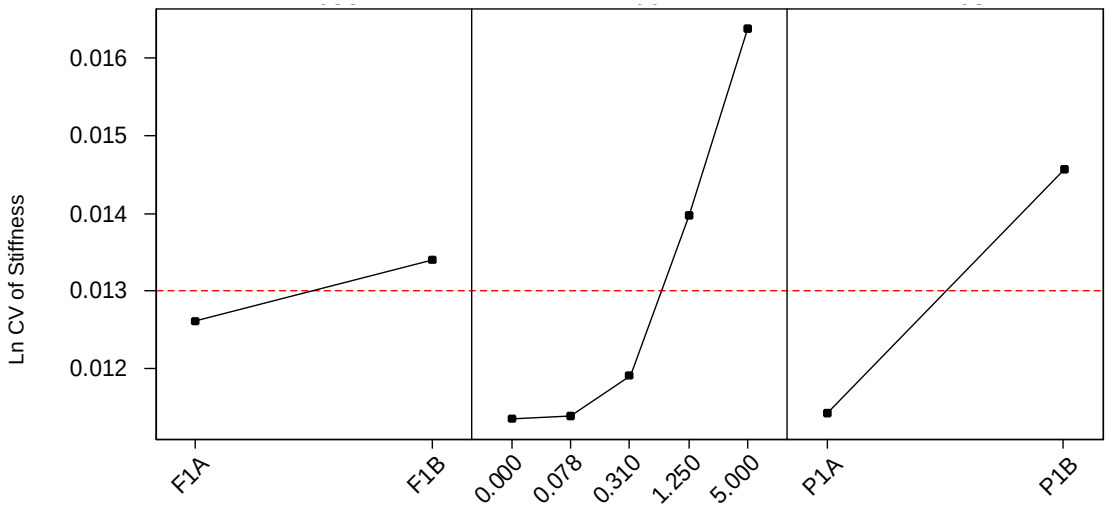


Figure 5.10-26. General Linear Model analysis of the effects of F1 exposure strategy (F1a, treated with parental diet; F1b, untreated), parental exposure scenario (P1A, dietary exposure to E2 from 3 weeks of age; P1B, dietary exposure to E2 after onset of egg laying), and parental dietary treatment group on Coefficient of Variation of the natural log-transformed shell stiffness. Data includes extra eggs from unmated hens. Nearly significant P1 dietary treatment (p=0.093) affecing the Coefficient of Variation of the shell stiffness. Doses in ppm.

5.11 Steroid Content in Eggs Laid by F1 Birds

Measurements were made of both 17ß-estradiol and testosterone in pooled egg yolks.

5.11.1 17ß-Estradiol

No significant differences in egg yolk concentrations of E2 were found between F1 exposure strategies, P1 dosing scenarios, or across parental dietary treatments ($p>0.43$) (Figure 5.11-1). When F1 designs were analyzed separately, mean E2 content of eggs laid by treated quail (F1a) was not significantly different between P1 exposure scenarios ($p=0.80$) or dietary concentration ($p=0.25$), nor were significant differences in E2 concentration due to P1 exposure scenario ($p=0.22$) or dietary concentration ($p=0.68$) detected in F1b eggs. Further, E2 concentrations in eggs laid by F1a-P1A birds were not significantly different across dietary concentrations ($p=0.35$). Similarly, E2 content in F1b-P1B eggs was unchanged by the P1 dietary treatment ($p=0.84$). Tables 5.11-1 and 5.11-2 show results for both estradiol and testosterone.

Table 5.11-1. Estradiol and testosterone in F1a-P1A egg yolks (n=1-4) [a].

Parental	Concentration (ppb)							
Dose (ppm)	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Estradiol								
0	0.32	0.30	0.10	0.24	0.44	0.24	0.44	32%
0.078	0.48	0.48	0.15	0.38	0.59	NC [b]	NC	31%
0.31	0.41	0.41	0.28	0.21	0.60	NC	NC	68%
1.25	0.20	0.20	0.051	0.17	0.24	NC	NC	25%
5	0.49	0.49	0.016	0.48	0.50	NC	NC	3%
Testosterone								
0	7.43	7.43	2.07	5.96	8.89	NC	NC	28%
0.078	9.07	9.07	0.25	8.89	9.24	NC	NC	3%
0.31	9.89	9.89	8.41	3.95	15.8	NC	NC	85%
1.25	3.67	3.67	2.61	1.82	5.52	NC	NC	71%
5	6.45	6.45	2.16	4.92	7.98	NC	NC	33%

[a] One to two composite samples per dietary concentration;1 or 4 eggs per composite.
[b] NC Not calculable because n is too small (n=2 or 3).

Table 5.11-2. Estradiol and testosterone in F1b-P1B egg yolks (n=1-4)[a].

Parental	Concentration (ppb)							
Dose (ppm)	Mean	Median	StDev	Minimum	Maximum	Q1	Q3	CV
Estradiol								
0	0.39	0.39	0.13	0.30	0.48	NC[b]	NC	32%
0.078	0.34	0.34	0.003	0.34	0.34	NC	NC	1%
0.31	0.46	0.46	0.17	0.34	0.58	NC	NC	36%
1.25	0.29	0.29	0.079	0.24	0.35	NC	NC	27%
5	0.37	0.37	0.16	0.25	0.48	NC	NC	45%
Testosterone								
0	6.45	6.45	0.34	6.21	6.69	NC	NC	5%
0.078	8.04	8.04	0.99	7.33	8.74	NC	NC	12%
0.31	7.48	7.48	4.49	4.31	10.7	NC	NC	60%
1.25	7.17	7.17	5.41	3.35	11.0	NC	NC	75%
5	5.68	5.68	0.44	5.37	5.99	NC	NC	8%

[a] One to two composite samples per dietary concentration;1 or 4 eggs per composite.
[b] NC Not calculable because n is too small (n=2 or 3).

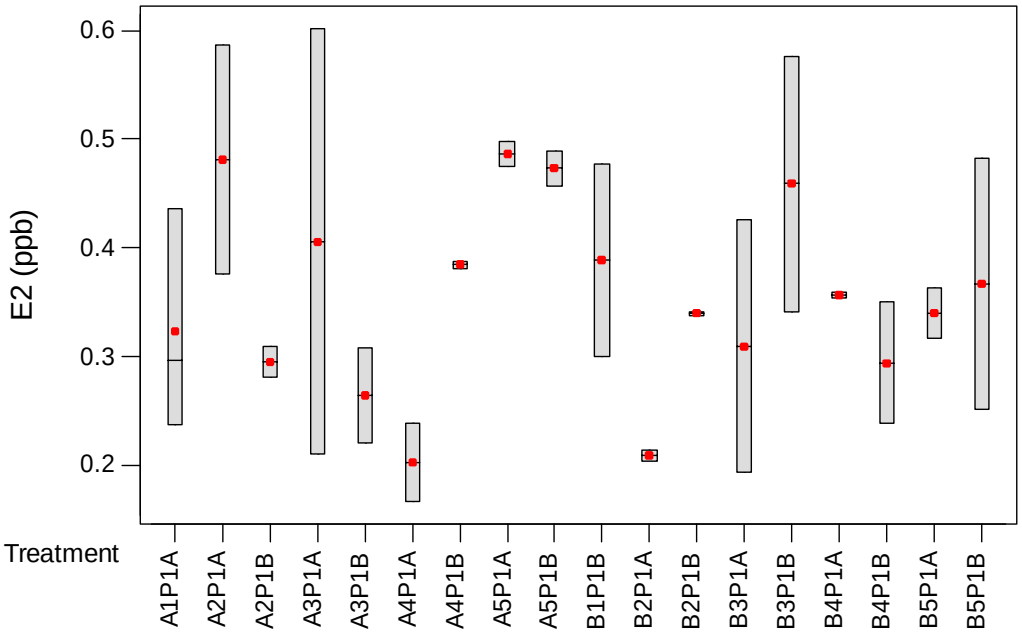


Figure 5.11-1. Box plots of estradiol (E2) concentrations (ppb in yolk) in eggs laid by birds exposed to E2 in diet under two F1 exposure strategies (F1a, treated diet; F1b, untreated diet) and two P1 exposure scenarios (P1A, exposure from pre-maturation, P1B exposure after onset of egg laying). Dietary treatments of E2 were 1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, and 5, 5 ppm. Means are indicated by solid circles.

5.11.2 Testosterone

As observed for E2, no significant differences in egg concentrations of this steroid were detected between F1 exposure strategies, P1 dosing scenarios or across parental dietary treatments ($p>0.68$) (Figure 5.11-2). No effects were found when the F1a and F1b eggs were analyzed separately ($p>0.46$), nor were there significant differences in testosterone content in eggs laid by the F1a-F1A ($p=0.40$) or F1b-P1B birds ($p=0.78$) (Tables 5.11-1 and 5.11-2).

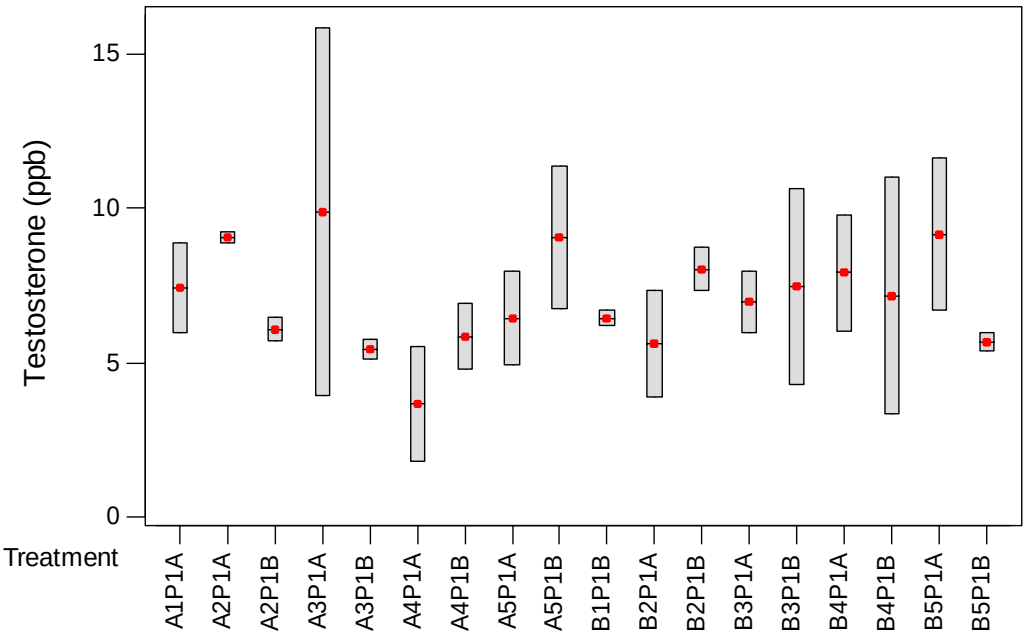


Figure 5.11-2. **Box plots of testosterone concentrations (ppb in yolk) in eggs laid by birds exposed to E2 in diet under two F1 exposure strategies (F1a, treated diet; F1b, untreated diet) and two P1 exposure scenarios (P1A, exposure from pre-maturation, P1B exposure after onset of egg laying).** Dietary treatments of E2 were 1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, and 5, 5 ppm. Means are indicated by solid circles.

5.12 Summary of the Results of the First Generation Offspring (F1)

Hatch and adult body weights were lower in female quail that were exposed to *in ovo* doses of E2 from parents under the P1A regime. Direct exposure to the treated diets (F1a) did not affect body weight in F1 hens, but did reduce tibiotarsus diameter. Significant increases in thyroid gland weights and nearly significant increases in spleen weights as a result of F1a exposure were observed in the females. Thyroid weights also responded to the P1B exposure history of their parents. In contrast, the pancreas and ovary weights tended to be lower in F1a hens. The number of active oocytes were increased in the F1a-P1A birds. Increased oviduct weight was found in birds with the greatest combined exposure (F1a-P1A). F1a females also matured later and had reduced lengths of female-type plumage. Day 15 viability of eggs and hatchling production over time were increased in F1a birds. An increase in the proportion of cracked shells was associated with P1B parental exposure. Eggshell thickness increased in eggs laid by F1a hens and the production of cracked shells decreased over time in this E2 treated population. Few of these growth or organ weight changes associated with a concentration-linear response. Generally, the variability in shell quality was greater in eggs laid by hens with P1B parents. Histologically, there were few changes in F1 female tissues. Increased mineralization of hepatic tissue was found in all treatment combinations except F1a-P1A. Diffuse hypertrophy of adrenal gland tissue was decreased in birds without the dietary exposure from hatch (F1b).

In F1 males, the interaction of F1a and P1A (*in ovo*) exposures resulted in increased terminal body weight, tibiotarsus weight, and pancreas, liver, and testis weights or weight indices. Mean cloacal gland weight tended to be smaller in males fed the high concentration E2 diet. When normalized to body weight the cloacal gland weight was smaller in F1a-P1A offspring that consumed the 5 ppm diet. However, the cloacal gland area was greater in F1a-P1A males. More incidences of dilatation of lumens of the submucosal glands of the cloacal gland occurred in birds with P1B *in ovo* exposure history, but the incidence was not concentration-linear. Diffuse hypertrophy was found in adrenal glands of untreated (F1b) males fed 5 ppm E2 and was absent in controls and F1a-5 ppm treated males.

Tables 5.12-1 (females) and 5.12-2 (males) summarize the results of the endpoint measurements obtained for the first generation offspring (F1).

Table 5.12-1. Summary of results for F1 females.

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Hatchling Body Weight	None	P1A lower weight	Not linear[a]
Terminal Adult Body Weight	None	P1A lower weight	Not linear
Growth Rate	F1a slower, 1st and 3rd segments[a]	None	None
Tibiotarsus			
Length	None	None	Not linear[a]
Diameter	F1a diameter reduced	None	None
Weight	Interaction: F1b-P1A reduced[a]	None	Not linear
Tarsometatarsus Length	None	None	None
Aggression			
Feather Loss	None	P1B greater[a]	None
Pecking Injury	F1b greater (but incidence low , unrelated to E2 concentration)[a]	P1B greater (but total incidence low, unrelated to E2 concentration)	None
Organ Weight			
Thyroid-Gross	F1a greater	P1B increased	None
Thyroid/Body Weight	F1a greater	None	None
Thyroid/Brain Weight	F1a greater[a]	P1B increased[a]	None
Adrenal Gland-Gross	None	None	None
Adrenal Gland/Body Weight	None	None	None
Adrenal Gland/Brain Weight	None	None	None
Pancreas-Gross	F1a lower	None	Not linear
Pancreas/Body Weight	Interaction:F1b-P1A increased[a]	None	Not linear
Pancreas/Brain Weight	F1a lower	None	Not linear[a]
Liver-Gross	None	None	None
Liver/Body Weight	None	None	Not linear[a]
Liver/Brain Weight	None	None	None

Table 5.12-1. Summary of results for F1 females (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Spleen-Gross	F1a greater[a]	None	Not linear[a]
Spleen/Body Weight	F1a greater[a]	None	Not linear[a]
Spleen/Brain Weight	Interaction F1a-P1A index increased[a]	None	Not linear[a]
Brain-Gross	None	None	None
Brain/Body Weight	None	P1A greater[a]	None
Ovary-Gross	F1a lower[a]	P1A lower[a]	None
Ovary/Body Weight	F1a lower	None	None
Ovary/Brain Weight	F1a lower[a]	None	None
Oviduct-Gross	Interaction-F1a-P1A greater	P1A greater[a]	None
Oviduct/Body Weight	Interaction-F1a-P1A greater	None	None
Oviduct/Brain Weight	Interaction F1a-P1A greater[a]	None	None
Number of Active Oocytes	Interaction F1a-P1A increased[a]	None	None
Gross Abnormalities			
Incidence of Rt. Ovary	None	None	None
Incidence of Rt. Oviduct	None	None	None
Neck Curvature	None	P1B greater incidence[a]	None
Foot/Leg	None	None	None
Sexual Maturation			
without extra hens	None	None	Not linear
with extra hens	F1a mature later[a]	None	Not linear
Plumage Dimorphism			
Female Phenotype	None	None	None
Length of Spotted Area	F1a less	None	Not linear[a]

Table 5.12-1. Summary of results for F1 females (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Reproductive Parameters			
Total Eggs	None	None	None
without extra hens	None	None	None
with extra hens	None	None	None
Total Eggs/Max	None	None	None
without extra hens	None	None	Not linear
with extra hens	None	None	None
Eggs Viable on Day 8	None	None	None
production over time	None	None	None
Eggs Viable on Day 15	None	None	None
production over time	F1a increased[a]	None	None
Hatchlings/Eggs Set	None	None	None
Hatchlings/Viable Day 8	None	None	None
Hatchlings/Max Eggs Set	None	None	Not linear
Hatchling Prod.OverTime	F1a increased		
Shell Quality			
Proportion Cracked Eggs			
without extra hens	None	P1B greater proportion cracked	Not linear
with extra hens	Interaction-F1b-P1B increased	None	Not linear
Prod. Cracked Over Time			
without extra hens	None	None	None
with extra hens	F1a decreased	None	None
Shell Thickness			
without extra hens	F1a greater[a]	None	Not linear[a]
with extra hens	None	None	Not linear [a]
Shell Thickness Over Time			
without extra hens	None	None	Concentration linear[a]
with extra hens	Interaction-F1a-P1A increased	None	None

Table 5.12-1. Summary of results for F1 females (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Breaking Strength			
without extra hens	None	None	Not linear
with extra hens	None	None	None
Breaking Strength Over Time			
without extra hens	None	P1B increased[a]	None
with extra hens	Interaction F1a-P1A greater[a]	None	None
Shell Stiffness			
without extra hens	None	None	Not linear [a]
with extra hens	None	None	Not linear
Shell Stiffness Over Time			
without extra hens	None	None	Not linear[a]
with extra hens	None	None	None
Coefficient of Variation			
Shell Thickness			
without extra hens	None	None	None
with extra hens	Interaction F1a-P1B greater[a]	None	None
Breaking Strength			
without extra hens	F1a less variable	None	Not linear[a]
with extra hens	Interaction F1b-P1B greater[a]	None	None
Shell Stiffness Over Time			
without extra hens	Interaction F1b-P1B greater[a]	None	Concentration linear[a]
with extra hens	Interaction F1b-P1B greater	None	Concentration linear[a]
Egg Steroid Content			
Estrogen	None	None	None
Testosterone	None	None	None

Note a. $p \leq 0.15$

Table 5.12-2. Summary of results for F1 males.

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Hatchling Body Weight	None	None	None
Terminal Adult Body Weight	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1[a]	None	None
Growth Rate	None	None	Not linear-days 34-51
Tibiotarsus			
Length	None	None	None
Diameter	None	P1A greater[a]	None
Weight	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1[a]	None	None
Tarsometatarsus Length	F1a greater (difference is small)[a]	None	None
Aggression			
Feather Loss	None	None	Not linear[a]
Pecking Injury	None	None	None
Organ Weight			
Thyroid-Gross	None	None	Not linear
Thyroid/Body Weight	None	None	Not linear
Thyroid/Brain Weight	None	None	Not linear
Adrenal Gland-Gross	None	None	None
Adrenal Gland/Body Weight	None	None	None
Adrenal Gland/Brain Weight	None	None	None
Pancreas-Gross	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1	None	None
Pancreas/Body Weight	Interaction: F1a-P1A > F1a-P1B[a]	None	Interaction of F1a-P1B and 5 ppm diet[a]
Pancreas/Brain Weight	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1	None	None
Liver-Gross	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1	None	None
Liver/Body Weight	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1[a]	None	None
Liver/Brain Weight	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1	None	None

Table 5.12-2. Summary of results for F1 males (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Spleen-Gross	F1a smaller[a]	None	Not linear[a]
Spleen/Body Weight	F1a smaller[a]	P1A greater[a]	Not linear[a]
Spleen/Brain Weight	F1a smaller[a]	None	Not linear[a]
Brain-Gross	None	None	None
Brain/Body Weight	Interaction F1a-P1A < F1a-P1B[a]; F1b not affected by P1	None	None
LeftTestis-Gross	Interaction F1a-P1A > F1a-P1B[a]; F1b not affected by P1	None	None
Left Testis/Body Weight	None	None	None
LeftTestis/Brain Weight	Interaction F1a-P1A > F1a-P1B[a]; F1b not affected by P1	None	None
Right Testis-Gross	None	None	None
Right Testis/Body Weight	None	P1A smaller[a]	Not linear[a]
Right Testis/Brain Weight	None	None	None
Testes Asymmetry	Interaction F1a-P1A > F1a-P1B[a]; F1b not affected by P1	None	None
Cloacal Gland-Gross	F1a smaller[a]	None	5 ppm smaller than controls[a]
Cloacal Gland/Body Weight	Interaction F1a-P1A < F1b-P1A;smaller in offspring that consumed 5 ppm diet[a]	None	None
Cloacal Gland/Brain Weight	None	None	None
Gross Abnormalities-Organ Lesions, Foot/Leg, Neck	None	None	None
Sexual Maturation-Day to First Foam	Interaction- F1a-P1A sooner than F1b-P1A (~2 days); F1b-P1B sooner than F1b-P1A (~5 days)	None	Not linear

Table 5.12-2. Summary of results for F1 males (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Plumage Dimorphism			
Incidence of Non-Male Phenotype	F1a greater	None	Increase
Length of Spotted Area	F1a greater	None	Increase
Cloacal Gland Size	Interaction F1a-P1A > F1a-P1B; F1b not affected by P1	None	None

Note a. $p \leq 0.15$

6.0 SECOND GENERATION OFFSPRING (F2) RESULTS

6.1 Body Weight, Tibiotarsus Growth, and Tarsometatarsus Growth of Hatchlings (F2)

Graphics shown below contain body weights of all chicks hatched, including those that died prior to 14 days of age; reported statistical values exclude extreme values.

Females

The hatch weight of females of the F2 generation was not affected by parental exposure design (F1a, treated vs. F1b, untreated), or the exposure scenario of the parents of the F1 generation (P1A or P1B) ($p \geq 0.25$). However, there was a highly significant ($p < 0.001$), but nonlinear effect of dietary concentration of the P1 generation on the F2 female hatch weights (Figure 6.1-1). Figure 6.1-2 shows the distribution of hatch weights of the F2 generation by dietary treatment within F1 and P1 exposure designs.

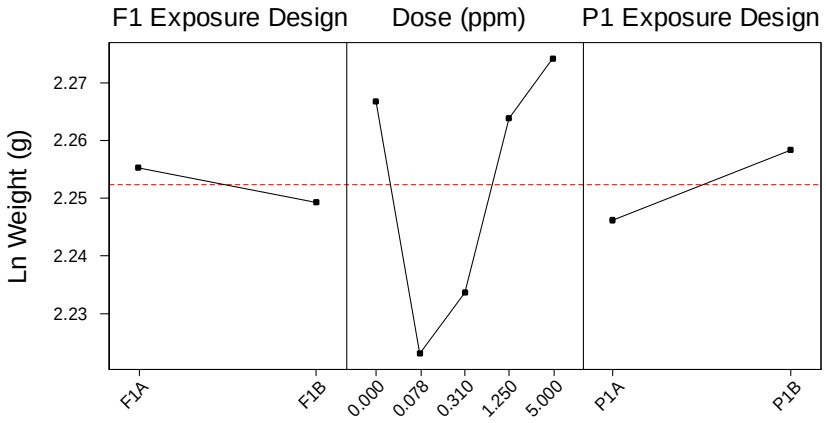


Figure 6.1-1. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed hatch weight of F2 females. General Linear Model analysis; highly significant differences between P1 dietary concentrations, $p < 0.001$. F1a birds were fed the same dietary treatments as their parents; all F2 chicks were untreated.

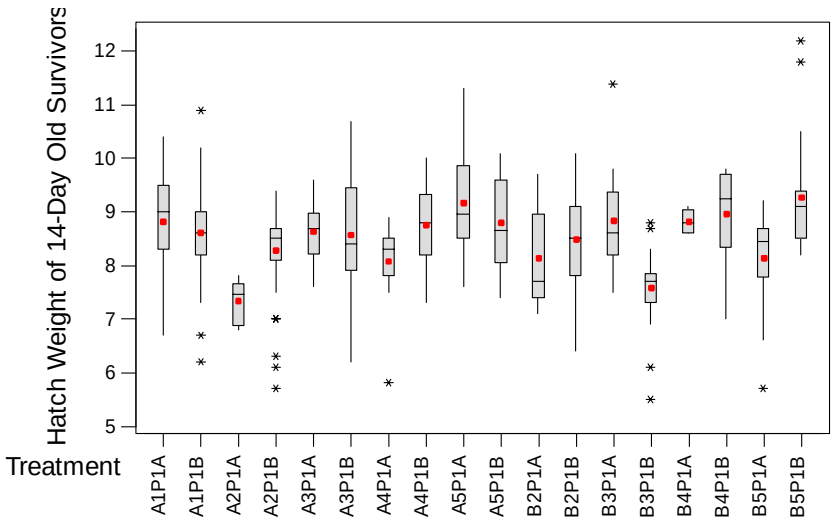


Figure 6.1-2. Box plots of the hatch weight (g) of female F2 chicks by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). (* = extreme value).

By 2 weeks of age the differences in body weight due to P1 dietary treatment seen at hatch were no longer significant (p=0.30) (Figure 6.1-3). No significant effects of F1 or P1 exposure design on 14-day old body weight were detected (p>0.31).

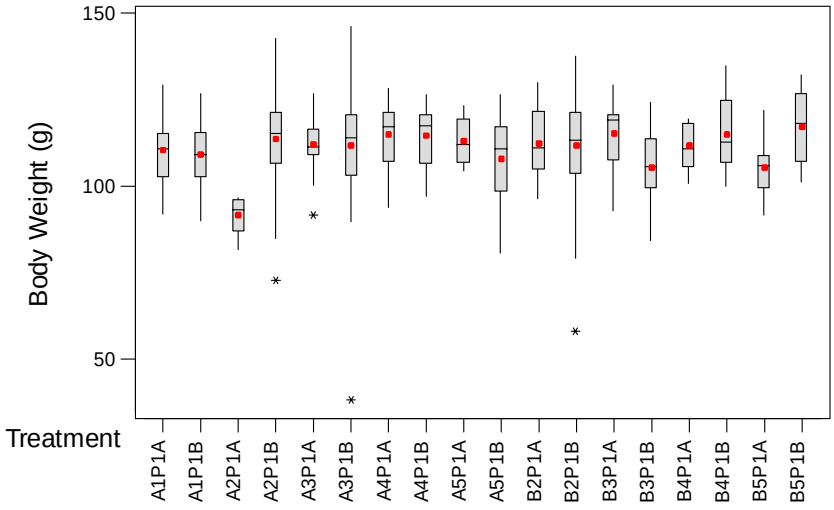


Figure 6.1-3. Box plots of the body weight of 2 week old female F2 chicks by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated).

Males

Significant differences between the effects of F1 exposure design (p=0.04) and P1 dietary treatment with E2 (p<0.02) were found in male F2 chick body weights at hatch (Figure 6.1-4). F2 hatchlings from F1a parents had lower body weights than hatchlings from F1b parents, but the difference between the overall means was small (about 2%). Diet concentration effects were non-dose linear. The effect of P1 exposure scenario on hatch weight was not significant (p=0.41).

At 2 weeks of age, the F1 design effect detected in the hatchlings was reduced (p=0.09), but the dietary treatment effect was still significant (p=0.02). P1 exposure scenario had no effect (p=0.76) on the F2 chick weights. The distributions of body weights of male F2 chicks at hatch and 2 weeks old by P1 dietary treatment within the F1 and P1 exposure scenarios are shown in Figure 6.1-5.

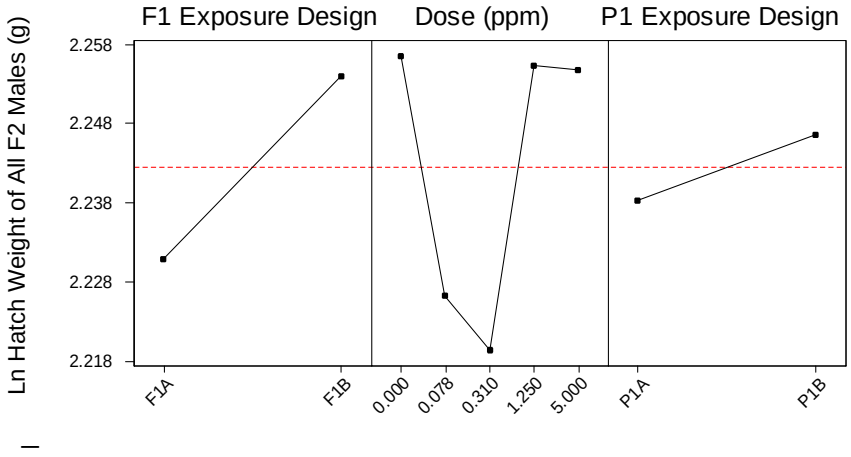


Figure 6.1-4. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on natural log-transformed hatch weight of F2 males. General Linear Model analysis; significant differences between F1 exposure designs (p=0.04) and P1 dietary concentrations (p<0.02). F1a birds were fed the same dietary treatments as their parents; all F2 chicks were untreated.

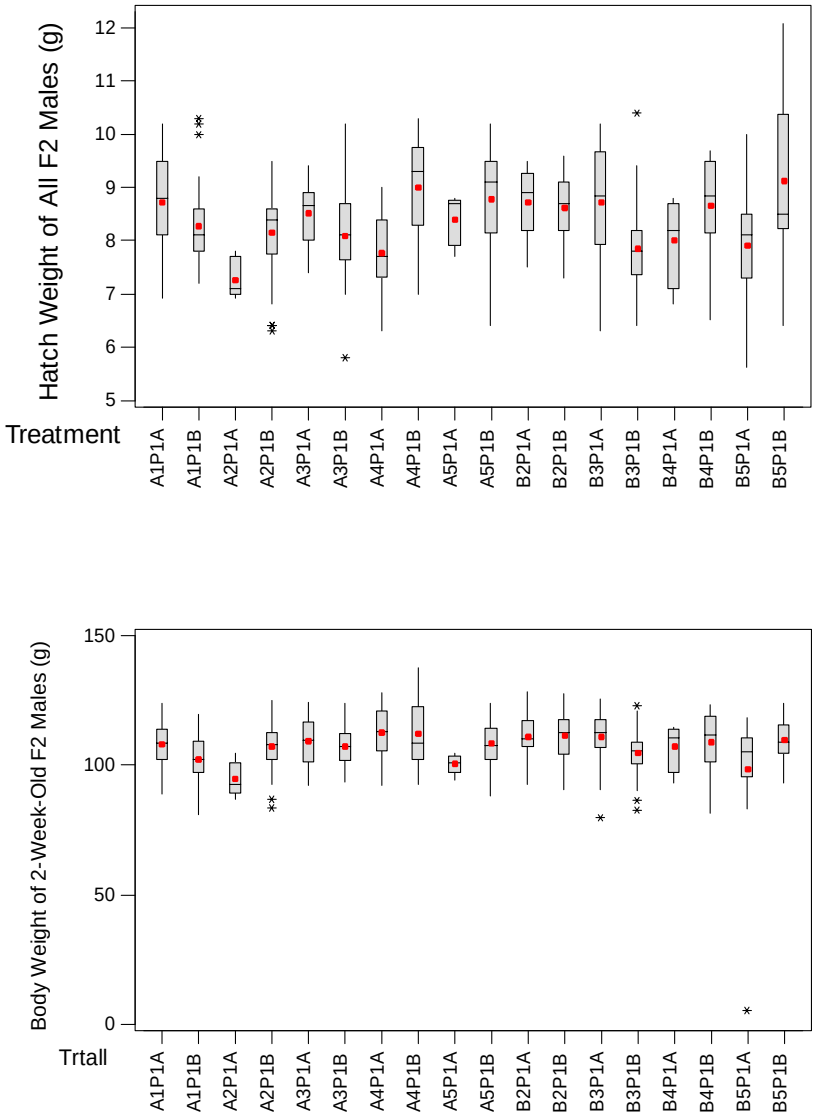


Figure 6.1-5. Box plots of male hatchling body weights in g (above) and body weights of 2 week old male F2 chicks (below) by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

Females

Tibiotarsus

Growth of the tibiotarsus in female F2 chicks was not affected by the exposure regime (F1a or F1b) of their parents (p=0.291), but tended to be affected by the P1 exposure scenario (p=0.083) and P1 dietary treatment (p=0.093) of their parents (Figure 6.1-6). Overall mean tibiotarsus length in chicks with P1A parentage was slightly shorter than the mean bone length of chicks with P1B parentage. P1 dietary concentration effects were non-concentration linear (Figure 6.1-6).

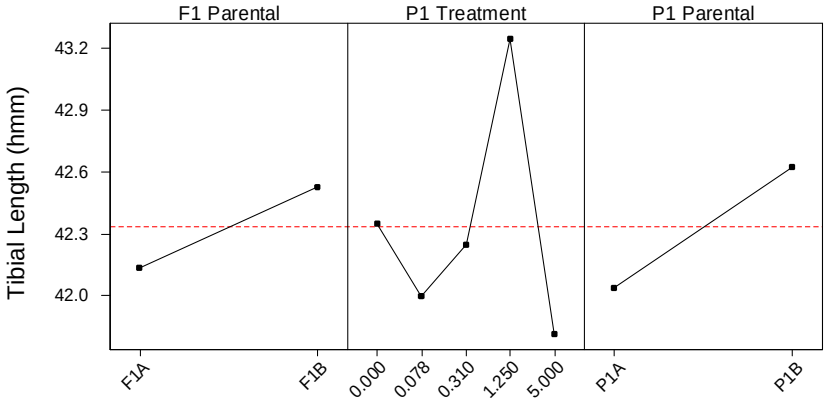


Figure 6.1-6. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on length of the tibiotarsus (mm) of F2 female chicks. General Linear Model Analysis, P1 exposure design and P1 dietary treatment effects, p=0.083 and p=0.093, respectively. Doses in ppm.

Tibiotarsus Diameter

The diameter of the tibiotarsus of F2 females was slightly, though significantly (p=0.048), affected by P1 dietary concentration, but effects were not linear (Figure 6.1-7). There was also a nearly significant interaction between the F1 and P1 exposure designs wherein F2 chicks of F1a-P1A parents had tibiotarsi diameters that were slightly smaller than those of the offspring of F1b-P1A parents (p=0.107). Mean tibiotarsus diameter of the F2 female chicks was unaffected by P1B parental exposure history (Figure 6.1-8)

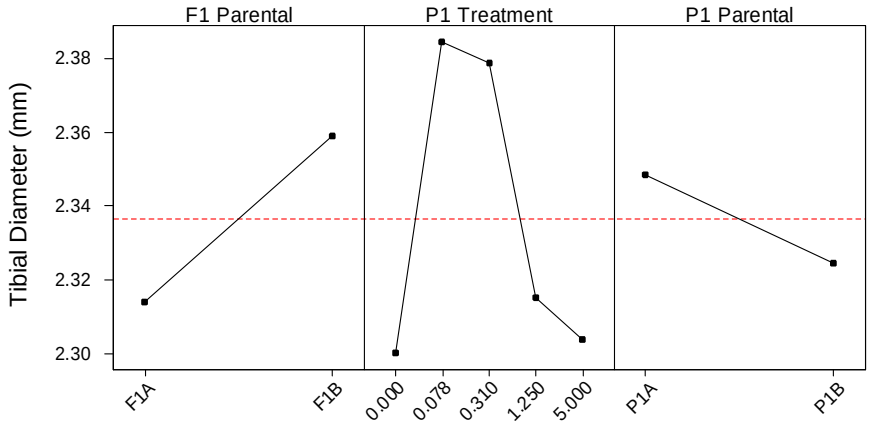


Figure 6.1-7. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the diameter (mm) of the tibiotarsus of F2 female chicks. General Linear Model Analysis, significant P1 dietary concentration effect (p=0.048). Doses in ppm.

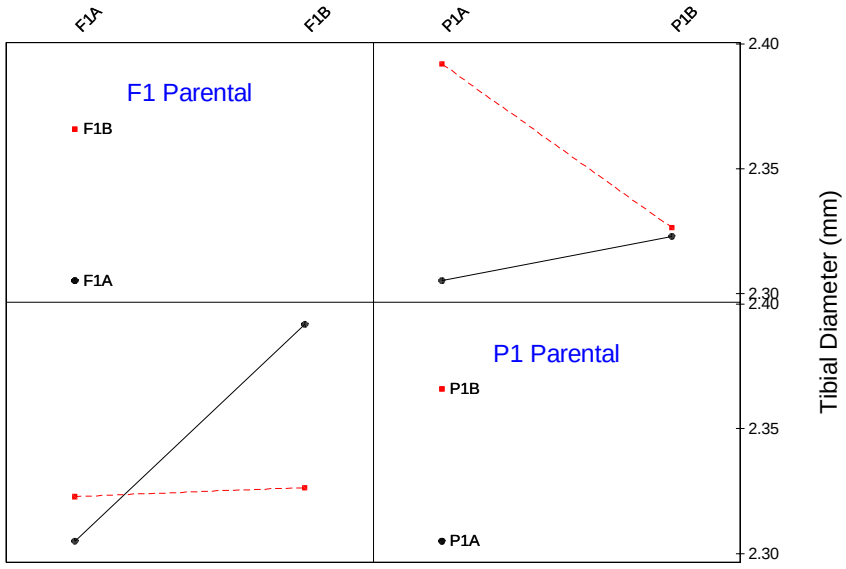


Figure 6.1-8. Interaction between F1 and P1 exposure designs affecting the tibiotarsus diameter (mm) of F2 female chicks. General Linear Model Analysis (p=0.107). F1a, treated; F1b, untreated; P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation.

Weight of the Tibiotarsus

A significant effect of P1 dietary treatment (p=0.030) on tibiotarsus weight was observed in the F2 female chicks. The effects were non-concentration linear (Figure 6.1-9). The F1 and P1 exposure designs under which the parents of the F2 chicks were exposed had no effect on their offspring (p>0.315)

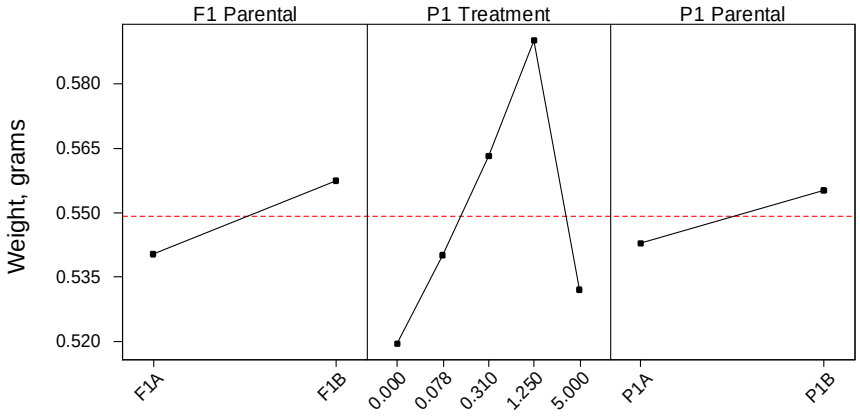


Figure 6.1-9. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the weight (g) of the tibiotarsus of F2 female chicks. General Linear Model Analysis, significant P1 dietary concentration effect (p=0.030). Doses in ppm.

Tarsometatarsus

An interaction between the F1 and P1 exposure designs of the parents of the F2 female chicks tended (p=0.094) to affect the tarsometatarsus length of the chicks. Chicks with F1a-P1A parents tended to have tarsometatarsi shorter in length than female chicks of F1b-P1A parents. Parental P1B exposure history did not appear to affect the length of the bone in the chicks (Figure 6.1-10).

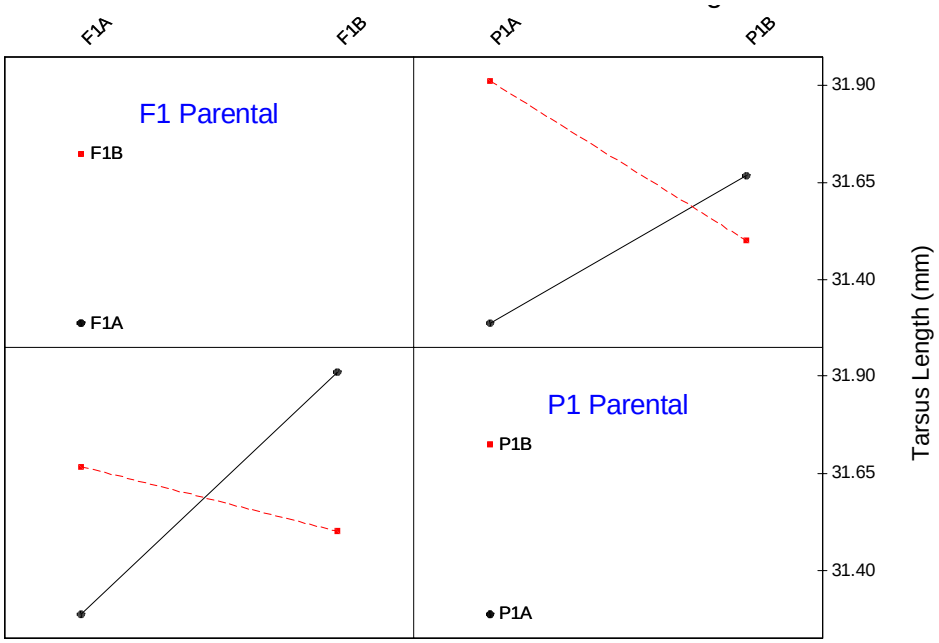


Figure 6.1-10. Interaction between F1 and P1 exposure designs affecting the tarsometatarsus length (mm) of F2 female chicks. General Linear Model Analysis (p=0.094). F1a, treated; F1b, untreated; P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation.

Males

Tibiotarsus

Significant non-linear differences in tibiotarsus length (p=0.013) and weight (p=0.002) across P1 dietary concentrations were detected in male F2 chicks (Figure 6.1-11). The F1 exposure strategy of the parents of the F2 males nearly significantly affected tibiotarsus length (p=0.102) and weight (p=0.102) (Figure 6.1-11). For both parameters, the tibiotarsus was reduced in chicks with F1a parents. The P1 exposure scenario history of their parents had no effect on the bone length or weight of the F2 chicks.

No differences in mean tibiotarsus diameter in response to F1 or P1 exposure design or P1 dietary concentration were found in F2 males (p>0.387).

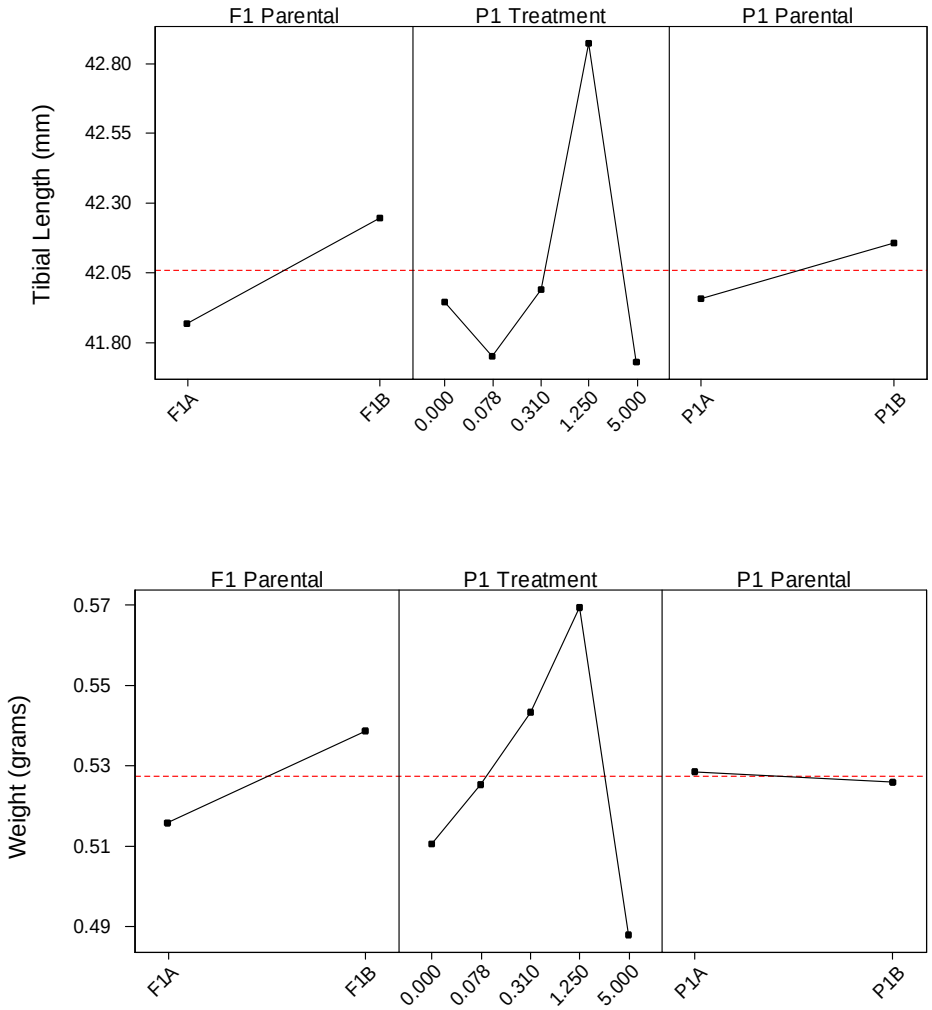


Figure 6.1-11. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the length in mm (above) and weight in grams (below) of the tibiotarsus of F2 male chicks. General Linear Model Analysis, significant P1 dietary concentration effects [p=0.013 (length) and p=0.002 (weight)]. Doses in ppm.

Tarsometatarsus

Mean tarsometatarsus lengths tended (p=0.121) to be smaller in male F2 chicks with F1a parents than in chicks with F1b parents (Figure 6.1-12). No significant effects of P1 dietary concentrations or P1 exposure design were detected (p>0.347)

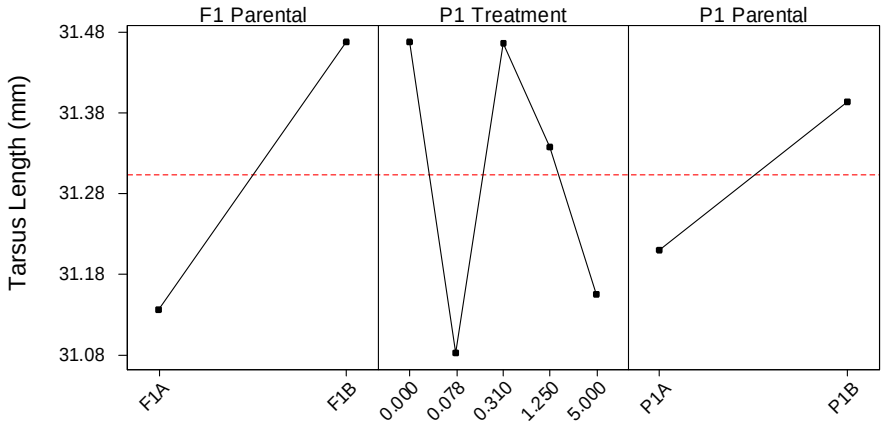


Figure 6.1-12. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the length (mm) of the tarsometatarsus of F2 male chicks. General Linear Model Analysis, nearly significant F1 effects (p=0.121). Doses in ppm.

6.2 14-Day Old Survivors

6.2.1 14-Day Old Survivors per Normal Hatchling (F2)

The number of 14-day old survivors divided by the number of normal hatchlings by hen was not affected by their parents' exposure design (F1a, treated vs. F1b, untreated), or the exposure scenario of the parents of the F1 generations (P1A or P1B) ($p>0.16$). However, there was a significant ($p<0.04$), but nonlinear effect of dietary concentration of the P1 generation on the number of 14 day old survivors of normal hatchlings (Figure 6.2-1). Figure 6.2-2 shows the distribution of 14 day old survivors of normal hatchlings by dietary treatment within F1 and P1 exposure designs.

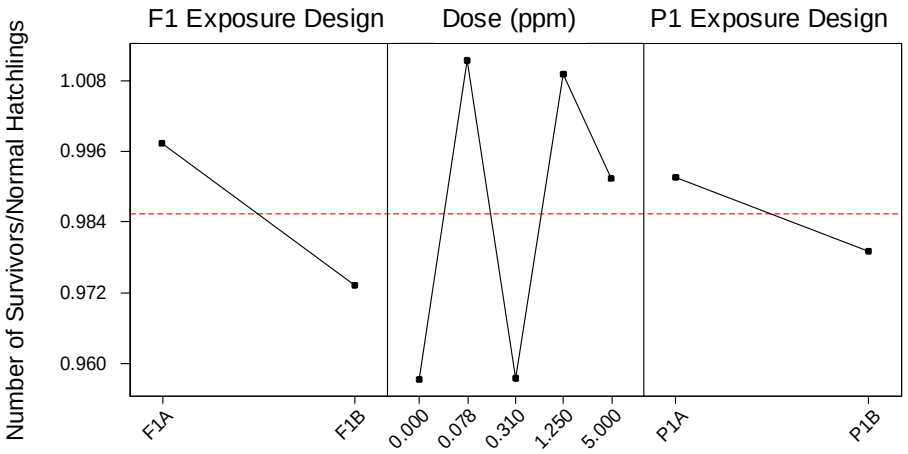


Figure 6.2-1. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on the number of 2 week old survivors out of the normal hatchlings per hen (General Linear Model analysis; significant differences between P1 dietary concentrations, $p<0.04$). F1a birds were fed the same dietary treatments as their parents; all F1b birds and F2 chicks were untreated.

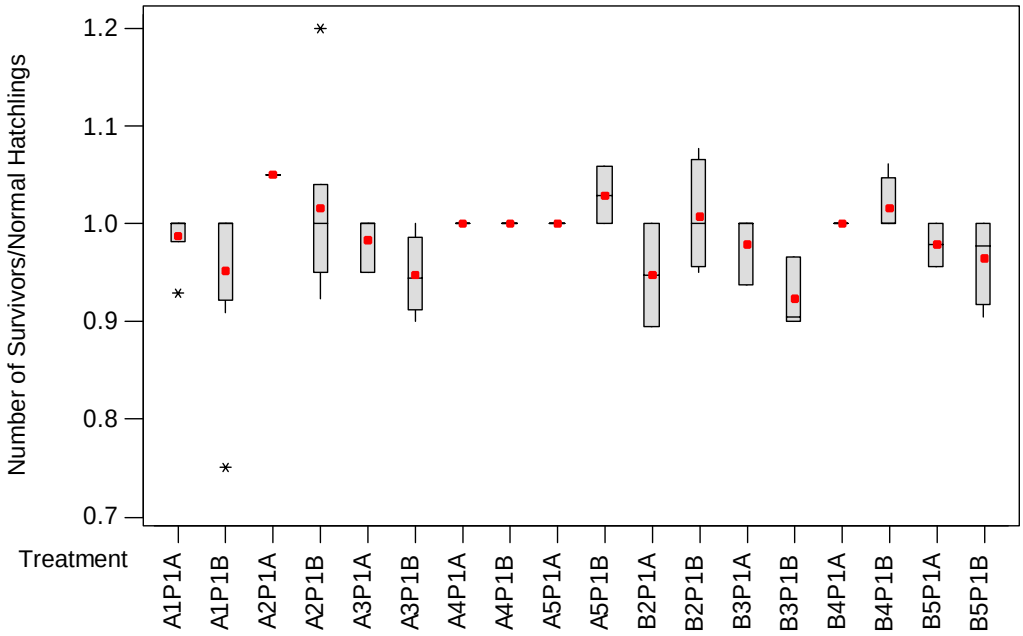


Figure 6.2-2. Box plots of the number of F2 14-day old survivors divided by the number normal hatchlings per hen by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

6.2.2 14-Day Old Survivors of Number of Eggs Set

A nearly significant interaction between F1 exposure strategies and P1 exposure scenarios (p=0.12) affected the number of 14-day old survivors divided by the total number of eggs set by hen (Figure 6.2-3). More F2 chicks with parentage from the F1a-P1A exposure designs survived than those with F1a-P1B parents, whereas the survival rate of F2 chicks appeared to be unaffected by F1b-P1A or F1b-P1B parentage. Dietary treatment of the P1 generation also appeared to have an affect, though nonlinear, on this measure of F2 survivability (p=0.13) (Figure 6.2-4).

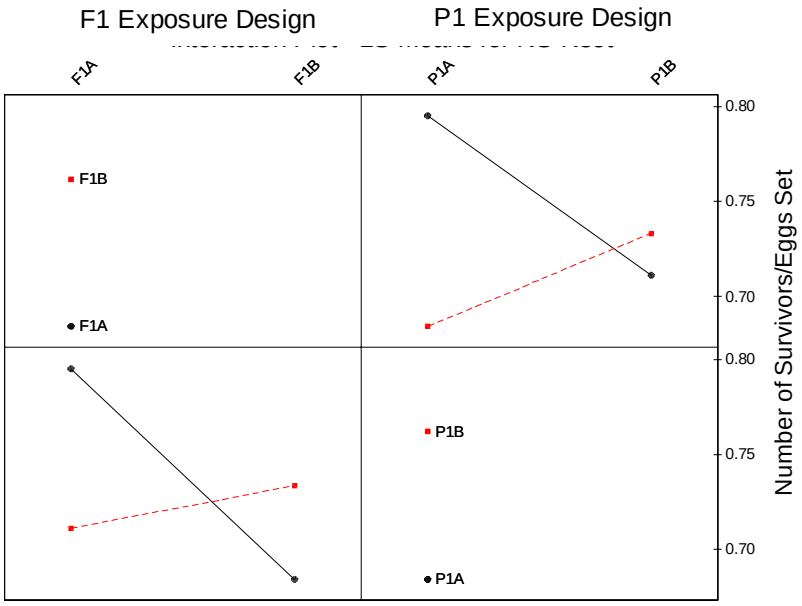


Figure 6.2-3. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the number of F2 14-day old survivors divided by the total number of eggs set by hen. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1b, parents exposed to E2 post-maturation.

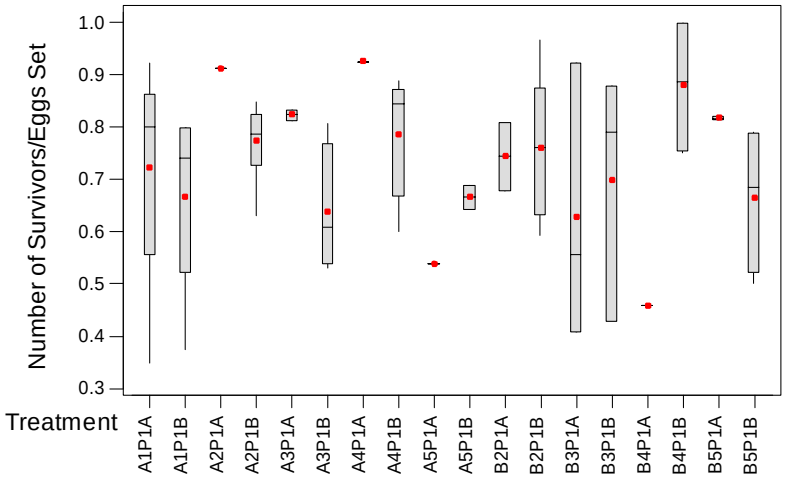


Figure 6.2-4. Box plots of the number of F2 14-day old survivors divided by the total number of eggs set per hen by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

6.2.3 14-Day Old Survivors of Maximum Number of Eggs Set

As observed in the other measures of 14-day old survivability, there was a non-linear effect (p=0.03) of P1 dietary concentrations on this measure of survivorship (Figure 6.2-5). The number of 14 day old survivors per pen divided by the largest number of eggs set was not affected by their parents' exposure design (F1a, treated vs. F1b, untreated), or the exposure scenario of the parents of the F1 generations (P1A or P1B) (p>0.38).

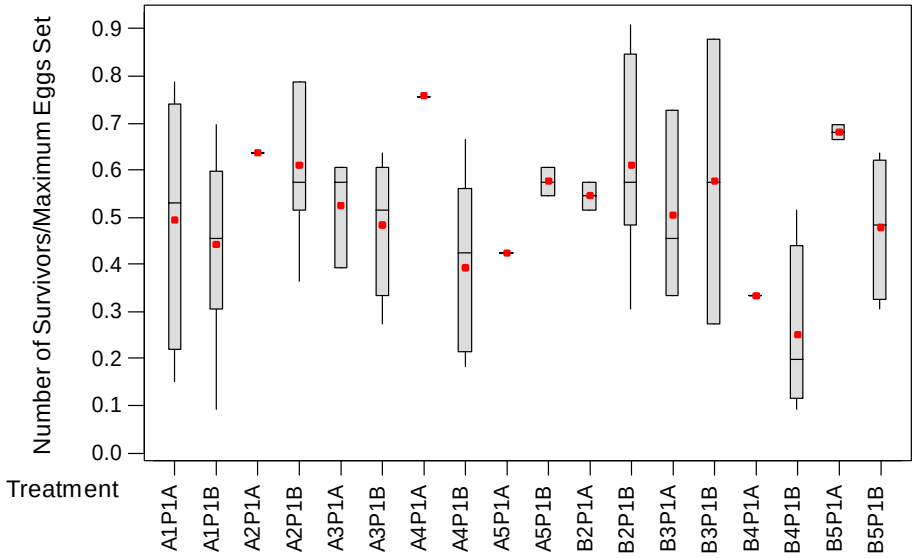
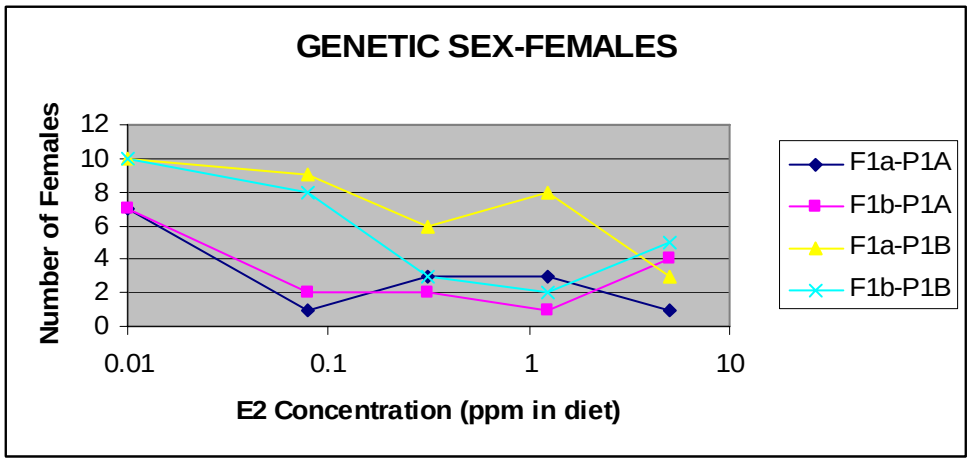


Figure 6.2-5. Box plots of the number of 14-day old survivors per pen divided by the largest number of eggs set by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentrations of E2 (1, 0 ppm, 2, 0.078 ppm, 3, 0.31 ppm, 4, 1.25 ppm, 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). Means are indicated by solid circles.

6.3 Genetic Sex Results and Sex Ratios (F2)

Blood samples were collected from a total of 180 F2 chicks to determine the genetic sex of the birds. Chicks were hatched from eggs collected during the eighth week of egg laying by the F1 generation. The genetic sex of each bird was compared to the sex of the bird determined by gross examination of the reproductive system at necropsy. Designation of gender by visual examination of the reproductive system in each bird examined was identical to the designation made by the genetic sexing technique. Comparison of genetic sex to the plumage phenotype could not be conducted because determination of gender could not be made with certainty at 2 weeks of age. Although sexes may be distinguished in some strains as early as the thirteenth day of age when the rufous feathers begin to appear along the ventral feather tract, gender was difficult to determine in untreated birds at 14 days of age in the strain used in this study.

Of the 180 chicks examined, 101 were determined to be males and 79 were determined to be females. Fewer chicks were produced by offspring of birds exposed to E2 under the P1A exposure scenario; therefore, the male-to-female ratios are based on smaller sample sizes than those determined for F2 chicks produced by offspring of birds from the P1B exposure design. For F2 chicks with treated (F1a) or untreated (F1b) parents from the P1B exposure scenario, the ratio of male to female chicks increased with P1 dietary concentration of E2, though in a nonlinear pattern. The sex ratio increased from 1.6 (control) to 2 and above for all test concentrations. F2 chicks with P1A parentage had reduced male-to-female ratios in the higher dietary treatment groups (1.25 ppm and 5 ppm E2). Indeed, no male F2 chicks were produced by treated (F1a) offspring of P1A parents from the 1.25 ppm and 5 ppm E2 treatment groups. Increased male to female ratios occurred at lower dietary concentrations. Figure 6.3-1 shows the distribution of males and females within the F1-P1 exposure scenarios of their parents by P1 dietary concentration. The male-to-female sex ratio of these chicks is shown within each treatment in Figure 6.3-2. A similar pattern of elevated male to female ratios in F2 chicks of F1a-P1A parents from the lower dietary treatment groups and a reduction in the sex ratio at higher treatment concentrations was observed when all surviving chicks from the F2 generation were considered. Sex ratios of the F2 generation regardless of F1 or P1 parental exposure were reduced below control ratios at higher dietary concentrations of E2 (Figure 6.3-3).



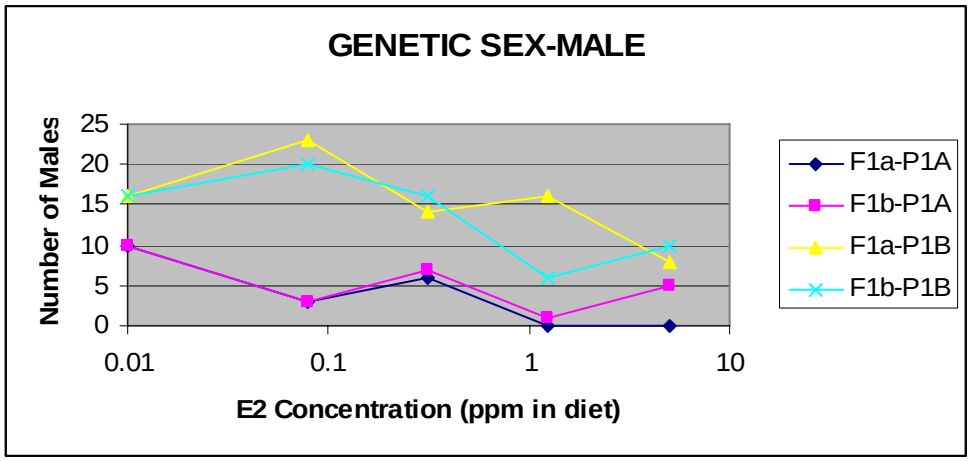


Figure 6.3-1. Number of 2 week old surviving quail from the eighth week of egg laying of the F1 generation confirmed to be females (above) and males (below) by genetic sexing. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents of F1 birds exposed to E2 post-maturation.) The number of control and 0.078 ppm DNA samples were randomly reduced by about 20% to reduce cost of analysis; all other groups were sampled in full.

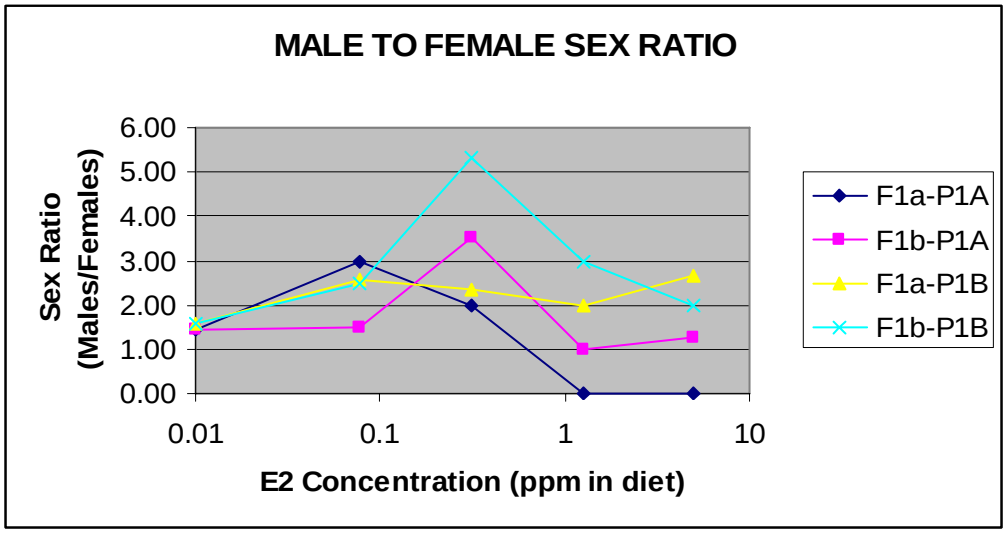


Figure 6.3-2. Male-to-female ratio in F2 chicks from Week 8 of egg laying by F1 generation for which genetic sex was determined. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents of F1 birds exposed to E2 post-maturation).

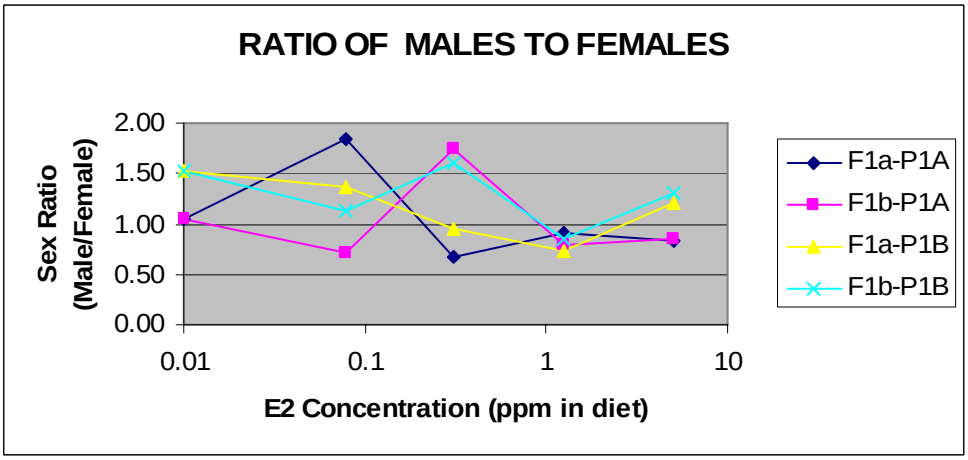


Figure 6.3-3. Male-to-female sex ratio of all F2 chicks surviving to 14 days of age. Sex determined morphologically at necropsy. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents of F1 birds exposed to E2 post-maturation.)

6.4 Clinical Observations, Early (Unscheduled) Deaths, and Abnormalities Observed at Necropsy (F2)

6.4.1 Clinical Observations

A total of 1105 F2 chicks hatched, of which 872 were necropsied and their sex determined when possible. Very young chicks that died after the last observation of the day often could not be sexed when found the next day because of tissue autolysis or pecking by cohorts. In addition to these chicks, a batch of chicks (week 7) was terminated at 14 days of age but not necropsied and about 20% of chicks from the 0 ppm and 0.078 ppm E2 treated parents in the larger hatches were not necropsied to reduce necropsy costs, resulting in a total of 233 birds for which the sex was not known. To determine the distribution of clinical observations, skeletal abnormalities and unscheduled deaths for the total hatch, all chicks for which the sex could not be determined were combined with all of the identified male and female chicks and reported in Table 6.4-1. Incidence of abnormalities is reported separately for male and female F2 chicks that were necropsied.

Orthopedic deformities comprised the majority of clinical observations. These deformities included spraddled leg, a pelvic socket deformity in which one or both legs deviate laterally from the pelvis, and curled or crooked toes. The distribution of these abnormities between treatment concentrations and exposure designs is discussed below. A few hatchlings exhibited a spinning behavior unrelated to parental treatment group. One chick with a 0.31 ppm parental exposure history had 2 sets of legs at hatch and one chick with a 5 ppm parental exposure history had both a mandibular and eye deformity.

6.4.2 Incidence of Unscheduled Deaths

Out of 1105 chicks that hatched, a total of 50 unscheduled deaths (4.5% of the total hatch) occurred (Table 6.4-1). With the exception of chicks from parents with a P1 exposure history of 1.25 ppm E2, the deaths were about evenly distributed among dietary treatment groups. The majority of unscheduled deaths were among chicks of parents with P1B exposure history; however, the incidence of death was also high in chicks of P1B control parentage. Overall, the proportion of premature deaths per number of hatchlings in all treatments was low (Table 6.4-1).

The distribution of premature deaths among treatment groups for chicks for which the sex could be determined is shown in Table 6.4-2 for female and Table 6.4-3 for male F2 chicks. In females, a total of 7 unscheduled deaths occurred out of 409 female F2 chicks (Table 6.4-2). None of the female chicks from control parents died early. The majority of the chicks that died (4/7) were of F1b-P1B parentage, i.e., chicks of untreated parents that received an *in ovo* dose under the P1B exposure scenario. Only two deaths occurred in chicks of treated (F1a) parents, but the dietary E2 concentrations of the parents were low. No clear pattern of P1 dietary treatment effect was detected, though most chicks identified as females (4/7) that died prematurely had P1 exposure histories of 0.31 ppm E2. Seven unscheduled deaths out of 463 male F2 chicks were recorded. The distribution of the unscheduled deaths among test groups did not appear to be related to treatment. Two of the chicks were from control parents. No female chicks of F1a-P1A parents that were exposed to E2 died early (Table 6.4-2).

Table 6.4-1. Overall incidence and percentage of unscheduled deaths and skeletal abnormalities in F2 chicks.

F1 Design	P1 Design	Dose (ppm)	N	Early Death		Incidence of Foot/Leg Abnormalities		Incidence of Crossbill	
				No.	%	No.	%	No.	%
F1a	P1A	0	104	5	4.8	3	2.9	0	0
F1a	P1A	0.078	20	0	0	1	5.0	0	0
F1a	P1A	0.31	53	1	1.9	0	0	0	0
F1a	P1A	1.25	25	0	0	0	0	0	0
F1a	P1A	5	15	1	6.7	1	6.7	0	0
F1a	P1B	0	120	7	5.8	6	5.0	0	0
F1a	P1B	0.078	142	6	4.2	11	7.7	1	0.7
F1a	P1B	0.31	69	5	7.2	4[a]	5.8	0	0
F1a	P1B	1.25	67	1	1.5	3	4.5	2	3.0
F1a	P1B	5	40	2	5.0	2	5.0	1	2.5
F1b	P1A	0.078	38	2	5.3	0	0	0	0
F1b	P1A	0.31	52	2	3.8	2	3.8	0	0
F1b	P1A	1.25	11	0	0	0	0	0	0
F1b	P1A	5	50	2	4.0	1	2.0	0	0
F1b	P1B	0.078	137	4	2.9	5	3.6	0	0
F1b	P1B	0.31	64	7	10.9	3	4.7	0	0
F1b	P1B	1.25	33	0	0	1	3.0	0	0
F1b	P1B	5	65	5	7.7	1	1.5	1	1.5

[a] Includes one chick with 4 legs (Figure 6.4-2 below).

Although the distribution of female and male unscheduled deaths are described above, it should be noted that the sex of the chicks was known in less than a third (14/50) of chicks that died prematurely.

6.4.3 Incidence of Abnormalities

Females

Few abnormalities were observed in female F2 chicks (Table 6.4-2). Of the abnormalities observed, only those of the feet and legs showed a significant difference between treatments. P1 dietary concentration significantly (p=0.041) affected the proportion of F2 female chicks that developed foot and/or leg malformations (Figure 6.4-1). However, the effect was largely limited to the chicks having parental *in ovo* exposure from the 0.078 ppm E2 diet. A nearly significant (p= 0.073) P1 design effect was also observed, with the mean proportion of foot and/or leg deformities greater in the chicks of parents that had *in ovo* exposure to E2 from P1B parents. Indeed, no incidence of these abnormalities was observed in chicks with P1A parentage. F1 exposure strategy had no affect on the occurrence of the foot/leg abnormalities (p=0.230). When the incidence of foot/leg deformities were combined with those of the males and chicks of undetermined gender, the pattern of greater incidence in 0.078 ppm dietary treatment and in P1B treatments is retained. However, the percentage of affected chicks in each treatment group was low (< 8%).

Table 6.4-2. Incidence of abnormalities observed in F2 females.

F1 Design	P1 Design	Dose (ppm)	N	Premature Death	Incidence of Foot/Leg Abnormalities	Incidence of Right Oviduct	Adrenal Gland Abnormality
F1a	P1A	0	39	0	0	1	1
F1a	P1A	0.078	6	0	0	0	0
F1a	P1A	0.31	24	0	0	0	0
F1a	P1A	1.25	11	0	0	0	0
F1a	P1A	5	6	0	0	0	0
F1a	P1B	0	36	0	0	0	0
F1a	P1B	0.078	48	1	6	1	0
F1a	P1B	0.31	26	1	1	1	0
F1a	P1B	1.25	30	0	0	1	0
F1a	P1B	5	14	0	0	0	0
F1b	P1A	0.078	17	0	0	0	0
F1b	P1A	0.31	16	0	0	1	0
F1b	P1A	1.25	5	0	0	0	1
F1b	P1A	5	22	1	0	0	0
F1b	P1B	0.078	51	0	2	2	0
F1b	P1B	0.31	21	3	1	0	0
F1b	P1B	1.25	14	0	0	0	0
F1b	P1B	5	23	1	1	0	0

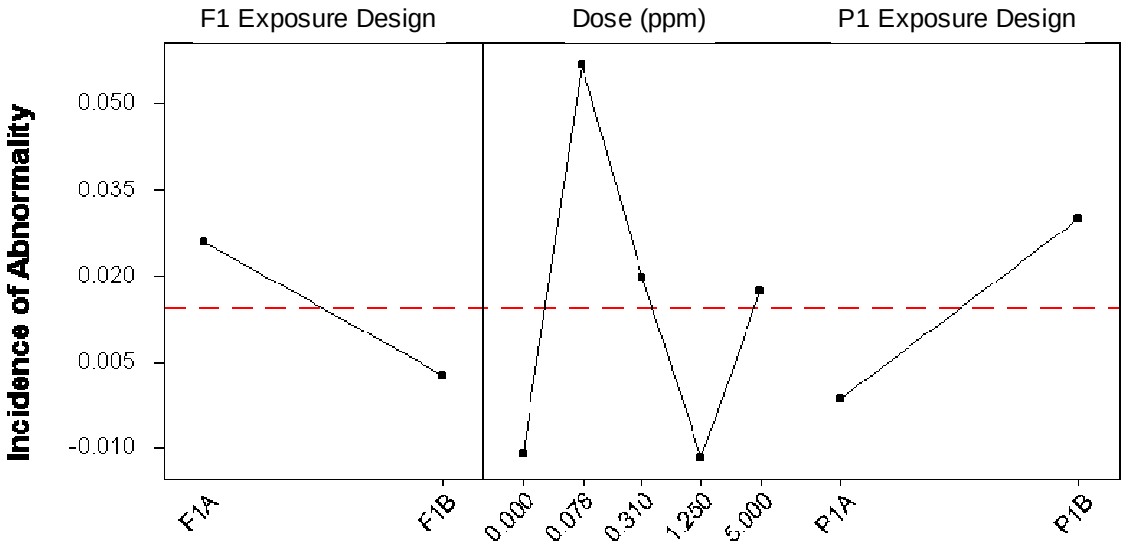


Figure 6.4-1. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), P1 dietary treatment with E2, and P1 exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on proportion of foot and/or leg abnormalities per group in F2 female chicks. General Linear Model analysis; significant difference between P1 dietary concentrations, nearly significant difference between P1 exposure scenario, p=0.073.

Males

As observed in females, few gross abnormalities were found in F2 male chicks (Table 6.4-3). Unlike female chicks, no significant increase in foot/leg malformations in any treatment combination was detected in males ($p>0.597$). Other morphological deformities included an incidence of crossbill and the absence of an eye. Both deformities occurred in the same chick, an offspring of an F1b-P1B-5 ppm E2 parent. No organ or tissue abnormalities observed during necropsy were significantly different ($p>0.15$) in male chicks between F1 or P1 exposure designs or across P1 dietary concentrations.

Table 6.4-3. Incidence of abnormalities observed in F2 males.

F1 Design	P1 Design	Dose (ppm)	N	Premature Death	Incidence of Foot/Leg Abnormalities	Incidence of Testis Abnormalities[a]	Incidence of Crossbill	Adrenal Gland Abnormalities
F1a	P1A	0	43	2	1	1	0	0
F1a	P1A	0.078	11	0	0	1	0	0
F1a	P1A	0.31	16	0	0	1	0	0
F1a	P1A	1.25	10	0	0	0	0	0
F1a	P1A	5	5	0	0	0	0	0
F1a	P1B	0	55	0	1	4	0	0
F1a	P1B	0.078	65	1	3	1	0	0
F1a	P1B	0.31	25	0	0	2	0	0
F1a	P1B	1.25	21	0	0	0	0	0
F1a	P1B	5	17	0	1	0	0	0
F1b	P1A	0.078	12	0	0	0	0	0
F1b	P1A	0.31	28	0	1	1	0	0
F1b	P1A	1.25	4	0	0	1	0	0
F1b	P1A	5	19	1	1	0	0	0
F1b	P1B	0.078	59	1	1	2	0	1
F1b	P1B	0.31	29	0	0	1	0	0
F1b	P1B	1.25	12	0	0	1	0	0
F1b	P1B	5	32	2	0	2	1[b]	1

[a] All abnormal testes were mottled and/or had dark foci.
[b] The right eye of this bird was missing at hatch.

Undetermined Gender

Many of the morphological abnormalities (limb and bill deformities) were recorded for chicks that died within the first day of brooding and could not be sexed due to tissue autolysis. Spraddled leg and curved or crooked toes were the most common deformities observed. One chick developed a double set of legs (Figure 6.4-2).

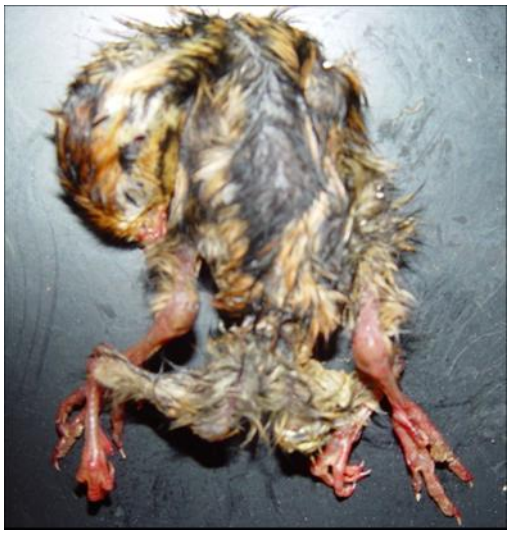


Figure 6.4-2. An F2 chick with four legs. The chick had an F1a-P1B-0.31 ppm parental exposure history.

6.5 Organ Weights (F2)

Females

Thyroid

The absolute weight of the thyroid glands of 2-week-old F2 females at necropsy was significantly affected by the exposure design of their parents ($p<0.04$), with female chicks from treated (F1a) parents greater in weight than those with untreated (F1b) parents (Figure 6.5-1). However, the difference in the overall means between the two treatments did not appear to be biologically important (<1%). No significant differences in thyroid weight between P1 exposure designs was observed ($p>0.84$) (Figure 6.5-1). However, a nearly significant effect ($p=0.109$) of P1 dietary concentrations on gross thyroid weight in F2 females was detected, but was not concentration-linear. After normalization of the thyroid weight to body weight, the effect of the F1 exposure design continued, but the dietary concentration effect was no longer significant ($p=0.24$). An interaction between F1 and P1 exposure designs appeared to affect ($p=0.129$) thyroid gland weight normalized to brain weight. When the F1 generations were analyzed separately, no significant difference ($p>0.48$) in thyroid-to-brain weight ratios between P1 exposure scenarios of F2 chicks from F1a parents was found (Figure 6.5-2). For offspring of untreated (F1b) parents, those with P1A parentage had thyroid weights greater than those with P1B parentage in response to an interaction between the P1 design and the 0.31 ppm P1 dietary concentration (Figure 6.5-2).

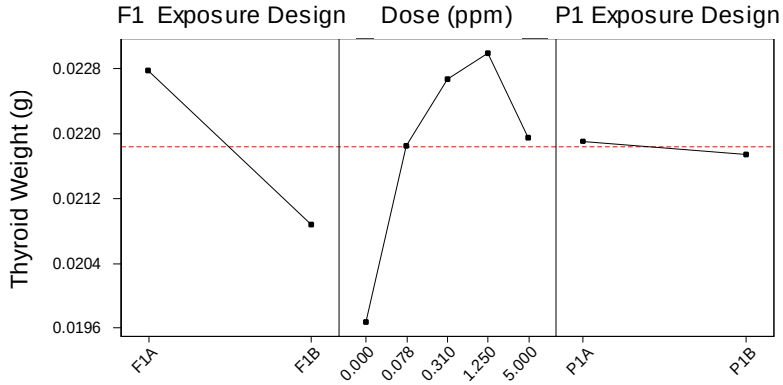


Figure 6.5-1. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1b, exposed post-maturation) on absolute thyroid gland weight of F2 females at 2 weeks of age (General Linear Model analysis; significant differences between F1 exposure design, $p<0.04$; trend ($p=0.109$) of parental dietary concentration effect). F1a birds were fed the same dietary treatments as their parents; all F1b birds and F2 chicks were untreated.

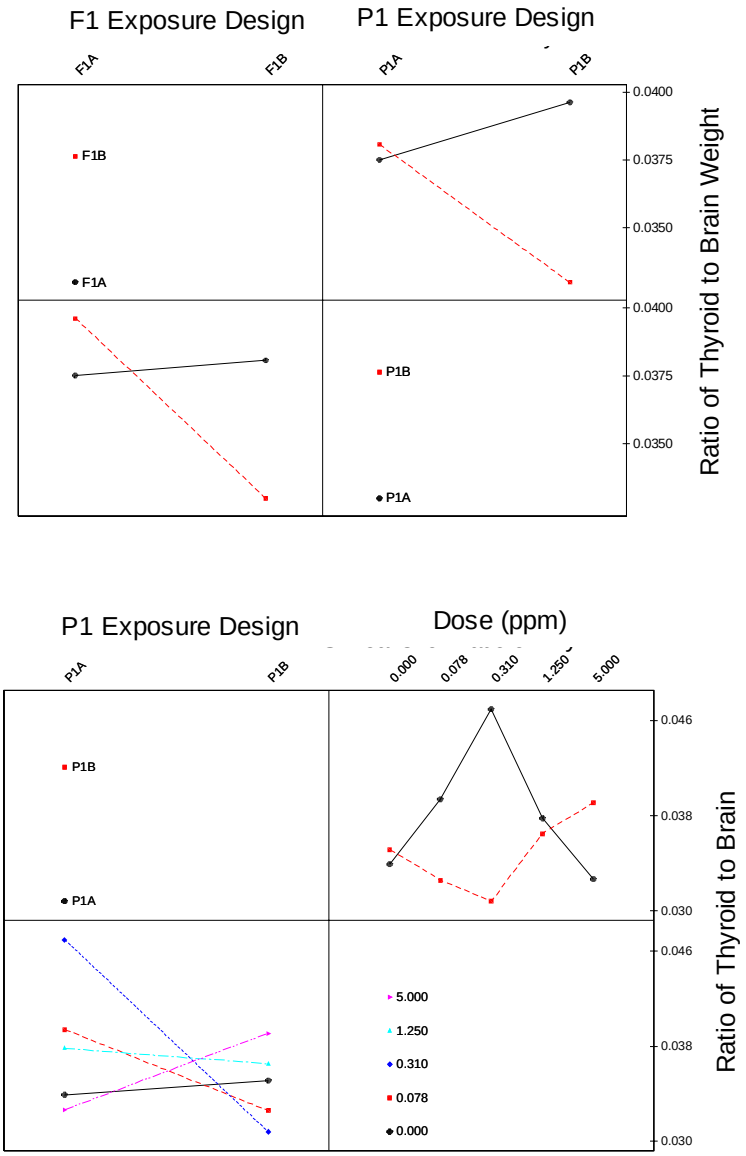


Figure 6.5-2. Interactions of F1 and P1 exposure designs from the General Linear Model analysis of the thyroid-to-brain weight ratios of all F2 female chicks at 2 weeks of age (above) and of the P1 exposure design and P1 diet concentration of the F2 female chicks from F1b parents (below). (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents of F1 birds exposed to E2 post-maturation.)

Adrenal Gland

An interaction between F1 and P1 exposure designs tended to affect gross adrenal gland weight (p=0.072) and adrenal gland-to-body weight ratios (p=0.068). F2 chicks with F1a-P1B parentage had greater adrenal gland-to-body weight ratios compared to chicks with F1a-P1A parentage. The relative adrenal weights of chicks of F1b parents were unaffected by the P1

exposure design. However, when normalized to brain weight, the interaction was significant (p=0.022), but nearly opposite in effect (Figure 6.5-3).

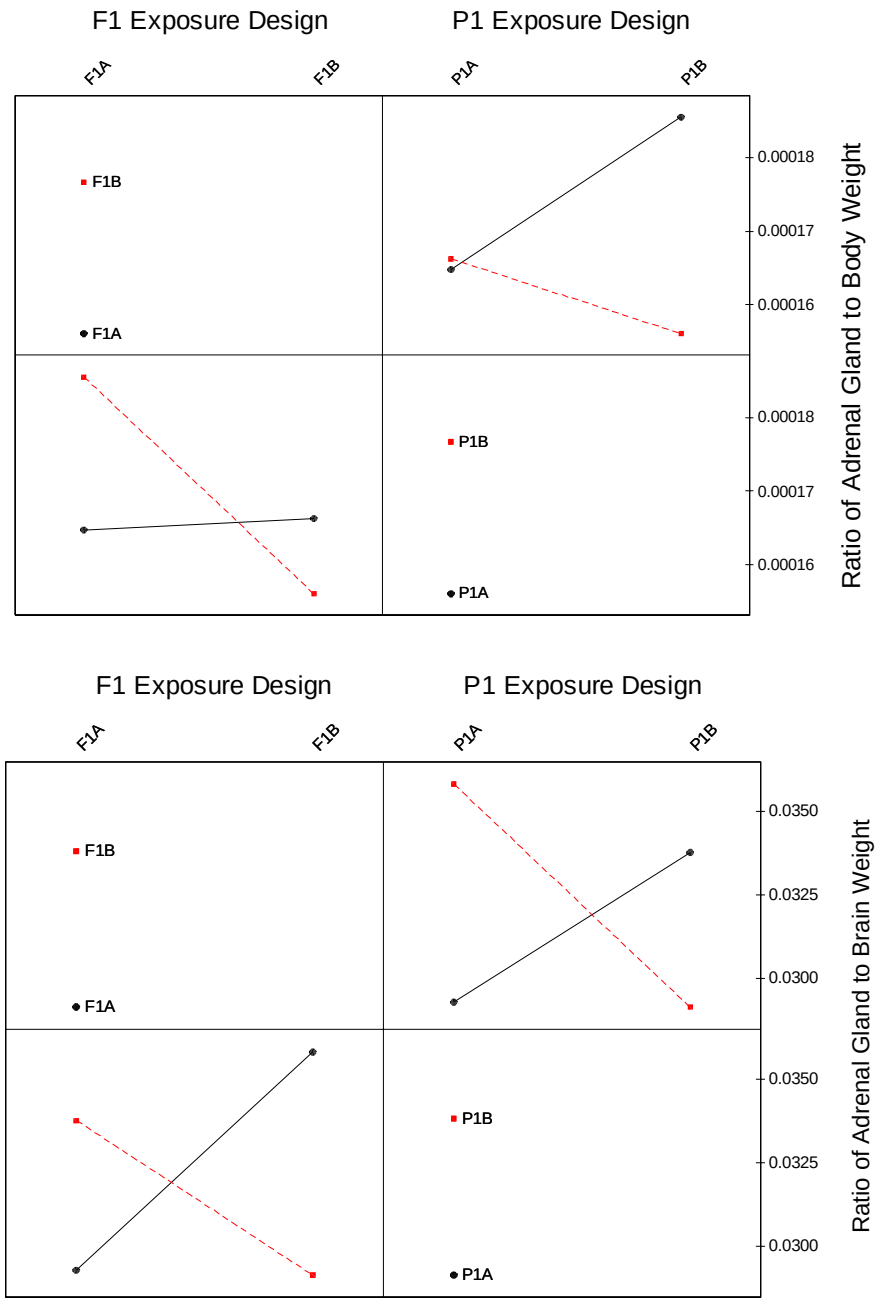


Figure 6.5-3. Interaction of F1 and P1 exposure designs from the General Linear Model analysis of the adrenal gland-to-body weight ratios (above) and adrenal gland-to-brain weight ratios (below) of F2 female chicks at 2 weeks of age. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents of F1 birds exposed to E2 post-maturation.)

Liver

A small (6% overall) but significant effect of F1 exposure regime was observed for the mean absolute liver weight ($p<0.003$; Figure 6.5-4) and the liver-to-body weight index ($p<0.001$). However, there was a nearly significant interaction between the F1 and P1 exposure designs affecting gross liver weight of the F2 generation ($p=0.053$). When gross liver weight was normalized to body weight, the interaction became a significant ($p=0.012$) (Figure 6.5-5). Analyzing the F1 generations separately, a significant interaction between the P1 exposure scenario and the P1 dietary concentrations was found (Figure 6.5-6). F2 chicks with F1a-P1A parentage had greater liver-to-body weight ratios if their parents received E2 treated diet compared to controls ($p=0.002$), whereas offspring of F1a-P1B parents were not significantly affected by diet ($p=0.50$). In chicks with F1b parents, those from the 5 ppm E2 diet in the P1A design tended to have enlarged livers ($p=0.07$). Chicks with F1b-P1B parentage tended to have increased liver-to-body weight ratios only in the 0.31 ppm P1 diet ($p=0.054$).

No significant effects of F1 exposure strategy, P1 exposure scenario or P1 dietary concentrations on liver weights normalized to brain weight were detected ($p\geq0.365$).

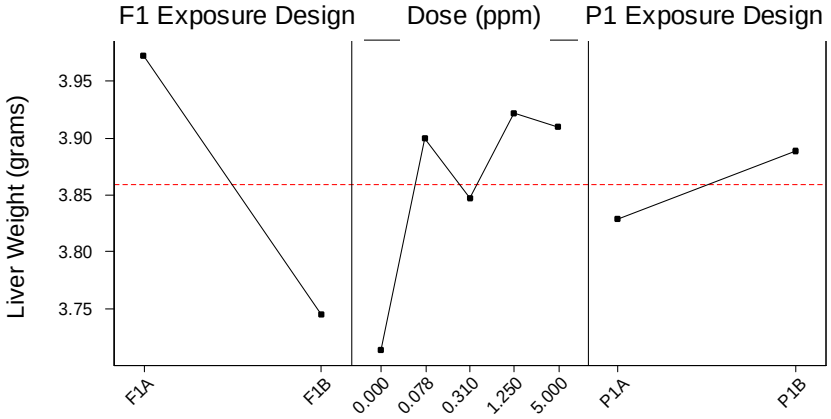


Figure 6-5.4. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on absolute liver weight (g) of F2 females at 2 weeks of age (General Linear Model analysis; significant difference between F1 exposure designs, $p<0.003$). F1a birds were fed the same dietary treatments as their parents; all F1b birds and F2 chicks were untreated.

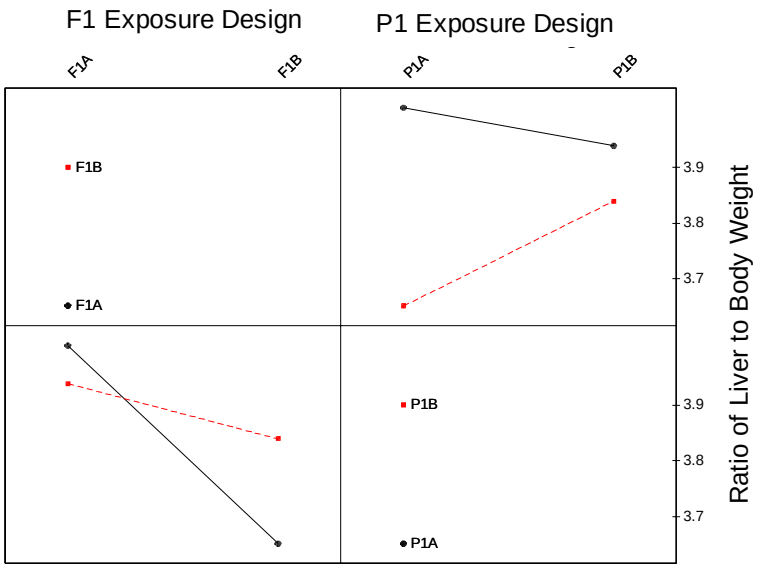


Figure 6.5-5. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the ratio of liver to body weights of F2 female chicks at 2 weeks of age. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.

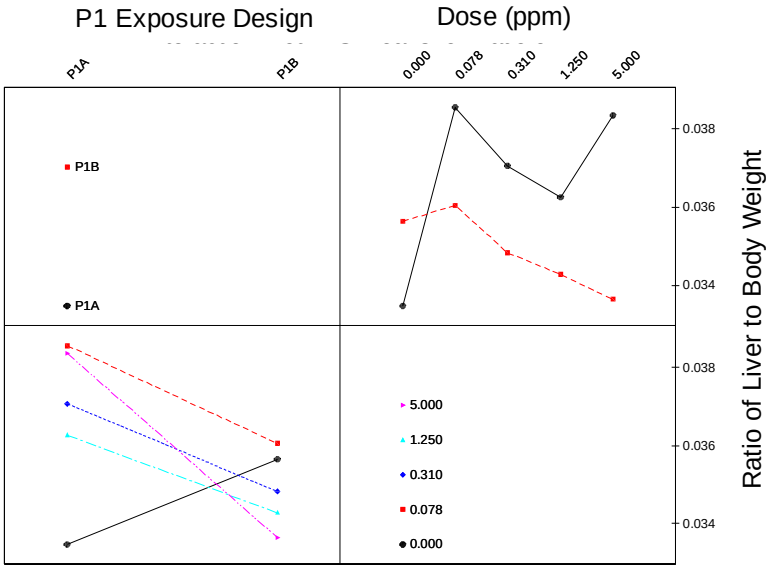


Figure 6.5-6. Interaction of P1 exposure design and P1 dietary concentration in 14-day-old F2 female chicks with F1a parentage. General Linear Model analysis of the liver-to-body weight ratios: F1a, dietary treatment with E2 in F1 generation; P1A, parents of F1 birds exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation. F1b birds and F2 chicks were untreated.

Pancreas

A significant interaction ($p=0.008$) between the F1 exposure strategy and P1 exposure scenario affected gross pancreas weights in F2 female chicks. The effect of P1 dietary treatment on pancreas weight was highly significant ($p<0.008$). When normalized to body weight, the interaction between the F1 and P1 exposure designs remained significant ($p=0.002$), as did the effect of P1 dietary concentration ($p<0.001$) (Figure 6.5-7). Analyzing the F1 generations separately showed that there was a significant difference in absolute and relative pancreas weight between the P1 exposure scenarios in F2 chicks with treated (F1a) parents ($p=0.007$); F2 female chicks from F1a-P1A parents having greater pancreas-to-body weight ratios than those from F1a-P1B parents (Figure 6.5-8). Relative pancreas weights of F2 chicks were also increased in response to dietary exposure of their treated parents, the pancreas-to-body weight ratios being significantly greater in those from F1a parents fed 5 ppm E2 ($p<0.001$) (Figure 6.5-8). Although relative pancreas weights of chicks from untreated (F1b) parents were significantly affected by an interaction between the P1 exposure scenario and dietary concentration of their parents ($p=0.01$), there was no meaningful relationship between P1 dietary concentration and the changes in relative weight (Figure 6.5-9).

The interaction between F1 and P1 exposure designs and the effect of dietary treatment on pancreas weight were not observed when the organ weight was normalized to brain weight ($p>0.28$). Only a nearly significant ($p=0.099$) difference between P1 exposure scenario effects was detected using this organ weight index.

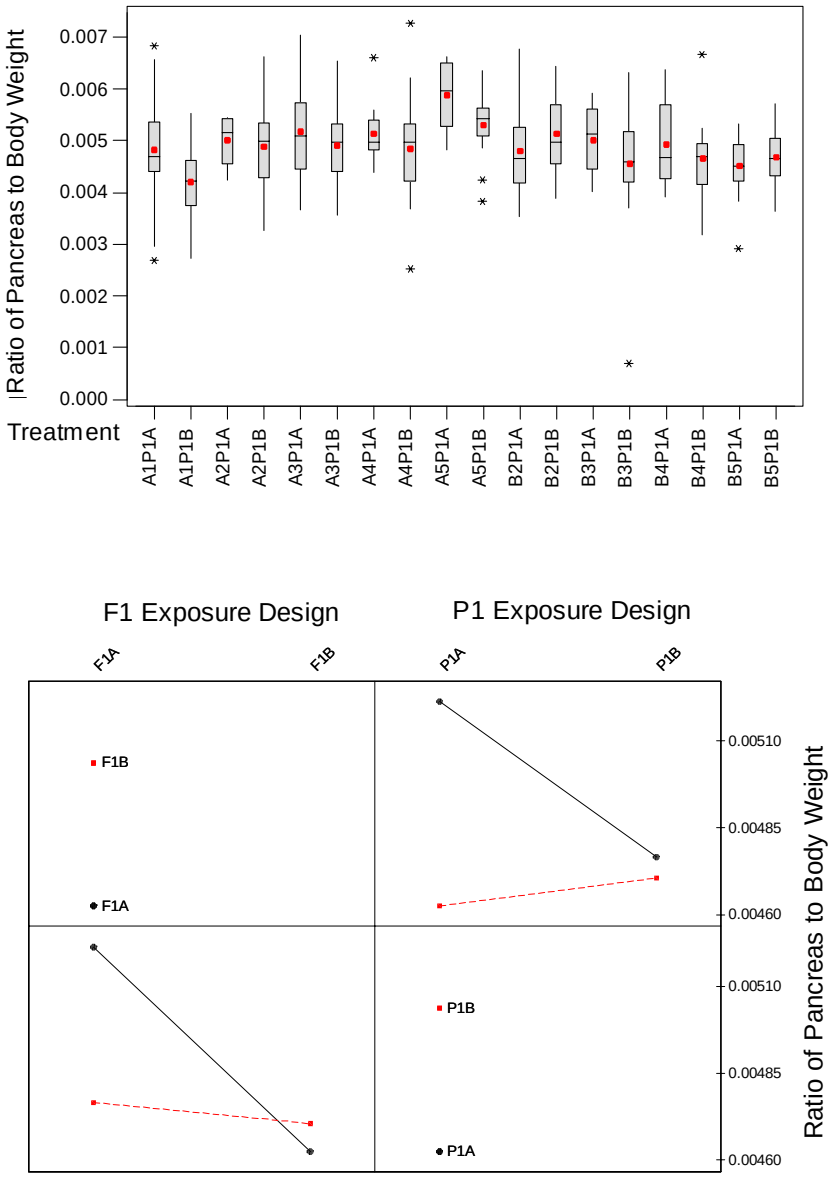


Figure 6.5-7. Box plots of the pancreas-to-body weight ratio (above) and interaction of F1 and P1 exposure designs from General Linear Model analysis of the pancreas-to-body weight ratios of F2 female chicks at 2 weeks of age (below). (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

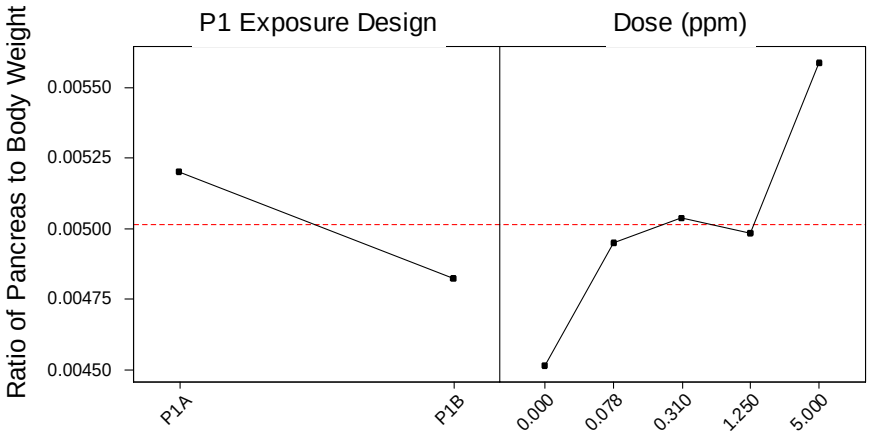


Figure 6.5-8. Effects of P1 dietary treatment with E2 and the P1 exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on pancreas-to-body weight ratios of F2 female 2-week-old offspring of treated (F1a) parents (General Linear Model analysis; significant effect of P1 exposure scenario, p=0.007 and highly significant differences between P1 dietary concentrations, p<0.001). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

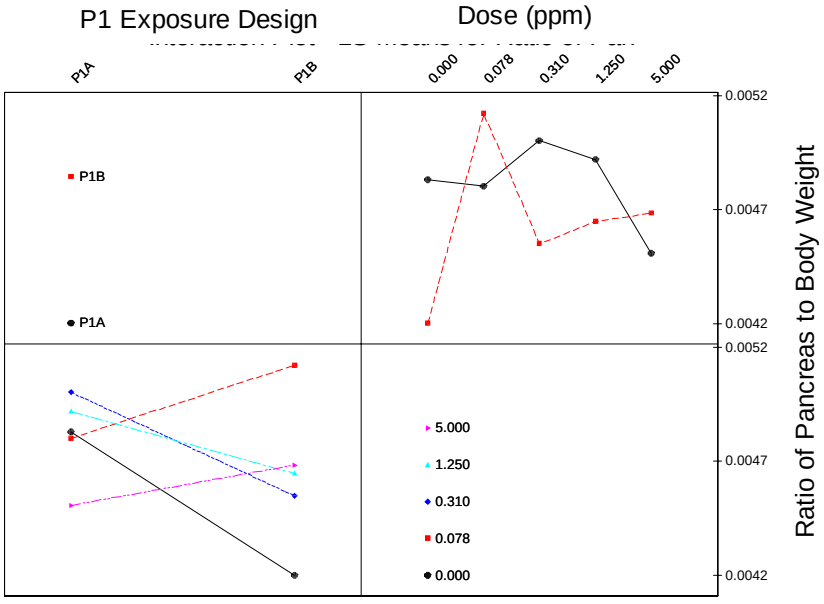


Figure 6.5-9. Interaction of P1 dietary concentrations and P1 exposure designs from General Linear Model analysis of the pancreas-to-body weight ratios of 2-week-old female F2 offspring of untreated (F1b) parents. (P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

Spleen

Absolute spleen weight and spleen-to-body weight ratios of F2 female chicks were significantly affected by both F1 exposure design ($p<0.002$) and P1 dietary concentration ($p<0.001$). F2 offspring of treated (F1a) parents had greater spleen weights than F2 females from untreated parents. Dietary concentrations above 0.078 resulted in elevated spleen weights compared to controls (Figure 6.5-10). When normalized to brain weight, only the dietary treatment effect remained significant ($p<0.001$). The distribution of spleen weights by dietary concentration within F1 and P1 exposure designs is shown in Figure 6.5-11.

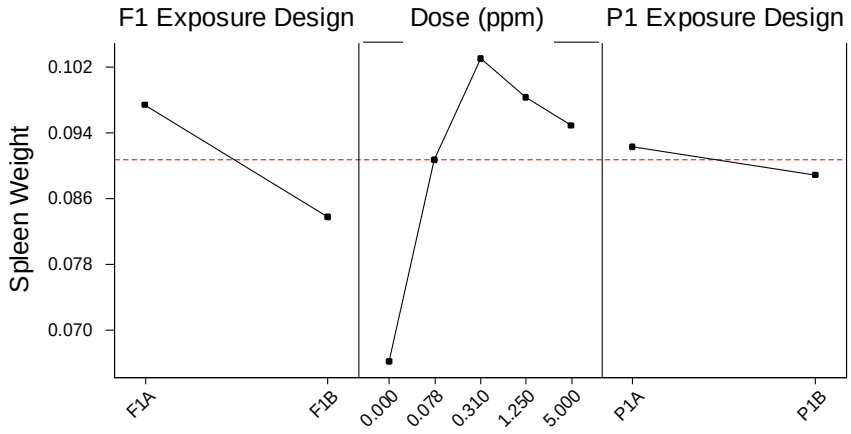


Figure 6.5-10. **Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on absolute spleen weight (grams) of F2 females at 2 weeks of age** (General Linear Model analysis; highly significant differences between P1 dietary concentrations, $p<0.001$; significant F1 design effect, $p<0.002$). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

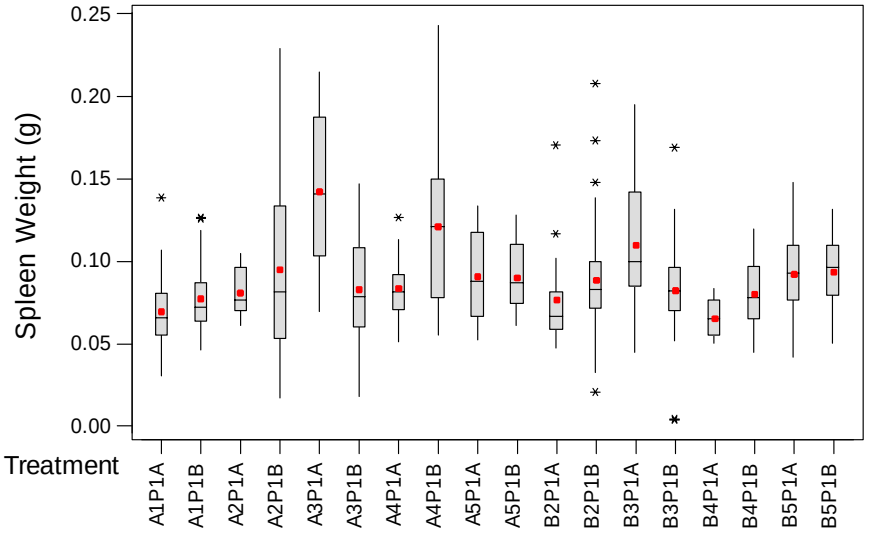


Figure 6.5-11. Box plots of the spleen weights of F2 female chicks by P1 exposure scenario (P1A, exposed prior to maturation through egg laying; P1B, exposed after onset of egg laying), P1 dietary concentration of E2 (1, 0 ppm; 2, 0.078 ppm; 3, 0.31 ppm; 4, 1.25 ppm; 5, 5 ppm) and F1 exposure strategy (a, treated with same diets as parents; b, untreated). All F1b and F2 chicks were untreated.

Brain

Absolute brain weight of F2 female chicks was not significantly affected by the direct and/or *in ovo* exposure history of their parents ($p<0.176$). When normalized to body weight, a nearly significant ($p<0.090$) P1 dietary concentration effect was detected, but the response was non-concentration linear (Figure 6.5-12).

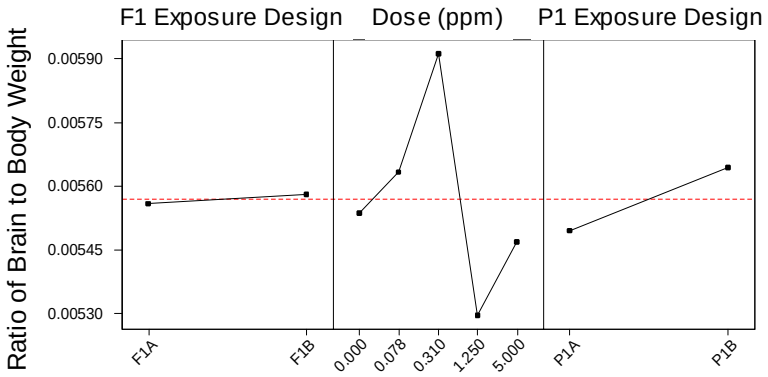


Figure 6.5-12. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on brain-to-body- weight ratio of F2 females at 2 weeks of age (General Linear Model analysis; nearly significant differences between P1 dietary concentrations, $p<0.090$). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Left Ovary

Left ovary weights were significantly different ($p<0.001$) between F2 chicks of parents with P1A vs P1B parentage. Chicks with parents that had P1A parents had reduced ovary weight (Figure 6.5-13). This effect was retained when the ovary weight was normalized to body weight ($p=0.002$), but was lost when a brain weight normalization was applied ($p=0.254$). Ovary-to-body weight ratios tended to show effects of an interaction between F1 and P1 exposure designs ($p=0.139$; Figure 6.5-13), the nearly significant F1 exposure design effect ($p=0.093$) was also detected when the ovary was normalized to brain weight.

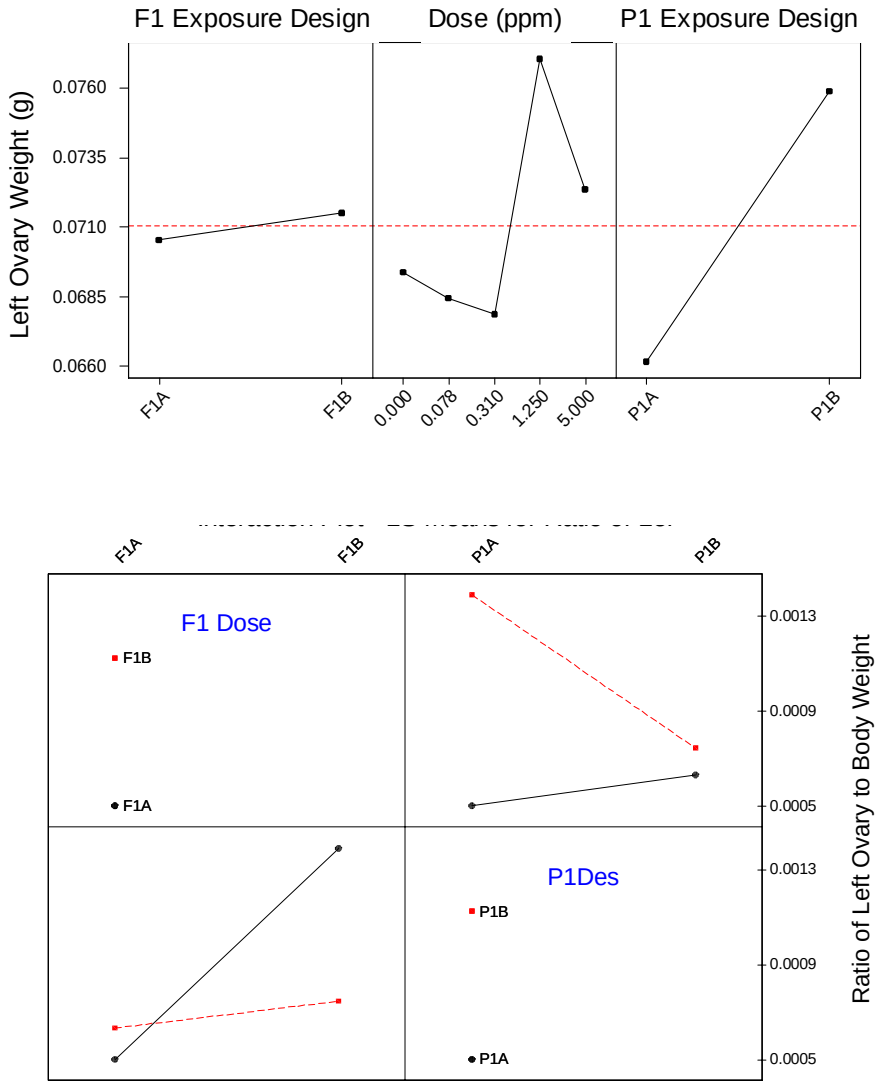


Figure 6.5-13. **Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on absolute ovary weight of F2 females at 2 weeks of age (above) and interaction of F1 and P1 exposure designs on ovary-to-body weight ratios** (General Linear Model analysis; significant difference between P1 exposure scenarios, $p<0.001$). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Left Oviduct

No significant effects on absolute or normalized oviduct weight of F2 chicks as a result of the F1 or P1 exposure designs or dietary treatment history of their parents were detected ($p<0.33$).

Males

Thyroid

Absolute thyroid weight and thyroid-to-body weight ratio of F2 male chicks at 2 weeks of age were unaffected by the exposure scenario or dietary treatment of the parents of their parents ($p<0.675$) (Figure 6.5-14). However, a nearly significant interaction between F1 exposure design and P1 exposure scenario affected thyroid weights of the F2 males ($p=0.089$). F2 chicks with F1a-P1A parentage tended to have larger thyroid glands than those with F1b-P1A, F1a-P1B, or F1b-P1B parentage (Figure 6.5-15). When thyroid weight was normalized to brain weight, this interaction was not retained ($p=0.556$).

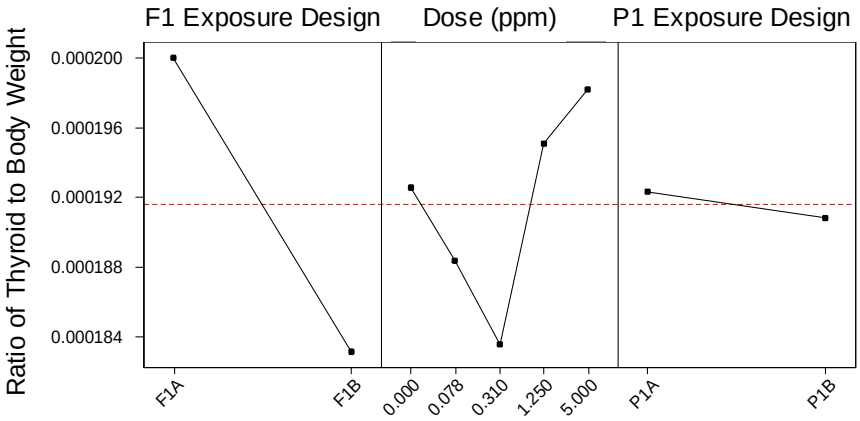


Figure 6.5-14. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on thyroid-to-body weight ratio of F2 males at 2 weeks of age (General Linear Model analysis: F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.)

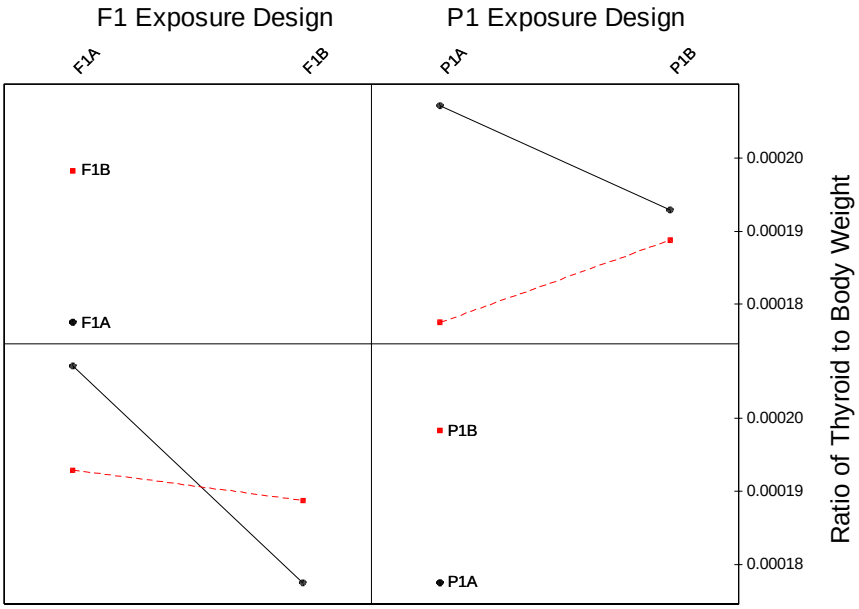


Figure 6.5-15. Interaction of P1 dietary concentrations and P1 exposure designs from General Linear Model analysis of the thyroid-to-body weight ratios of 2-week-old male F2 offspring of untreated (F1b) parents. (P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

Adrenal Glands

The only effect observed in the gross or relative weights of adrenal glands was a tendency for the gross adrenal weight in F2 male chicks to be affected by P1 design scenario ($p=0.144$) (Figure 6.5-16). This effect was absent when normalized weights were analyzed ($p>0.156$).

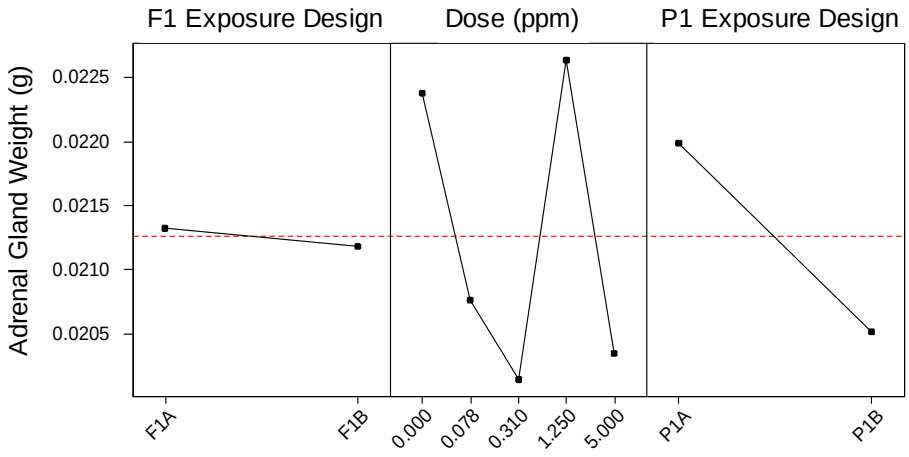


Figure 6.5-16. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on adrenal gland weight of F2 males at 2 weeks of age (General Linear Model analysis: P1 exposure effects, p=0.144). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Liver

A significant effect of P1 dietary concentrations on liver weight in F2 males was detected (p=0.002), but the effects were not dose linear (Figure 6.5-17). When normalized to body weight, the effect was reduced (p=0.099) and lost entirely when normalized to brain weight (p=0.707). The F1 exposure strategy under which the parents of the F2 male chicks were exposed to E2 had a small, but significant effect on gross liver weight (p=0.009) (Figure 6.5-17). Analysis of liver weights normalized to body weight detected a highly significant (p<0.001) increase in the relative liver weights of offspring of F1a parents compared to offspring of F1b parents. A nearly significant F1*P1 exposure design interaction affecting gross liver weights was found for both the absolute weight and liver-to-body weight ratios (p=0.124). As seen in Figure 6.5-18, the greatest effect is manifested in F2 chicks that have parents with the greatest direct and *in ovo* exposure history (F1a-P1A). This interaction was not detected in liver-to-brain weight ratios (p=0.458).

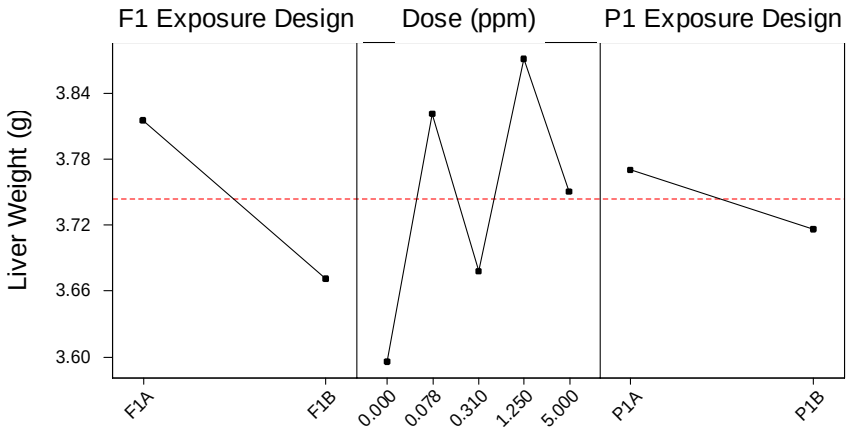


Figure 6.5-17. **Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on gross liver weight of F2 males at 2 weeks of age** (General Linear Model analysis: significant difference between P1dietary concentrations, p=0.002). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

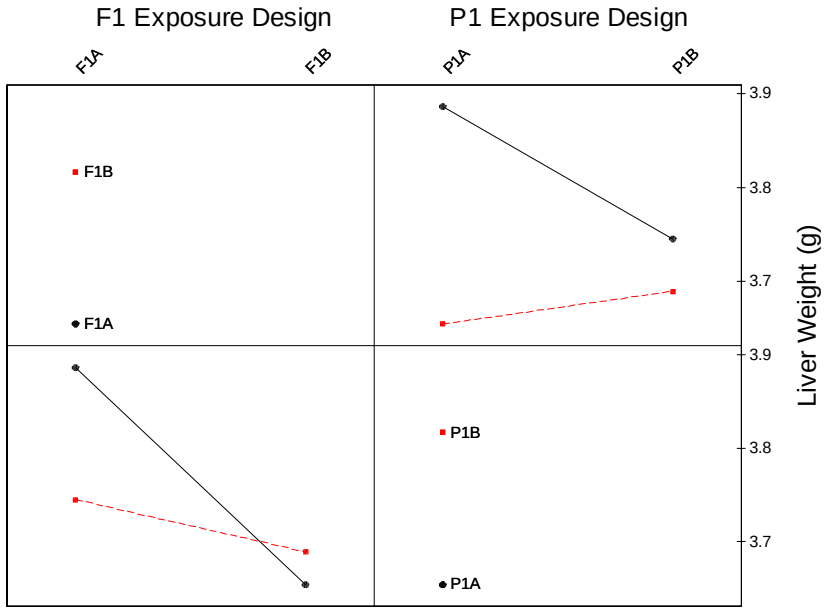


Figure 6.5-18. **Interaction of F1 and P1 exposure designs from General Linear Model analysis of the gross liver weight of F2 male chicks at 2 weeks of age.** (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

Pancreas

Significant differences in both the gross (p=0.003) and body weight normalized (p=0.008) pancreas weights across P1 dietary concentrations were observed in the F2 male chicks, but the differences were not dose-linear (Figure 6.5-19). A significant P1 design effect was observed for both the absolute and relative pancreas weight ($p \leq 0.013$); however, a nearly significant interaction between the P1 exposure scenario and the F1 exposure strategy affecting gross and relative pancreas weight was also detected (p=0.058 and p=0.058, respectively) (Figure 6.5-20). When the F1 generations were analyzed separately, pancreas weights of F2 chicks from treated (F1a) parents were found to be significantly affected by an interaction between the P1 exposure scenario and dietary concentration of their parents (p<0.03). In F2 chicks with F1a-P1B parentage, pancreas weights were significantly increased in parents exposed to the 5 ppm E2 diet (p<0.001). Chicks with F1b-P1B parentage also showed significant changes in pancreas weight; however, no meaningful relationship between P1 dietary concentration and pancreas weight was observed (Figure 6.5-21). No significant dietary effect on pancreas weight was observed in F2 chicks with F1a-P1A or F1b-P1A parentage.

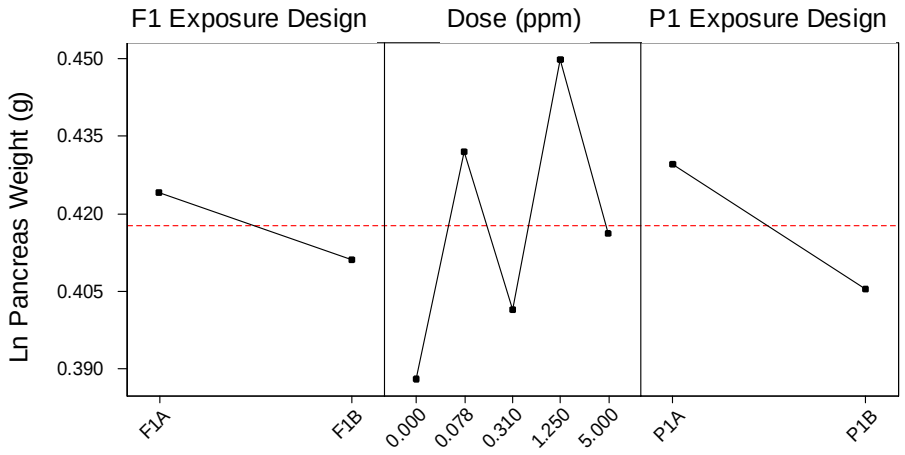


Figure 6.5-19. **Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on natural log-transformed pancreas weight of F2 males at 2 weeks of age** (General Linear Model analysis: significant difference between P1 exposure scenarios, p=0.013 and between P1 dietary concentrations, p=0.003). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

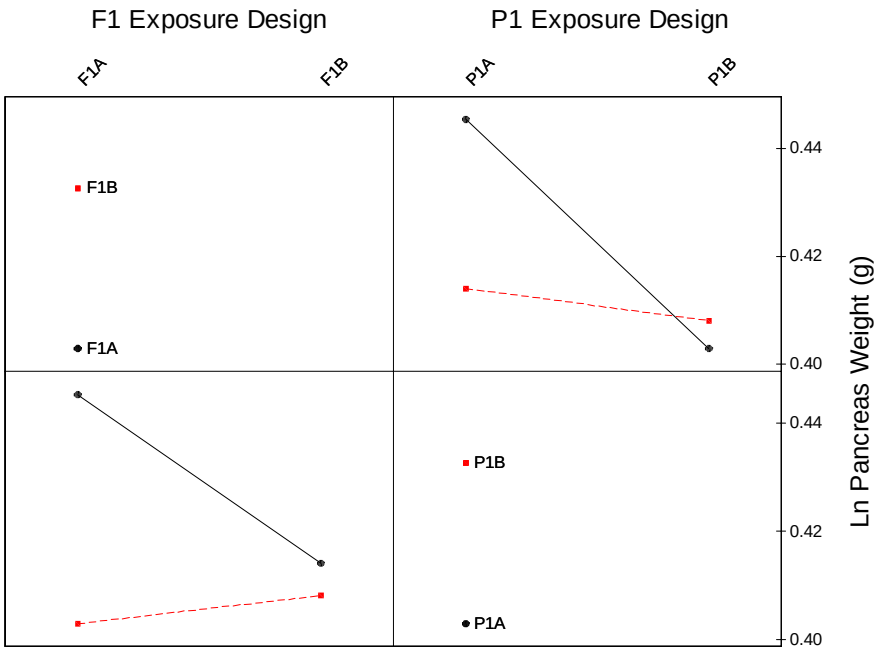


Figure 6.5-20. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the natural log-transformed pancreas weight of F2 male chicks at 2 weeks of age. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

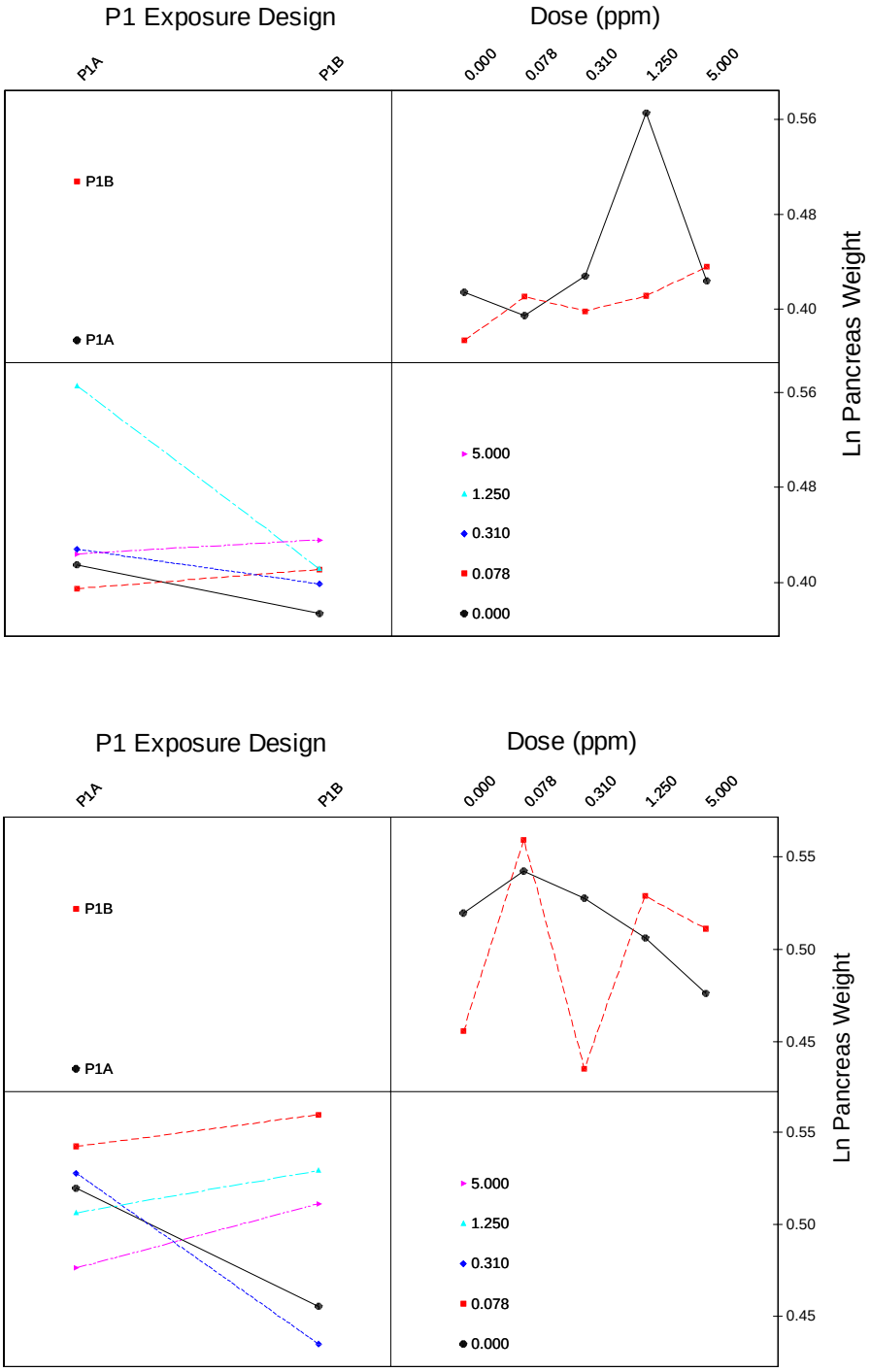


Figure 6.5-21. Interaction of P1 dietary concentrations and P1 exposure designs from General Linear Model analysis of the natural log-transformed pancreas weight of 2-week-old male F2 offspring of untreated (F1a) parents (above) and F1b parents (below). (P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

Spleen

P1 dietary concentrations significantly affected absolute spleen weights in F2 male chicks (p=0.036), but not linearly (Figure 6.5-22). The dietary treatment effect was reduced (p=0.138) when the spleen weights were normalized to body weight. No dietary E2 effect was observed in spleen-to-brain weight ratios (p=0.871). No other effects on absolute or relative spleen weights in F2 male chicks were significant (p>0.30).

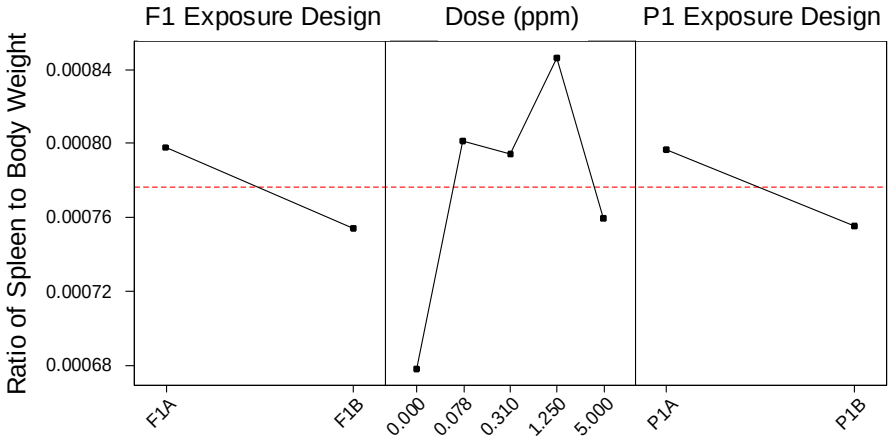


Figure 6.5-22. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on spleen-to-body weight ratios of F2 males at 2 weeks of age (General Linear Model analysis: P1 dietary concentration effects, p=0.138). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Brain

Absolute brain weights in F2 male chicks were significantly affected by the F1 exposure (F1a or F1b) of their parents (p=0.040). Nearly significant P1 dietary concentration and P1 exposure design effects on gross brain weight in the chicks were also detected (p=0.133 and p=0.08, respectively). However, when the brain weights were normalized to body weight, F1 exposure design differences was no longer significant (p=0.569) and the P1 exposure design effect was also lost (p=0.214). In contrast, the nearly significant dietary concentration effect became a significant effect (p=0.039) after normalization of the brain weight to body mass (Figure 6.5-23). The dietary dose-response was nonlinear.

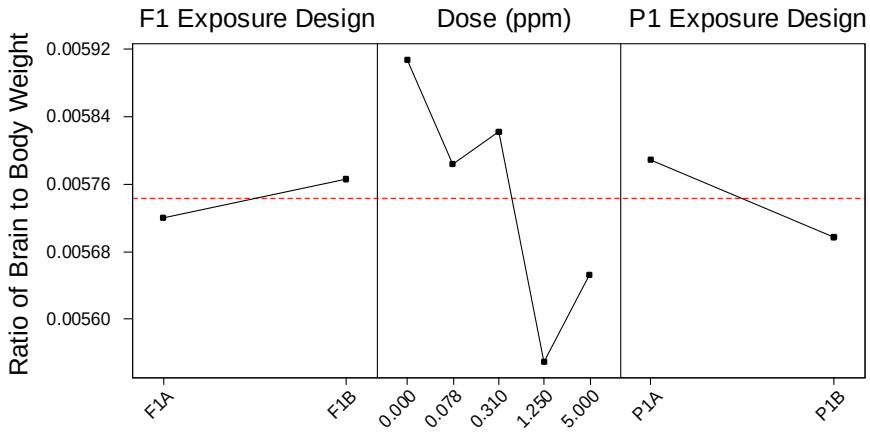


Figure 6.5-23. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on brain-to-body weight ratios of F2 males at 2 weeks of age (General Linear Model analysis: significant difference between P1 dietary concentrations, p=0.039). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Testis

Gross weights of the left testis of F2 males were not affected by F1 or P1 exposure designs of their parents (p>0.157), but a nearly significant (p=0.144) interaction between the F1 and P1 designs was detected when the testis weight was normalized to body weight. The F2 offspring of F1 parents with the greatest combined direct and *in ovo* E2 exposure (F1a-P1A) had reduced left testis-to body weight ratios (Figure 6.5-24) compared to F2 chicks of untreated (F1b) parents or those with parents having P1B parents. Dietary treatment concentration also tended to affect left testis absolute and relative weights (p=0.104 and p=0.121, respectively), but the response was not linear (Figure 6.5-25).

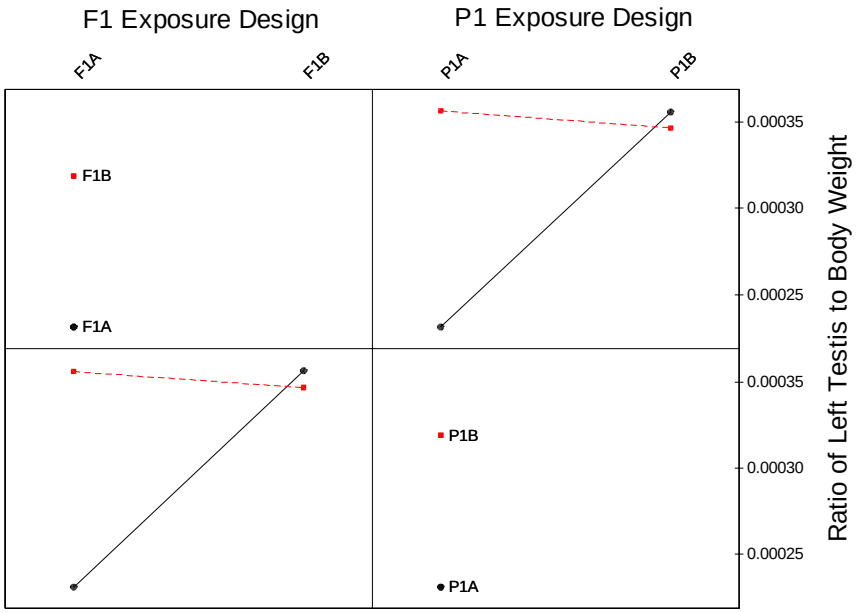


Figure 6.5-24. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the left testis-to-body weight ratios of F2 male chicks at 2 weeks of age. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

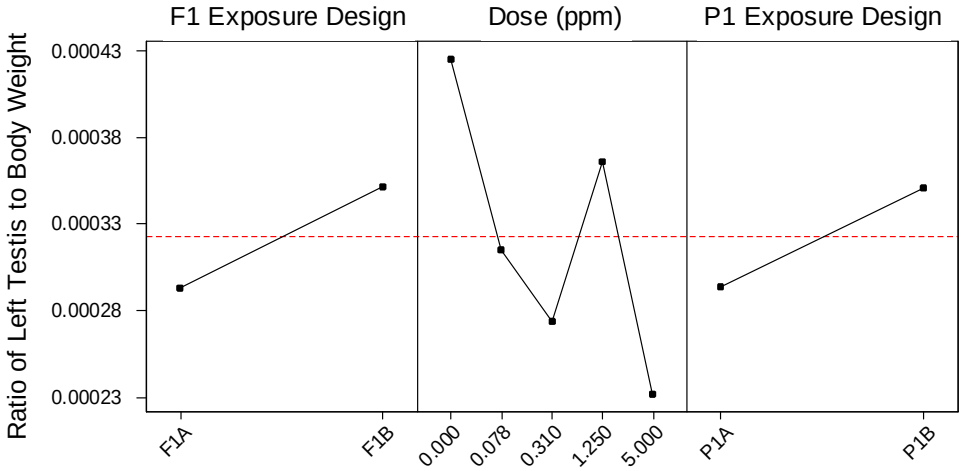


Figure 6.5-25. Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on left testis-to-body weight ratios of F2 males at 2 weeks of age (General Linear Model analysis: nearly significant difference between P1 dietary concentrations, p=0.144). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

Absolute weights of the right testis of F2 chicks were significantly affected (p=0.001) by dietary concentration history of their parents, but not by F1 or P1 exposure design (p>0.23). After normalization of the right testis weight to body weight the dietary concentration effect was nearly significant (p=0.051). In both measures, the concentration-response was not linear (Figure 6.5-26). A nearly significant (p=0.137) interaction between the F1 and P1 exposure designs affecting testicular weight ratio similar to that seen for the left testis-to-body weight ratio was also detected (Figure 6.5-27). F2 chicks with F1a-P1A parents tended to have reduced right testis-to-body weight ratios compared to other exposure design combinations.

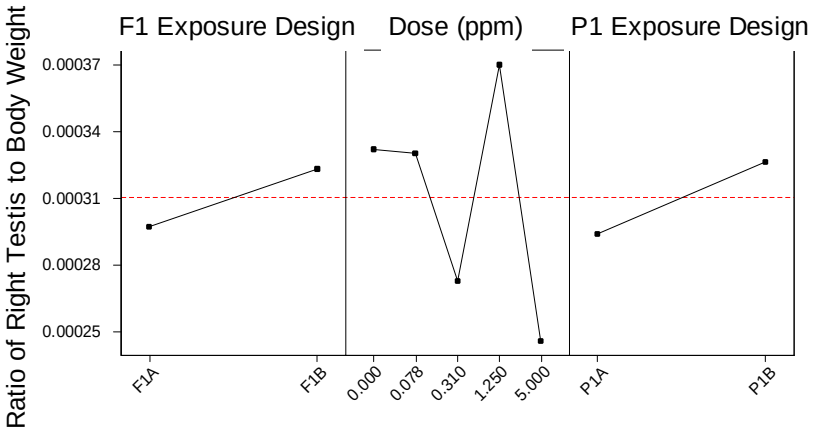


Figure 6.5-26. **Effects of F1 exposure strategy (F1a, treated; F1b, untreated), dietary treatment with E2, and the parental exposure scenario (P1A, exposed to dietary E2 from pre-puberty through reproduction; P1B, exposed post-maturation) on right testis-to-body weight ratios of F2 males at 2 weeks of age** (General Linear Model analysis: nearly significant difference between P1 dietary concentrations, p=0.051). F1a birds were fed the same dietary treatments as their parents; all F1b and F2 chicks were untreated.

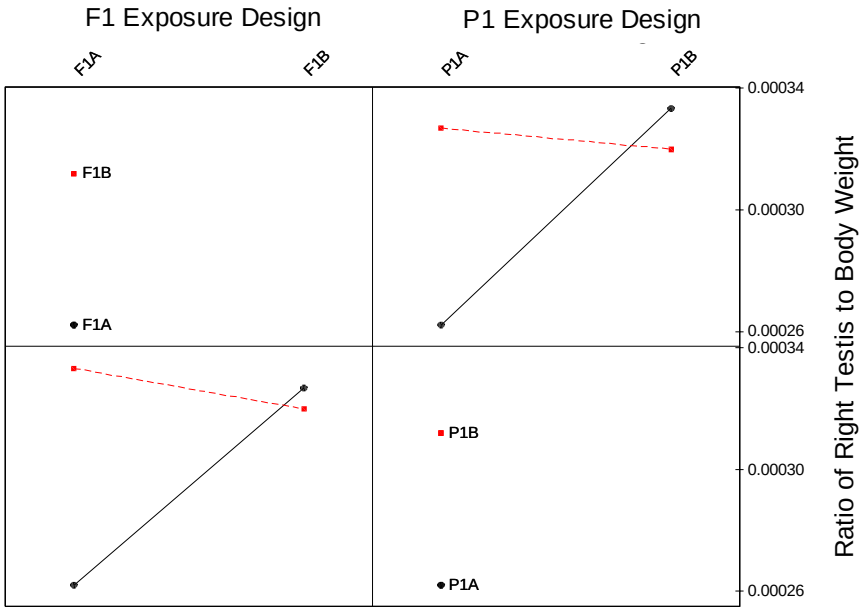


Figure 6.5-27. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the right testis-to-body weight ratios of F2 male chicks at 2 weeks of age. (F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.) All F1b and F2 chicks were untreated.

No significant difference in the ratio of left testis weight to right testis weight in F2 chicks was found between the F1 exposure strategy (p=0.565), P1 exposure scenario (p=0.768), and the dietary treatment (p=0.807) history of their parents.

Cloacal Gland

Gross cloacal gland weight of 14-day-old F2 chicks were nearly significantly affected (p=0.089) by an interaction of the F1 and P1 exposure designs of their parents. No parental dietary concentration effect was apparent (p=0.83). When the gland weights were normalized to body weight, the interaction was statistically significant (p=0.007), but the interaction was reduced (p=0.119) with normalization to brain weight (Figure 6.5-28). Offspring of treated (F1a) parent whose parents were exposed prior to puberty (P1A) had cloacal glands of greater weight than offspring of untreated parents (F1b) under the same P1 exposure. The P1B exposure scenario did not appear to interact with the F1 scenario (Figure 6.5-28).

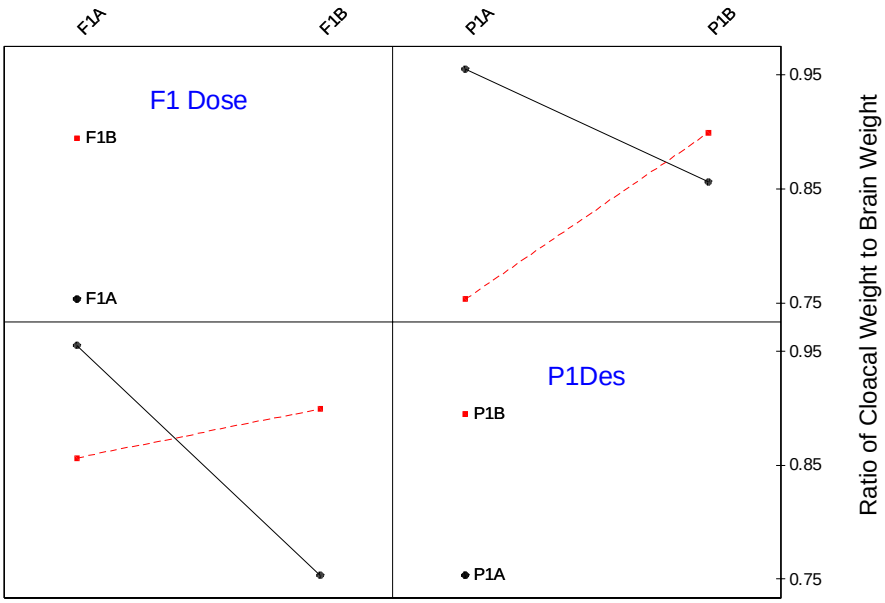


Figure 6.5-28. Interaction of F1 and P1 exposure designs from General Linear Model analysis of the ratio of cloacal gland weights to brain weight of F2 male chicks at 2 weeks of age. F1a, treated in F1 generation; F1b, untreated in F1 generation; P1A, parents exposed to dietary E2 from pre-puberty through reproduction; P1B, parents exposed to E2 post-maturation.

6.6 Histology (F2)

No treatment-related effects were found in any of the organs examined in the F2 generation. Reproductive organs (testes, epididymis, cloacal gland, ovary, and oviduct) were all immature and the rapidly growing cells of the ovary and testes had not begun to proliferate. Histopathology of thyroid tissue from all chicks was not remarkable. Appendix J contains the pathology evaluation and incidence data.

6.7 Summary of the Results of the Second Generation Offspring (F2)

Body weight of females of the F2 generation at hatch and at 2 weeks of age was not altered by *in ovo* exposure to E2. One measure of survivorship (number of 14-day-old survivors/number of eggs set) was affected by an interaction of the F1and P1 exposure scenarios of their parents, with more surviving offspring produced by treated (F1a) parents having *in ovo* E2 exposure from P1A parents. The number of offspring per maximum number of eggs increased over time for hens exposed to dietary E2 (F1a). F1 and P1exposure designs interacted to affect the mass of several organs of the second generation offspring. Chicks receiving *in ovo* exposure from F1a-P1A parents were the most affected, having significantly or nearly significantly reduced measures of growth (tibiotarsus diameter and tarsometatsus length) ovary-to-body weight ratios and increased gross and body weight-adjusted pancreas weights of the F1a 5 ppm treatment groups. F2 chicks of F1a parents also had greater absolute and relative thyroid weights and greater gross spleen weight. *In ovo* exposure from F1a-P1B parents increased adrenal gland mass of the F2 females. Mean gross and relative liver weights were significantly increased in offspring of F1b-P1b parents. P1A exposure significantly lowered gross and body weight-normalized ovary weights. Chicks with P1B parentage had increased incidence of limb malformations. No histological changes of organs were found in female F2 chicks.

In contrast to the F2 female chicks, body weight of second generation male offspring were affected by the F1 exposure strategy of their parents. F2 male chicks of F1a parents weighed less than male offspring of untreated (F1b) parents. Measures of bone growth were also smaller in F2 males with F1a parents. An interaction of the F1a and P1A exposure scenarios of their parents affected gross or relative organ weights of F2 males from F1a parents, increasing the mean weight of the thyroid, pancreas, liver and cloacal gland. The same interaction decreased testis weight. Significantly greater mean gross and relative weights of the liver were detected in males from F1a-P1A parents, and gross and relative weights of the pancreas were found in chicks with P1A parentage. Gross weights of the adrenal gland and brain were nearly significantly increased in chicks from P1A exposed (*in ovo*) parents. Brain weight was significantly decreased in F2 males of F1a parents. When normalized to body weight, no effect of P1 exposure scenario on adrenal gland or brain weight was found. None of the effects observed were concentration linear. Histological changes in the F2 generation were not remarkable.

Tables 6.7-1 (females) and 6.7-2 (males) summarize the results of the endpoint measurements obtained for the second generation offspring (F2).

Table 6.7-1. Summary of results for F2 females.

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Hatchling Body Weight	None	None	Not linear
2-Week-Old Body Weight	None	None	None
Tibiotarsus			
Length	None	P1A slightly smaller[a]	Not linear[a]
Diameter	Interaction F1a-P1A smaller than F1b-P1A[a]; P1B no affect on F1	None	Not linear
Weight	None	None	Not linear
Tarsometatarsus Length	Interaction F1a-P1A smaller than F1b-P1A[a]; P1B no effect on F1	None	None
Organ Weight			
Thyroid-Gross	F1a greater (difference small)	None	Not linear[a]
Thyroid/Body Weight	F1a greater	None	None
Thyroid/Brain Weight	Interaction F1b-P1B smaller than F1b-P1A (due to interaction of P1A and 0.31 ppm diet)[a]	None	None
Adrenal Gland-Gross	Interaction F1a-P1B >F1a-P1A[a]; P1A no effect on F1	None	None
Adrenal Gland/Body Weight	Interaction F1a-P1B >F1b-P1B[a]; P1A no effect on F1	None	None
Adrenal Gland/Brain Weight	Interaction-F1a-P1A < F1b-P1A; F1b-P1A > F1a-P1B	None	None
Pancreas-Gross	Interaction-F1a-P1A > F1a-P1B	None	F1a-5 ppm
Pancreas/Body Weight	Interaction-F1a-P1A > F1a-P1B	None	F1a-5 ppm
Pancreas/Brain Weight	None	P1A greater[a]	None
Liver-Gross	F1a increase (Interaction F1b-P1B > F1b-P1A[a])	None	None
Liver/Body Weight	F1a increase (Interaction- F1b-P1B > F1b-P1A)	None	None
Liver/Brain Weight	None	None	None
Spleen-Gross	F1a greater	None	> 0.078
Spleen/Body Weight	None	None	None
Spleen/Brain Weight	None	None	Not Linear

Table 6.7-1. Summary of results for F2 females (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Brain-Gross	None	None	None
Brain/Body Weight	None	None	Not linear[a]
Ovary-Gross	None	P1A lower	None
Ovary/Body Weight	Interaction-F1a-P1A lower[a]	P1A lower	None
Ovary/Brain Weight	F1a lower[a]	None	None
Oviduct-Gross	None	None	None
Oviduct/Body Weight	None	None	None
Oviduct/Brain Weight	None	None	None
Gross Abnormalities			
Incidence of Rt. Ovary	None	None	None
Incidence of Rt. Oviduct	None	None	None
Incidence of Neck Curvature	None	None	None
Incidence of Foot/Leg	None	P1B greater [a]	Not linear
14-Day-Old Survivors			
Survivors/Normal Hatchlings	None	None	Not linear
Survivors/Number Eggs Set	Interaction F1a-P1A > F1a-P1B [a]; F1b not affected by P1	None	Not linear[a]
Survivors/Max Eggs Set	None	None	Not linear
production over time	F1a increased[a]	None	None

Note a. $p \leq 0.15$

Table 6.7-2. Summary of results for F2 males.

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Hatchling Body Weight	F1a smaller (small difference)	None	Not linear
2-Week-Old Body Weight	F1a smaller [a]	None	Not linear
Tibiotarsus			
Length	F1a smaller [a]	None	Not linear
Diameter	None	None	None
Weight	F1a smaller [a]	None	Not linear
Tarsometatarsus Length	F1a smaller [a]	None	None
Organ Weight			
Thyroid-Gross	Interaction F1a-P1A greater than all other treatment combinations[a]	None	None
Thyroid/Body Weight	F1a greater	None	None
Thyroid/Brain Weight	None	None	None
Adrenal Gland-Gross	None	P1A greater[a]	None
Adrenal Gland/Body Weight	None	None	None
Adrenal Gland/Brain Weight	None	None	None
Pancreas-Gross	P1A increase (Interaction-F1a-P1A > F1a-P1B[a])	P1A greater	Not linear
Pancreas/Body Weight	P1A increase (Interaction-F1a-P1A > F1a-P1B[a])	P1A greater	F1a-P1B 5 ppm greater
Pancreas/Brain Weight	P1A increase (Interaction-F1a-P1A > F1a-P1B[a])	None	None
Liver-Gross	F1a increase (Interaction F1a-P1A > F1a-P1B [a]; F1b not affected by P1)	None	Not linear
Liver/Body Weight	F1a increase (Interaction F1a-P1A > F1a-P1B [a]; F1b not affected by P1)	None	Not linear[a]
Liver/Brain Weight	None	None	None
Spleen-Gross	None	None	Not linear
Spleen/Body Weight	None	None	Not linear[a]
Spleen/Brain Weight	None	None	None
Brain-Gross	F1a smaller	P1A greater[a]	Not linear[a]
Brain/Body Weight	None	None	Not linear

Table 6.7-2. Summary of results for F2 males (continued).

Parameter	F1 Effect	P1 Effect	Dietary Concentration Effect
Left Testis-Gross	None	None	Not linear[a]
Left Testis/Body Weight	Interaction-F1a-P1A <F1b-P1B[a]	None	Not linear[a]
Left Testis/Brain Weight	None	None	None
Right Testis-Gross	None	None	Not linear
Right Testis/Body Weight	Interaction F1a-P1A smaller than all other treatment combinations[a]	None	Not linear[a]
Right Testis/Brain Weight	None	None	None
Testis Asymmetry	None	None	None
Cloacal Gland-Gross	Interaction-F1a-P1A greater[a]	None	None
Cloacal Gland/Body Weight	Interaction-F1a-P1A greater	None	None
Cloacal Gland/Brain Weight	Interaction-F1a-P1A greater[a]	None	None
Gross Abnormalities			
Incidence of Organ Lesions	None	None	None
Incidence of Foot/Leg	None	None	None

Note a. $p \leq 0.15$

7.0 DISCUSSION

Mating the P1 generation birds post-puberty resulted in a high level of aggression of females towards their male pen mates resulting in the separation of all pairs and in deaths from aggression that contributed to reduced pair numbers in the P1A exposure scenario. However, a greater number of males were injured and died as a result of female aggression under the P1B exposure scenario than under the P1A scenario. Because P1A males had been exposed to E2 for several weeks prior to pairing and the P1B males had not yet been exposed to the estrogen, less female aggression towards males in the P1A scenario may have been a result of some degree of behavioral feminization and/or the plumage feminization of the P1A males. Lacking the direct measurements of a behavioral test, this observation could not be confirmed. Because of the post-maturation exposure of the P1B population, loss at pairing was ameliorated by the substitution of non-injured pairs and underscores the potential statistical advantage of this exposure regime. Note that birds were paired prior to maturation in the F1 generation and pecking injuries and deaths of males were greatly reduced. However, increasing feather loss and pecking injuries of females from male mounting attempts necessitated separation of pairs in the F1 generation as well.

The loss of pairs to aggression in the P1A groups and the subsequent reduction in hatchlings from which to draw the F1 generation compounded with the treatment effects to greatly reduce the pairs in some groups of the F1 generation. This is further discussed in the Sex Ratio section below.

Of the endpoints measured in the P1 generation, few showed dose-response relationships. In birds exposed under the P1A regime, a linear dose-response relationship was detected only for egg shell breaking strength, female-type plumage length in males and females, and the proportion of males with mixed plumage (for concentrations greater than 0.078 ppm). Those endpoints that had an increasing or decreasing effect or trend with increasing dietary concentration in birds under the P1B exposure scenario occurred in decreased males' fecal-urate testosterone levels and increased incidence of hypertrophy of the adrenal gland (based on 0 ppm and 5 ppm treatments only). There was also a trend for increased egg steroid content, increasednumber of eggs cracked per number of eggs laid over time, increased breaking strength over time, and increased production of viable eggs (Day 8) over time to change with increasing dietary concentration under both P1 exposure scenarios. A tendency for a non-linear decrease in tibiotarsus length in P1A males was also detected. In the F1 generation, even fewer effects were observed that increased or decreased with increasing dietary concentration. These effects included increased incidence and length of non-male plumage in F1a males, and trends for decreased gross cloacal gland weight in F1a males, and decreased pancreas to body weight index in treated male offspring of P1B birds. In females, trends of increased shell thickness over time and increased variability in shell stiffness over time were detected. However, many more non-linear responses were observed in the F1 birds than in the P1generation and included some measures of growth, sexual maturation, aggression, plumage dimorphism, and shell quality, and the gross and/or relative weights of several organs (pancreas, liver, and spleen weights in females; thyroid, right testis, and spleen in males). F2 chicks were not fed treated diet; therefore their exposure to E2 was from *in ovo* levels of the steroid. Only two endpoints displaying increased effect with increased dietary concentration of E2 were found in the F2 generation. In offspring of F1a parents, the mean gross weight of the spleen in females and the mean pancreas

body weight index in both male and female chicks was increased. Non-linear responses to the dietary exposure history of their parents was observed in several gross and/or relative organ weights in both sexes of the F2 chicks and in the tibiotarsus length and weight of male and female chicks. The lack of linear dose-response may be due to low test concentrations, wherein the 5 ppm concentration appears to be only minimally effective in inducing measurable changes in many endpoints. Although steroid receptor-mediated dose-response relationships may not be monotonic (Welshons et al. 2003), the non-linear dose-response patterns in the F1 and F2 offspring of P1A parents observed in this study appear to be impacted by reduced F1 pairs in some groups caused by aggression losses, reduced production of males, and lack of non-sibling mates.

Those endpoints for which a significant or nearly significant response was detected are discussed below relative to exposure scenario and generation affected.

Secondary Sex Characteristics and Sexual Maturation

In the P1 generation, plumage changes were detected in both male and female quail. Significantly increased incidence of males with phenotypic female plumage or plumage with characteristics of both male and female feathers occurred in males that began E2 treatment prior to maturation (P1A). Dietary treatment with E2 in the F1 generation (F1a) also resulted in increased incidence of males with non-phenotypic male plumage compared to untreated (F1b) males.

The length of female-type coloration (spotted area) covering the breast was significantly increased in female birds of the parental generation fed the 5 ppm diet under the P1A exposure regime. In the F1 generation, the F1a (treated) exposure strategy resulted in increased length of the spotted area in males with the length of the feminized plumage increasing linearly as a function of increasing dietary concentration. In contrast, the length of the spotted area in F1 females did not respond linearly to increasing dietary concentration and was decreased in treated hens. The P1 exposure scenario (*in ovo* dose) of their parents had no effect on either the proportion of F1 males with non-male plumage characteristics or the length of the feminized feathers. Size of the female-type coloration area has been previously used to quantify the degree of feminization in male Japanese quail (Hutchison 1978) and appears, from the current study, to also have application in monitoring exposure to exogenous estrogens in females.

Two other secondary sex characteristics, body size over time and functional maturation of the cloacal gland, also appeared, statistically, to be affected by E2 exposure in male birds of the P1 generation, but the effects were biologically inconclusive at the E2 concentrations tested. Growth rate of maturing P1A males was nearly significantly increased over the P1B population which had yet to receive dietary treatments under their exposure scenario, with the 5 ppm E2 diet group having the greatest mean weight. However, increased growth rate was not maintained post-maturation, and adult males did not attain increased (female-typical) size at the dietary E2 concentrations tested. While cloacal gland size also did not appear to be affected by dietary exposure to E2, functional maturation of the gland as indicated by secretion of proteinaceous foam occurred earlier in the males exposed prior to maturation. The detected difference between the two populations, however, was small and the differences between the test groups of the treated population at that age (P1A) were statistically and biologically insignificant. Still, when only males with feminized plumage were considered, maturation of P1A males occurred 2 days

earlier compared to P1B males. Sexual maturation and development of secondary sex characteristics other than plumage attributes of the P1females were unaltered by E2 exposure under both exposure scenarios.

While increased body size after maturation and subsequent initiation of treatment of the P1B males was not maintained in P1A treated males, offspring of P1A birds that were fed E2 treated diets from hatch (F1a) had increased body weight at necropsy. Male offspring of P1B birds did not have increased body weights at necropsy. Mean cloacal gland area at necropsy was also significantly increased in treated male offspring of P1A parents (F1a-P1A), though the size of the gland was not different from controls or other treatment combinations prior to or at maturation. An interaction between F1 and P1 designs also affected the functional maturation of the cloacal gland (first foam production) in F1 males, with offspring of the P1A parents reaching sexual maturation several days later than offspring of P1B parents. Treated (F1a) females tended to mature (begin egg laying) later and grow slower post-puberty than untreated F1 females (when extra treated but unmated birds were included in the analysis).

Body weight of the two-week-old F2 female chicks were not affected by the exposure history of their parents, but the body weight of male F2 chicks were affected by the F1 exposure strategy of their parents with male offspring of F1a parents weighing less than male offspring of untreated (F1b) parents.

Bone Growth

No clear effect of E2 exposure on growth measures of the tibiotarsus or the tarsometatarsus of both males and females of the P1 generation prior to or post-puberty could be discerned from the exposure data at the test concentrations used. In the F1 generation, the diameter of the tibiotarsus was significantly decreased in treated F1a females. Tibiotarsus weight tended to be affected by interaction of the F1 and P1 exposure designs in both male and female F1 quail. In males, treated offspring of P1A parents that consumed 5 ppm E2 had increased bone weights; in females a tendency for a reduction in bone weight in F1b-P1A hens was detected. Measures of growth of the tibiotarsus (length and weight) and tarsometatarsus (length) were smaller in F2 males with F1a parents. In F2 female chicks, the offspring of F1a-P1A parents tended to have decreased tibiotarsus diameter and tarsometatarsus length. For females, tibiotarsus length tended to be slightly decreased in offspring of F1 parents with P1A exposure history.

Histological Changes

Microscopic evaluation of tissue changes in reproductive organs of female quail treated with E2 showed significant alterations between control and treated birds exposed under the P1A regime, but differences attributable to exposure scenario (P1A or P1B) were not clear. No histological changes of reproductive organs were found in the female offspring of the P1 generation.

In males, histological changes of the reproductive organs were detected with greater incidence and severity in the P1A exposure scenario compared to the P1B design. Reduced numbers of primary and secondary spermatocytes and increased cytoplasm granularity in seminiferous tubules were evident in all groups given E2 treated diets from pre-puberty through adulthood and after the lengthy exposure (13 weeks) appeared to equally affect all groups. Post-puberty

exposure to E2 for 5 weeks induced the degenerative lesions only in the 5 ppm treatment group (Table 4.6-6), indicating that E2-induced changes of the testis were detected at much lower treatment levels under the P1A exposure scenario. Reduction of mature spermatids in the epididymes was similarly high in incidence and detected in all but control males in the P1A exposure design. No clear induction of hypospermia by E2 was detected in the post-puberty exposure scenario. Also, early exposure (P1A) to E2 appeared to cause cellular atrophy in the cloacal gland in a concentration dependent manner. The lesion was absent in birds exposed after puberty (P1B). Incidence and severity of gonadal degeneration and hypospermia were much lower in male offspring of the P1 birds whether they received dietary treatment from hatch (F1a) or not (F1b). Cloacal gland lesions observed in the F1 generation were largely found in the offspring of P1B parents.

Of the remaining organs examined histologically, only the adrenal gland, the most susceptible of the endocrine organs to chemically induced structural alteration, was found to be affected by dietary E2 in either male or female quail in the P1 generation. In both sexes, diffuse hypertrophy of the cortical and medullary cells was observed. However, in males, the lesion was detected only in those males exposed for 13 weeks to E2 (only the control and 5 ppm diet groups were examined in both sexes), whereas in females the hypertrophy was found in the 5 ppm treatment groups of both the P1A and P1B exposure scenarios, but with over twice the incidence rate in birds exposed after sexual maturation. In the F1 generation, the adrenal hypertrophy was observed in untreated males of either P1A or P1B parents, whereas the incidence of hypertrophy was decreased in F1b females.

An additional tissue was found to be affected by E2 exposure in females of the F1 generation. In all groups of female offspring fed 5 ppm E2, more than half the birds had had mineralized hepatic tissue except the F1a-P1A group, which had no incidence of mineralization. Interestingly, an opposite outcome would be anticipated as mineralization of liver and other soft organs in birds often accompanies over administration of vitamin D_3, which plays an important role in mineral dynamics, an effect that can be duplicated by administration of exogenous estrogen (Howard et al. 2002). Exogenous estrogen increases the production of 1α–hydroxylase in the kidney, thereby increasing the production of the active form of vitamin D_3.

Histological changes of all organs examined for the F2 generation were not remarkable.

Organ Weights and Gross Morphology

Size and morphological changes of reproductive organs and their accessory structures are primary endpoints for assessing the impact of chemicals on the development and integrity of the reproductive system. At the concentrations tested, no changes in the mass of these organs in response to dietary E2 under either P1 exposure scenario were detected in male or female quail. Notably, testicular weight, often used as an indicator of possible changes in reproductive condition (Zenic and Clegg 1989), was not altered by E2 exposure in adult male quail despite the pronounced reduction of spermatocyte production in E2 treated birds. However, the mean number of fertile eggs per pair, as measured by their viability at Day 8 of incubation, was not decreased by exogenous E2 under either the P1A or P1B exposure design, indicating that the observed spermatocyte reduction was not severe enough to alter reproductive condition. The only gross alteration observed in reproductive tissues of the P1 generation was increased

occurrence of right oviducts in females. Overall, hens exposed to E2 after the onset of puberty (P1B) had over 4 times the incidence of right oviducts compared to those exposed from prior to maturation (P1A), but the greatest increases were found in the groups consuming the two lowest E2 concentrations.

In contrast to the P1 generation, weights of reproductive organs were altered by E2 exposure in the F1 generation. In females, the gross and relative weights of the left ovary tended to be reduced in F1a birds and oviduct gross weight and body weight index were significantly increased in treated offspring (F1a) of P1A parents. The number of active oocytes in the ovary were also increased in the F1a-P1A birds, but incidence of right oviducts were not related to F1 or P1 exposure designs. Testicular weight tended to increase in E2 treated F1a-P1A males. Absolute cloacal gland weight also tended to be affected by the F1a dietary exposure (decreased in the 5 ppm treatement groups). Normalized to body weight, the cloacal gland tended to be decreased in weight in treated male offspring of P1A birds.

Female F2 chicks of F1a or F1b parents with P1A parentage had significantly lower mean gross ovary weights and mean ovary-to-body weight ratios than F2 female offspring of parents with P1B *in ovo* exposure history. An interaction of the F1a and P1A exposure scenarios of their parents affected gross and/or relative organ weights of F2 males, decreasing the mean weight of the left and right testes and increasing the mean gross and relative weights of the cloacal gland.

The adrenal gland was the only organ that exhibited weight change in response to exposure scenario in the parental generation. The diffuse hypertrophy of adrenal tissue in the P1B females was reflected in the increased weight of the gland in the birds in the P1B exposure scenario; however, no increase in adrenal gland weight was observed in P1A males that also exhibited the lesion. No weight changes of the adrenal gland were induced by E2 exposure in the F1 generation. *In ovo* exposure from F1a-P1B parents tended to increase adrenal gland mass and gland-to-body-weight index of F2 females. In male F2 chicks, the gross weights of the adrenal gland were nearly significantly increased in chicks from P1A exposed (*in ovo*) parents, but when normalized to body weight, the increase was no longer detected.

Weight change was observed in a number of organs of F1 birds in addition to those of the reproductive tract. As observed for the reproductive tissues, changes in organ weights were induced under the F1a exposure strategy in both male and female quail. In F1a females, the gross and relative thyroid and spleen weights tended to be increased, whereas pancreas weights were decreased under this exposure regime. Organ weights of the pancreas, liver, and brain were altered in male F1a offspring of P1A parents.

The same organs that were affected by dietary treatment under the F1a exposure design in F1 females were affected in the F2 generation by *in ovo* exposure from F1a parents. F2 female chicks of F1a parents had greater absolute and relative thyroid weights and greater gross spleen weight. The mean gross and body-weight-adjusted pancreas weights of F2 female offspring of F1a parents were significantly increased in the 5 ppm treatment group. Additionally, the mean absolute and relative weights of the liver of F2 female chicks of F1a parents were significantly increased. F2 females with P1B parentage had increased incidence of limb malformations. In F2 males, gross weights of the brain were significantly decreased in offspring of F1a exposed parents and nearly significantly increased in chicks from P1A exposed (*in ovo*) parents. The

liver was significantly increased in gross and relative weight in offspring of F1a parents with a tendency for chicks of F1a-P1A parents to have greatest liver weight. Gross and relative weights of the pancreas of F2 male chicks were increased in those with P1A parentage. A tendency for elevated weights from F1a-P1A parents was also detected. Mean thyroid-to-body weight ratio of the thyroid of male offspring of F1a treated parents was increased.

Fecal-Urate Steroid Concentration

Fecal-urate concentrations of estrogen increased as a function of dietary E2 and were similar whether the birds were exposed for 13 weeks (P1A) or 5 weeks (P1B) or were male or female. A significant reduction in median fecal-urate testosterone concentration with increasing dietary exposure to E2 was observed for P1B males, but not in males from the P1A exposure population. However, when averaged over the exposure scenarios (i.e., P1A and P1B populations), mean testosterone levels tended to decrease with increasing E2 concentration. Steroid excretion in offspring of the P1 birds was not monitored.

Steroid Content of Eggs

Estrogen levels in egg yolk increased with increasing dietary concentration of E2 and were significantly affected by the exposure scenario. The long (13 week) exposure initiated prior to maturation (P1A) had E2 burdens in eggs up to twice that found in birds exposed post-maturation. Therefore, the dose to the developing embryos of the F1 generation was relatively maximized within the P1A exposure scenario. Testosterone levels in eggs laid by P1A or P1B birds did not respond linearly to the dietary treatments; however, E2 treatment appeared to increase testosterone concentrations in eggs laid by hens under the P1A exposure scenario and decrease testosterone concentrations in eggs laid by E2-treated hens exposed under the P1B design.

In contrast, median E2 concentrations in eggs laid by treated hens (F1a) from the F1 generation were not increased over dietary concentration nor were they greater than concentrations found in eggs from untreated (F1b) hens of that generation. The *in ovo* dose available to the F2 generation in all treated groups was greater (1.1 to 4.3 fold) than the *in ovo* exposure of their parents. The E2 concentrations in control eggs laid by the F1 generation were also substantially elevated (2.3 to 3.5 fold) over concentrations in eggs of the controls from the P1 hens. It is not known why the control levels of estrogen were greater in the F1 generation. Cross contamination of samples during the study was below the MDL, indicating no contamination of the test diets in the feed troughs. With the higher background values of the estrogen in eggs laid by the F1 birds and the relatively small increases in estrogen content of the yolk with dietary exposure as observed in the P1 yolk levels, it is likely that small concentration increases would not be discernable above the variability within the treatment groups.

Testosterone levels in eggs (including controls) laid by F1 hens were also elevated (1.5 to 4.7 fold) over levels detected in the eggs of the P1 populations.

Egg Shell Quality

Of the egg shell quality measures made, only shell thickness showed a difference between exposure scenarios. Eggshell thickness decreased over time in eggs laid by P1B hens consuming control or the lowest E2 concentration diet, whereas eggs laid by P1B birds on higher E2 diets tended to increase in thickness over time. Shell thickness of eggs laid by P1A birds did not change over time. Dietary exposure to E2 in the F1 generation (F1a) resulted in increased shell thickness (when unmated extra hens were not considered in the analysis). The proportion of eggs with cracked shells laid per treated F1a hen also decreased over time. In contrast, the proportion of cracked eggs per hen increased in untreated hens from P1B parents. This appears to be in keeping with the increased mobilization of calcium that can occur when exogenous estrogen increases the production of 1 α–hydroxylase and thereby the production of the active form of vitamin D_3 which plays a key role in calcium mobilization during egg laying. (Howard et al. 2002).

Reproductive Parameters

No difference in the reproductive endpoints of egg and hatchling production and viability were detected for either of the P1 exposure scenarios. However, Day 15 viability of eggs over time and hatchling production over time were increased in eggs and F2 hatchlings produced by hens raised under the F1a exposure design. Increased hatchability and production have been related to elevated levels of parathyroid hormone (PTH) effects on circulating calcium levels in birds (Yagil et al. 1993). PTH stimulates production of the active form of vitamin D_3 and subsequently increasing absorption of calcium, an effect that is also induced by exogenous estrogen (Norris 1997).

Whereas female offspring of P1A parents had lower hatch weights than those from P1B parents, males were unaffected by P1 exposure scenario. For F2 chicks, the F1 and P1 exposure history of their parents had no effect on hatch weights of female offspring of the F1 generation, but male offspring of F1a parents had decreased body weights. However, the overall mean difference between the F1a and F1b exposure scenarios was small (~2%) and may have been detected because of the large degrees of freedom (over 400) of the analysis.

The number of 14-day-old survivors was affected by dietary exposure history of their parents, but the responses were non-linear. Only one measure of survivorship (number of 14-day-old survivors/number of eggs set) was affected by E2 exposure. An interaction of the F1and P1 exposure scenarios of their parents was observed, with more surviving offspring produced by treated (F1a) parents having *in ovo* E2 exposure from P1A parents. The production of F2 offspring per maximum number of eggs that survived to 14 days of age increased over time for hens exposed to dietary E2 (F1a).

Sex Ratio

A pattern of elevated male to female ratios in F2 chicks of F1a-P1A parents from the lower dietary treatment groups and a reduction in the sex ratio at higher treatment concentrations was observed in the chicks hatched from eggs collected during the eighth week of egg laying and for which their genetic sex was determined. Genetic sex did not differ from sex determined by gross organ examination at necropsy.

The same pattern was observed when all surviving chicks from the F2 generation were considered and in the offspring of P1A parents in the F1 generation. Sex ratios in the F2 generation regardless of F1 or P1 parental exposure were reduced below control ratios at higher dietary concentrations of E2 when all birds were considered. However, sex ratios at the higher concentrations in F2 chicks of P1B parentage from the eighth week of egg laying did not decrease below control ratios. In the offspring of the P1B birds of the F1 generation, only the 5 ppm E2 diet exhibited a reduced sex ratio.

The reduced proportion of males produced in the two high concentration groups of offspring of P1A birds greatly limited the number of pairs that could be formed for these test groups in the F1a-P1A and F1b-P1A populations. Further limiting the number of pairs formed was the complication of preventing sibling crosses and the difficulty in visually sexing some feminized males. Although genetic sex data did not provide more accurate information in the F2 generation than could be determined visually at necropsy, application of this technique to optimize pairing of the F1 generation could be beneficial.

8.0 CONCLUSIONS

Two P1 exposure scenarios (P1A receiving treated diet prior to sexual maturation and P1B receiving treatment after proven egg-laying ability has been established) and two F1 exposure options for each P1 scenario (F1a chicks receiving treated diet from hatch through egg laying and F1b birds receiving no dietary treatment) were compared to determine the relative importance of the timing of onset of treatment of the P1 generation and the most appropriate exposure regimen for the F1 generation.

Test concentrations of E2 used in the study appeared to be too low to elicit a significant response in many endpoint measures, with the highest concentration (5 ppm) often inducing only a small effect. However, four endpoints were clearly impacted by the E2 concentrations used, identifying these measures as sensitive indicators of estrogenic effect in birds. These endpoints are

1. Male to female sex ratio in F1 and F2 generations—P1A exposure history inducing greatest effect

2. Sexual maturation of males in the F1 generation—Offspring of P1A parents affected

3. Histological response of male reproductive organs in the P1 generation—Effect was greatest under the P1A design

4. Incidence or length of feminized plumage in males in the P1 and F1 generation—The impact was greatest in the P1A and F1a exposure scenarios, respectively. The length of female plumage also appeared to be sensitive to exogenous estrogen and may provide means to monitoring exposure of females to estrogenic compounds.

Body weight, cloacal gland size, sexual maturation of females, and hatchling production over time also showed a difference between exposure designs in at least one generation during the study and were all affected by the P1A or F1a exposure scenarios. Reproductive organs of both the male and female of the F1 and F2 generations were sensitive to effects from an F1a exposure history or F1a-P1A interaction. With the exception of the adrenal gland, which was changed by P1B and/or F1a-P1B exposures, all remaining organ weights were changed by P1A and/or F1a exposure.

Determination of genetic sex in the F2 generation did not provide more information than could be attained from necropsy at two weeks of age at the test concentrations tested. However, application of this technique to optimize pairing of the F1 generation could be beneficial

9.0 RECOMMENDATIONS

Though sexual maturation was not strongly affected by the E2 exposures in the first generation, suggesting that the P1A scenario may not be necessary, many of the other important impacts were manifested in P1A birds or their offspring. Therefore, the P1A dosing regime is recommended for the P1 generation. P1A exposure can reduce the sex ratio in its offspring, making selection of initial number of pairs an important consideration in order to provide sufficient number of pairs for the F1 generation and subsequent measures. For the F1 and F2 generations, most effects were detected in F1a or F1a-P1A birds and their offspring, indicating that the F1 generation should be exposed to the test substance from hatch through egg laying.

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