Report to the Endocrine Disruptor Methods Validation Advisory Committee

AVIAN SPECIES COMPARISON STUDY—A PROTOCOL DEVELOPMENT STUDY FOR THE AVIAN 2-GENERATION TIER II ASSAY

Mary Ann Ottinger
Department of Animal and Avian Sciences
University of Maryland, College Park, MD 20742
November 1, 2005

Executive Summary:

A two-generation dietary test provides a method for determining multigenerational effects of endocrine disrupting chemicals (EDCs), with varied exposures over the stages in the life cycle. The Japanese quail has been developed as a model for precocial avian species; however, northern bobwhite quail have been the standard for toxicological testing. Our purpose was to compare relevant end points in these species, in a two-generation dietary study. In addition the to species comparison, a range of potential measurement end points were examined, including appraisal of potential sensitivity, constraints, and reliability for regulatory application. A modification of the OECD one generation protocol was followed with each generation for definition of the adult experimental phase of the study. The first generation (parental generation) was synchronized by raising both species under short photoperiod until mature. Specifically, Japanese and northern bobwhite quail (parents; P) were raised under short photoperiod, then placed on diet containing low concentrations of methoxychlor (MXC; 0, 5ppm, and 10ppm) and simultaneously photostimulated. A range of measures were monitored including feed intake, egg production, egg quality, fertility, offspring viability. In addition, other measures were selected for their potential to detect endocrine disruption. Chicks (F1) were raised on the same diet as their parents, paired as adults, and sexual maturation, reproductive behavior and endocrine measures were examined, similar to the parent generation. F2 chicks were raised on control feed and assessed for the same end points as the F1 birds.

There were some inherent species differences, including slower maturation and lower productivity in bobwhite quail. These differences were observed consistently over all generations in both species. No treatment effects were observed in either species for body weight, feed intake, egg quality, or viability. Effects of 5ppm MXC were similar in both species, and no treatment effects were observed for egg production, shell quality, fertility, and chick growth. However, both species showed delayed sexual maturation with treatment. This was more pronounced in the 10ppm MXC treatment, with significant ($p<0.05$) reduction in the number of birds achieving optimal reproductive performance. Sexual behavior was significantly ($p<0.05$) impaired by MXC exposure in both F1 and F2 Japanese quail males; behavioral assessment was not conducted in bobwhite males due to species differences in courtship and mating behavior. A modified open field test was developed and pilot data was collected to assess potential impairment of motor and behavioral responses in young chicks. This testing method has potential for Japanese quail, but not for bobwhite quail, again due to species differences in behavioral responses. Although variable, plasma and fecal steroid hormones were significantly ($p<0.05$) different with treatment. In the 5ppm MXC treatment, there was an

apparent bimodal response, especially in maturing birds. This is potentially an important marker because gonadal steroids typically are variable among individuals during sexual maturation whereas treated birds showed reduced and uniformly lower plasma steroid hormones. This variability in gonadal steroids may provide an index of EDC adverse effects on maturation, which would be an important for field populations where timing of reproduction is likely to be a critical factor. This maturation related relationship between reproductive status and steroid hormones was weaker in bobwhite quail even though MXC exposure was associated with delayed sexual maturation, similar to the Japanese quail. Hypothalamic neurotransmitters and GnRH-I reflected stage in maturation and reproductive capability; however these measures were variable and warrant further characterization to link individual responses. Moreover, the response of these measures is likely to differ with the mechanism of action of classes of EDCs. There are a number of specific items that we have considered, including number of eggs to incubate, hatch, and sampling paradigms. We have provided some specific recommendations in our summary. Finally, there are measures not conducted in this study that should be considered in the two-generation diet study. These include morphological, histological (gonad, bursa), immune, and hormonal (thyroid, vitellogenin, gonadotropins) measures as some examples.

In summary, a two-generation diet protocol has been developed that includes refinement of methods for sampling, consideration of species specific modifications, and comparison of Japanese quail and bobwhite quail. Overall, the two species had similar responses to the low levels of dietary MXC treatments. Japanese quail offer a fast maturing precocial avian model that is practical relative to time for completion of a two-generation dietary regimen. The list of possible measures is long and we have collected data that will be useful in discerning appropriate measurement end points for selected classes of EDCs. These end points will incorporate measures to detect toxicological effects and for evaluation of endocrine disruption that will be applicable for regulatory applications.

Introduction and Overview of the Study:

Endocrine disrupting chemicals (EDCs) have been defined functionally due to their influence on endocrine systems.. Furthermore, the exposure of wildlife at field relevant exposures is often at sublethal concentrations, making detection of effects of EDCs a challenging, especially when attempting to separate other interacting factors in the animal's environment (Ottinger and vom Saal, 2002). These additional factors may include environmental conditions, food availability, disease, or confounds such as simultaneous exposure to several chemicals. It is therefore, important to develop reliable and sensitive measurement end points that are appropriate for the endocrine or organ system that is the target of the EDC. A number of workshops and symposia addressed these issues, generating publications and reports that raised awareness to the potential effects of EDCs on wildlife and humans (Fox, 1992; Kavlock et al., 1996; NRC, 1999). Other publications have documented impacts of EDCs in laboratory studies and in wild populations in avian species (Fox, 1992; Fry, 1995; Fairbrother et al., 1998; Fox and Grasman, 1999; Rattner et al., 2000; Golden et al., 2003). In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) submitted a report that recommended a tiered approach to testing suspected EDCs, which involved screening, followed by selected bioassays, and subsequent single and multigenerational was indicated for EDC evaluation in birds (http://www.epa.gov/oppfead1/cb/csb_page/updates/endocrin.htm). A parallel activity has been ongoing through the Organization for Economic Cooperation and Development, which established an Expert Working Group on Avian Reproduction Toxicity Testing. We have been considering the testing parameters and measurement end points that are reliable and sensitive indices of EDC exposure in birds and have generated a discussion document, which served as a contribution to the Avian DRP. The Expert Working Group has revised the Avian Reproduction Toxicity Test guideline, which is under review and is drafting a protocol for a Multigenerational Reproduction Toxicity Test guideline, to be reviewed by the OECD member nations.

Toxicity Testing with End Points that Detect Endocrine Disruption

A basic issue is the development of the appropriate testing paradigm, including reliable end points. One source of confusion has been that avian toxicology tests currently in use generally include reproductive end points as a measure of the fitness of the adults. Because these reproductive end points, such as fertility and hatching success are included in the testing paradigm, it has been assumed that these measures are sufficient. However, these reproductive end points may not necessarily be appropriate, reliable, or sensitive for detecting an endocrine disrupting chemical. Therefore, it is important to distinguish the toxicology test from a test designed to detect endocrine disruption. A number of studies are underway in laboratories around the world; many of these laboratories have participation in the OECD Expert Group on Avian Reproduction Testing. At this time, the measurement end points for consideration include neuroendocrine, behavioral, endocrine, and whole organism measures which relate to the health of the bird.

Avian Species Selected for Models

In most of the toxicological applications for regulatory monitoring, species indigenous to the United States have been used for testing. This means that the species of choice have been the mallard duck and northern bobwhite quail (Bennett et al., 1990). Although the toxicological responses of these species have been tested with a variety of compounds, few background or baseline data exist for the reproductive endocrinology of these birds especially relative to EDC

impact (Ottinger et al., 2001; 2002). Conversely, there are data available on the reproductive and metabolic endocrinology and on the neural regulation of reproductive behavior in Japanese quail, zebra finches, and doves (Adkins-Regan et al., 1990; Ottinger et al., 2001; Abdelnabi et al., 2003). Moreover, in addition to data on endogenous patterns in gonadal steroids during ontogeny and in adults, the effects of exogenous steroids have been examined in many of these species (Rissman et al., 1984; Ottinger et al., 2001) there are data on the effects However, these species were not considered for toxicological testing until recently. Japanese quail have been under intense study recently with the recognition that multigenerational approaches are necessary to detect some of the more subtle effects of EDCs. Moreover, since field exposures are often at low levels or in an unpredictable frequency, the impact may be more significant over several generations. As such, multigenerational testing is necessary to detect this type of low exposure, long term outcome. Further, maternal deposition is a primary route of exposure to the avian embryo, especially for precocial species (Adkins-Regan et al., 1995). Figure 1 a and b show the pattern of deposition into the egg yolk with exposure of the hen to methoxychlor (MXC) or to soy isoflavones.

The purpose of this two-generation test was to conduct a two-generation dietary study with Japanese quail and northern bobwhite quail in a parallel manner in order to compare the response of the two species to the same dietary treatments. Fortunately, these species are relatively similar in size and their housing and management requirements are therefore similar (Ottinger and Rattner, 1999). There is a difference in their maturation rate in that Japanese quail mature by 3 months of age; whereas northern bobwhite quail require approximately 6 months. Because the time required for maturation differs, both species were hatched in our lab and then reared on a short photoperiod (9 hr light; 15 hr dark) to prevent induction of reproduction during somatic maturation. At the time that both species were somatically mature (bobwhite quail=6 months; Japanese quail=3 months), birds were switched to a long photoperiod (15 hr light; 9 hr dark) to stimulate reproductive maturation and the treatment diets were initiated. Our study design and findings are detailed below.

Study Design:

This study was based on research that we conducted in Japanese quail as part of STAR grant funded research. That project included egg injection studies to ascertain the consequences of EDC exposure as well as one and two generation dietary studies. This initial two generation study (see below) used lower dietary concentrations of MXC. Results from that study have been published (please see attached papers).

SUMMARY OF TWO GENERATION STUDY: (STAR grant research; Ottinger et al., 2001; 2002, and 2005)

In this study, dietary methoxychlor (MXC; 0, 0.5 and 5 ppm) was initiated after the parents (P1) were sexually mature and with proven reproductive pairs. The OECD one-generation paradigm was followed in that there was a 2 week pretreatment period to allow equilibration and adjustment to the treatment diets as well as collection of baseline reproductive data (in addition to the data collected during identification of proven breeding pairs).

- Fertility, body weights, egg production, shell strength, plasma hormones, gonad weights, monoamines, and GnRH-I. Feed intake estimated for pairs.

F1 chicks sampled at hatch or at maturity (constant exposure to treated feed)

- Sexual maturation, body weights, fertility, reproductive behavior, motor behavior, egg production, shell strength, plasma hormones, brain monoamines, and GnRH-I. Feed intake estimated.

F2 chicks sampled at hatch or at maturity (control feed)

- Sexual maturation, body weights, fertility, reproductive behavior, motor behavior, egg production, shell strength, plasma hormones, brain monoamines, and GnRH-I.

DESIGN OF THE AVIAN SPECIES COMPARISON STUDY (Figures 2a and b)

Incubation and Rearing

Fertile Japanese quail eggs were obtained from the University of Maryland Japanese quail colony and fertile northern bobwhite quail eggs were obtained from Wildlife International, Ltd, Easton, MD. Eggs were incubated in Petersime incubators, hatched, and chicks were brooded in temperature controlled brooders. Heaters were turned off when the chicks were 3-4 weeks of age and no longer required additional heat. All birds were maintained on a short photoperiod (9L:15D) with feed (Wildlife International Quail Diet) and water available ad lib. Birds were transferred caging for pairs and housed there throughout the rest of their portion of the study.

Initiation of Dietary MXC, F1 and F2 Dietary Treatments and Sampling Regimen:

Dietary MXC (0, 5 and 10ppm) began at the same time as the photoperiod was changed to long days (15L:9D). This occurred when the Japanese quail were 3 months of age and the northern bobwhite quail were 6 months of age. Therefore, these birds were somatically mature, but were not reproductive because they were housed on a short photoperiod (9L: 15D). As outlined below (also see Figures 2a and b), F1 birds were fed the same diet as their parents and all F2 birds were fed control diet. An adjustment was made in the timing of treatment for the P1 bobwhite quail due to a slower rate of sexual maturation compared to the Japanese quail.

- P1 birds were hatched and reared in heated brooders until 6 weeks of age; pairs were transferred into cages and a subset of animals were housed singly
- At 3 months of age (Japanese quail) and 6 months of age (northern bobwhite quail), long photoperiod and treatment diets began
- Body weight, feed intake, egg production, shell quality, fertility, hatching success, chick viability, and general health were monitored
- Samples for analysis of plasma hormones, fecal hormones, gonad weights, monoamines, and GnRH-I were taken at the end of the experimental period. Fixed tissue collected for later immunocytochemistry (tissues are being analyzed by GianCarlo Panzica, University of Torino for arginine vasotocin). Some samples taken for possible histology if any abnormalities were noted.
- F1 offspring were fed treated feed. Samples collected at hatch and in adults following experimental treatment period.
- F2 offspring fed control feed and raised to maturity.
- Same samples collected and parallel determinations made in the Japanese and bobwhite quail.

- Although the photoperiod was changed for both bobwhite and Japanese quail at the same time, the experimental period for the bobwhite quail began 3 weeks after the Japanese quail due to slower sexual maturation

Results and Interpretations:

Feed Intake:

No differences were detected in average feed intake between treatments over the experimental period (Figure 3a). There appeared to be differences between generations (Figure 3b) for both species; however, there were no treatment differences were detected in either species.

Body Weight:

No significant differences were detected in weight of Japanese quail chicks at hatch in any generation (see data for F1 generation shown in Figure 4). However, three appeared to be lower body weights in both males and females during the experimental period. Error bars are only shown for the male bobwhite quail to illustrate the variability that was observed. Similarly, there were substantial error measurements in all treatment and control birds. It would be of interest to further analyze these data on an individual basis to determine if smaller individuals had fewer eggs or reduced hormone levels, etc.

Egg Shell Strength:

No differences were observed in egg shell strength or thickness (data not shown) for either species in the eggs from P1 or F1 females (Figure 5). Peak load and break load were intriguing, but need more refinement.

Sexual Maturation:

Sexual maturation was monitored in females by the production of the first egg (Figure 6). No difference was detected within a generation with treatment. However, there was a decrease in the age of onset of egg production, due to the timing of photostimulation in the bobwhite quail. In the Japanese quail, they were kept on short photoperiod until the P1 bobwhite quail were ready for photostimulation. It should be noted that P1 females of both species were switched to long photoperiod at the same time, but the bobwhite females required about 3 weeks longer to production of their first egg.

Egg Production

No treatment or generation differences were observed for egg production in bobwhite females in response to dietary MXC (Figure 7). The P1 females averaged 3-5 eggs/week and the F1 females had a slightly higher average egg production/week of 4-6 eggs. In female Japanese quail, little difference was observed in the P1 and F1 generations (Figure 8). However, the F2 females showed more variability in egg production. Egg production averaged about 5 egg/hen/week, except in the F2 females in which there was lower productivity. These differences were also reflected in the total number of eggs produced by all females in a week.

Ovarian Maturation and Ovary Weight in Adults:

In Japanese quail, there appeared to be a delay in ovarian maturation, with a more pronounced effect in the F1 high MXC females and in both F2 MXC exposed females (Figures 9

and 10). Interestingly, the low MXC had more females that appeared delayed, whereas more of the high MXC females were maturing. This may have been due to differential survival of chicks in these groups. Studies in other labs have shown that MXC can be lethal at higher exposure levels (Eroschenko et al., 2002). In addition, females that never became reproductive were not used in the experimental pairs in order to have eggs and chicks produced.

No difference was observed in ovarian weight in the adult bobwhite females (Figure 10). However, samples taken of a subset of females at puberty (week 10 of photostimulation) showed that the high MXC females appeared to be among the hens that matured more quickly, resulting in higher average ovary weight in this treatment group. However, we must determine the relative mortality of chicks in this group to make certain that this is not due to a survivor effect, meaning that the less sensitive birds are represented in this group of females.

Testicular Maturation and Sexual Behavior:

Male Japanese quail were delayed in sexual maturation by the dietary MXC (Figure 11). There were no significant differences in testes weight in adult Japanese or bobwhite quail. In addition, the mount latency of the low MXC F1 males was longer compared to the other groups; both low and high MXCF2 males showed longer latency to mount. More detailed behavioral data are shown over the 3 days of testing for the F1 and F2 Japanese quail (Figure 12). A number of studies have shown that reproductive behavior in male quail is sensitive to exogenous steroid or EDC exposure during embryonic development (Berg et al., 1999; Halldin et al., 1999; McGary et al., 2001; Ottinger et al., 2001; 2002; in press). The cloacal gland is an androgen sensitive accessory sex structure that enlarges in adult male Japanese quail and produces foam that is thought to facilitate sperm transfer and function as a territorial marker. The size of the gland and the foam production by the gland is shown in Figure 12 for the F2 males. The size of the cloacal gland is estimated from the length X width of the gland and the foam is quantified on a scale of 1 to 5 (least to most). There was some indication of effects during maturation in the 7 week old males; however, we did not detect significant differences in the mature males relative to gland size or foam production.

Behavioral testing was not possible in the male bobwhite quail due to species differences in mating behavior.

Fertility and Hatchability:

No significant differences were observed in fertility and hatchability of chicks over the generations or treatments in Japanese quail (Figures 13a, b, c, and d). The pattern of development differed in Japanese quail compared to the bobwhite quail in that the Japanese quail generally showed an impact on fertility, and chicks that hatched generally survived. In contrast, nearly 100% of the bobwhite quail eggs were fertile and hatched; however, the two week survival of the chicks appeared to be the critical factor for this species.

Plasma and Fecal Steroid Hormones:

Figure 14 presents plasma steroid hormones in box plots as an example of the wide individual variability that often occurs in plasma hormones. Interestingly, the early pubertal (EP) samples showed the normal variation that occurs during sexual maturation in males, whereas both MXC treatment groups had lower variability presumably associated with the delay

observed in sexual maturation in these groups. Treated females also had reduced estradiol, which would be expected with delayed ovarian development.

Figure 15 shows average plasma steroid hormone levels for the P1 generation of Japanese quail. Because we were not sure if there was sufficient plasma available for the assays, a subset of samples was assayed for each hormone. Fecal steroid hormone assays were conducted just to test this technique. As may be seen from Figure 15, these measures did not match up with the plasma steroid hormones, probably due to the collection of feces from pairs so that the male and female samples were mixed. We did validate the fecal steroid assay and found it to be a stable method. In the F1 high MXC female Japanese quail, plasma estradiol was elevated whereas the fecal estrogen was reduced, perhaps indicating slower turnover and usage of the steroid hormone. F1 low MXC males had higher circulating androgen levels while the fecal steroids were highest in samples from the high MXC males (Figure 16). In the F2 birds, both male and female low MXC birds had elevated plasma steroid hormones, with reduced steroids in the fecal samples (Figure 17). Again, analysis of individual records may resolve some of the variability that accompanies plasma hormone determinations to ascertain if an individual is consistent in plasma and fecal steroid hormones.

Both androgen and estradiol assays were validated for bobwhite quail plasma (data not shown). Plasma androgens are shown for adult bobwhite quail males over 3 generations and with treatment (Figure 18). The P1 generation did not show any difference; however, the plasma levels were consistently low which is surprising. We checked these data and did not find any assay error to explain the low levels. Plasma androgens in F1 males appeared to reflect dietary MXC and F2 males showed higher levels in low MXC males similar to the data in Japanese quail. Data for the females were similar among treatments, again there was variability within some of the groups.

Brain Neurotransmitters,GnRH-I, and aromatase enzyme:

Hypothalamic monoamines (NE, DA, E, 5-HT, 5-HIAA) were analyzed by HPLC in hatchlings and the adult hypothalamus was divided into two sections. The anterior region microdissection includes the preoptic and septal regions (POA-SL) and contain the GnRH-I cell bodies, aromatase enzyme and vasotocin containing neurons, and the neural elements that regulate behavioral and GnRH-I cell responses. The posterior region microdissection included the median eminence, posterior hypothalamus, and tuberal infundibular regions, with the GnRH-I axonal projections and neuroendocrine regulators.

Figure 19 shows some of our data, which demonstrate some of the life stages in which we found significant differences in monoamine levels, including F1 male and females at 3 weeks of age, F1 females at week 6, and hatchling F2 females. More extensive presentation of data is shown in Figure 20, with the anterior (A) and posterior portions (B) of the hypothalamus shown. Figure 21 shows NE and DA in bobwhite quail with generation and MXC treatment. NE and DA are important for reproduction because these neurotransmitters stimulate production and release of GnRH-I in both males and females and reproductive behavior in the male.

Figure 22 shows the levels of GnRH-I in the same samples analyzed for monoamines. Levels in the F2 were relatively lower because these individuals were sampled early in maturation. GnRH-I levels are also shown for bobwhite quail. Please note that the overall content of GnRH-I appears to be lower in the bobwhite quail as compared to Japanese quail.

Figure 23 shows levels of serotonin (5-HT) and its metabolite (5-HIAA) in the microdissected hypothalamus.

Figure 24 shows an integrated presentation of catecholamines and GnRH-I.

Figure 25 shows hypothalamic aromatase enzyme in F1 Japanese quail. Interestingly, there was a MXC treatment effect, which was consistent in males and females. In the low MXC group, there appeared to be some differences in individual response to the treatment. These data are interesting in light of data from our laboratory and other labs that have shown an association of aromatase enzyme and reproductive behavior in male quail (Dellovade et al., 1995; Aste et al., 1996). In addition, vasotocin is also sexually dimorphic and occurs in relatively higher concentrations in the male POA-SL region. As such, this neuropeptide may be another measure that would be an indicator of endocrine disruption of sexual differentiation due to embryonic EDC exposure.

Acknowledgements: Please see Figure 26.

Data identified for further analyses or not presented in this report:

There are additional data that we will present in papers to be published from this study which include the following:

- Relate all measures on an individual basis to detect differential sensitivities to the dietary treatments,
- Hatching of chicks relative to the females that showed delayed ovarian maturation,
- Number of females that never came into reproduction.

Endpoints that were not examined in this study include vitellogenin, liver enzymes (such as cyp 1A1), immune system measures including cell mediated immunity, bursal size and histology, and thymus size and histology, thyroid system measures including hormones, enzymes, and histology, adrenal system hormones and adrenal histology, pituitary gland hormones and histology, sperm count and morphology. There is literature that support the response of some of these variables as potential measurement end points (Novotny et al., 1983; Olah et al., 1986; Glick, 1991; Grasman and Fox, 2001; Stanton et al., 2003;

Conclusions

Japanese quail and northern bobwhite quail appeared to have similar sensitivity to low level dietary MXC exposure.

In terms of regulatory applications, we would recommend the following:

- the study design which provides the most complete assessment of impact at a variety of life stages includes initiating treatment diets at the onset of sexual maturation in the paired P1 birds, maintaining the treatment diets for the F1 birds throughout breeding, and control feed for all F2 birds,
- the Japanese quail produce so many eggs that all eggs should not be incubated for assessment of fertility and hatchability data; therefore subsets of eggs should be used for:
    a) eggshell quality,
    b) fertility and not hatching,
    c) chemical transfer from the hen,
    d) other contaminants,
    e) steroid hormone content

- pairs used in each generation as the n=16 pairs/treatment must reproduce; therefore it is necessary to have a surplus of pairs (we usually maintained 20 pairs/treatment group) in order to have the 16 pairs for the study,
- use fecal steroids as a measure for circulating steroid hormones as they are less variable and will mirror the reproductive status of the individual,
- the bobwhite quail dietary study, which also began with hatching the parent generation, required approximately 24 months to complete the in life portion of the study. This becomes a prohibitive time frame for use in regulatory applications.

➢ REFERENCES

Abdelnabi, M.A. and M.A. Ottinger. (2003). Hypothalamic indolamines during embryonic development and effects of steroid exposure. Gen Comp Endocrinol **130(1)**: 13-19.

Adkins-Regan, E., M. Abdelnabi, M. Mobarak and M.A. Ottinger (1990). Sex steroid levels in developing and adult male and female zebra finches (Poephila guttata). Gen. Comp. Endocrinol., **78**:93-109.

Adkins-Regan, E. K., Ottinger, M. A., and Park, J. (1995). Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. *J. Exp. Zool.* **271**, 466-470.

Aste, N., Panzica, G. C., Aimar, P., Viglietti-Panzica, C., Foidart, N., and Balthazart, J. (1996). Morphometric studies demonstrate that aromatase-immunoreactive cells are the main target of androgens and estrogens in the quail medial preoptic nucleus. *Exper. Brain Res.* **101**, 241-252.

Bennett, R. S., Bentley, R., Shiroyama, T., and Bennett, J. K. (1990). Effects of the duration and timing of dietary methyl parathion exposure on bobwhite reproduction. *Environ. Toxicol. Chem*. **7**, 1473-1480.

Berg, C., Halldin, K., Fridolfsson, A.-K., Brandt, I., Brunstrom, B. (1999). The avian egg as a test system for endocrine disrupters: effects of dietylstilbestrol and ethynylestradiol on sex organ development. *Science Total Environ.* **233**, 57-66.

Dellovade, T. L., Rissman, E. F., Thompson, N., Harada, N., and Ottinger, M.. A.. (1995) Co-localization of aromatase enzyme and estrogen receptor immunoreactivity in the preoptic area during reproductive aging. *Brain Res*. **674**, 181-187.

Eroschenko, V.P., Amstislavsky, S. Y., Schwabel, H., Ingermann, R.L. (2002). Altered behaviors in male mice, male quail, and salamander larvae following early exposures to the estrogenic pesticide methoxychlor. Neurotox. Teratol. **24(1)**, 29-36.

Fairbrother, A., Landis, W. G., Dominquez, S., Shiroyama, T., Buchholz, P., Roze, M. J., and Matthews, G. B. (1998). A novel nonmetric multivariate approach to the evaluation of biomarkers in terrestrial field studies. *Ecotoxicology* **7**, 1-10.

Fox, G. A. 1992. Epidemiological and pathobiological evidence of contaminant-induced alterations in sexual development in free-living wildlife. *In* "Chemically-induced alterations in sexual and functional development: the wildlife / human connection." (T. Colborn and C. Clement, Eds.), pp.147-158. Princeton Scientific Publishing Co., Inc., Princeton.

Fox, L.I. and K.A. Grasman. (1999). Effects of PCB 126 on primary immune organ development in chicken embryos. Journal of Toxicology and Environmental Health, Part A, **58**: 233-244.

Fry, M.. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **103(Suppl. 7)**, 165-171.

Glick, B. (1991). How it all began: The continuing story of the bursa of Fabricius, in *Avian Immunology: Basis and Practice*, CRC Press, Boca Raton, Florida, A. Toivanen and P. Toivanen, ed.

Golden NH, BA Rattner, PC McGowan, KC Parsons, MA Ottinger. (2003). Concentrations of metals in feathers and blood of nestling black-crowned night-herons (*Nycticorax nycticorax*) in Chesapeake and Delaware Bays. Bull Environ Contam Toxicol **70(2)**: 385-393.

Grasman, K.A. and G.A. Fox. (2001). Associations between altered immune function and organochlorine contamination in young Caspian terns (*Sterna caspia*) from Lake Huron, 1997-1999. *Ecotoxicology*, **10**: 101-114.

Halldin, K., Berg, C., Brandt, I., and Brunstrom, B. (1999). Sexual behavior in Japanese quail as a test end point for endocrine disruption: Effects of *in Ovo* exposure to ethinylestradiol and diethylstilbestrol. *Environ. Health Perspect.* **107(11)**, 861-866.

Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaatari, S., Lucier, G., Luster, M., Mac, M. J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D. M., Sinks, T., and Tilson, H. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* **104(Suppl 4)**, 715-740.

Lin, F, Wu, J Abdelnabi, MA, Ottinger, MA, Guisti MM. (2004). Effects of dose and glycosylation on the transfer of genistein into the eggs of Japanese quail (*Coturnix japonica*). Journal of Agric. Food Chem. **52**: 2397-2403.

McGary, S., P.F. P. Henry, M. A. Ottinger. (2001). Impact of vinclozolin exposure on Japanese quail reproduction. Environ. Toxicol. Chem. Vol. **20, No. 11**, pp. 2487-2493.

Novotny, E.A., Raveche, E.S.. Sharrow, S. Ottinger, M.A., and Steinberg, A.D. (1983). Analysis of thymocyte subpopulations following treatment with sex hormones. Clinical Immunology and Immunopathology, 28: 205-217.

NRC (1999). Hormonally Active Agents in the Environment. Washington, D.C., National Academy Press.

Olah, I., Glick, B., and Toro I. (1986). Bursal development in normal and testosterone-treated chick embryos. Poultry Science, **65**: 574-588.

Ottinger, M.A., S. Pitts, and M.A. Abdelnabi. (2001). Steroid hormones during embryonic development in Japanese quail: Plasma, gonadal, and adrenal levels. Poultry Sci. **80**: 795-799.

Ottinger, M.A., M.A. Abdelnabi, P. F.P. Henry, S. McGary, N. Thompson, and J. Wu. (2001). Neuroendocrine and behavioral implications of endocrine disrupting chemicals in quail. Horm. Behavior. **40**: 234-247.

Ottinger, M.A., M. A. Abdelnabi, M. Quinn, N. Golden, J. Wu, and N. Thompson. (2002). Reproductive consequences of EDCs in birds: What do laboratory effects mean in field species? Neurotoxicology Teratology. **24 (1)**: 17-29.

Ottinger, M.A. and B. Rattner. (1999). Husbandry and care of quail. Poultry and Avian Biology Reviews **10(2)**: 117-120.

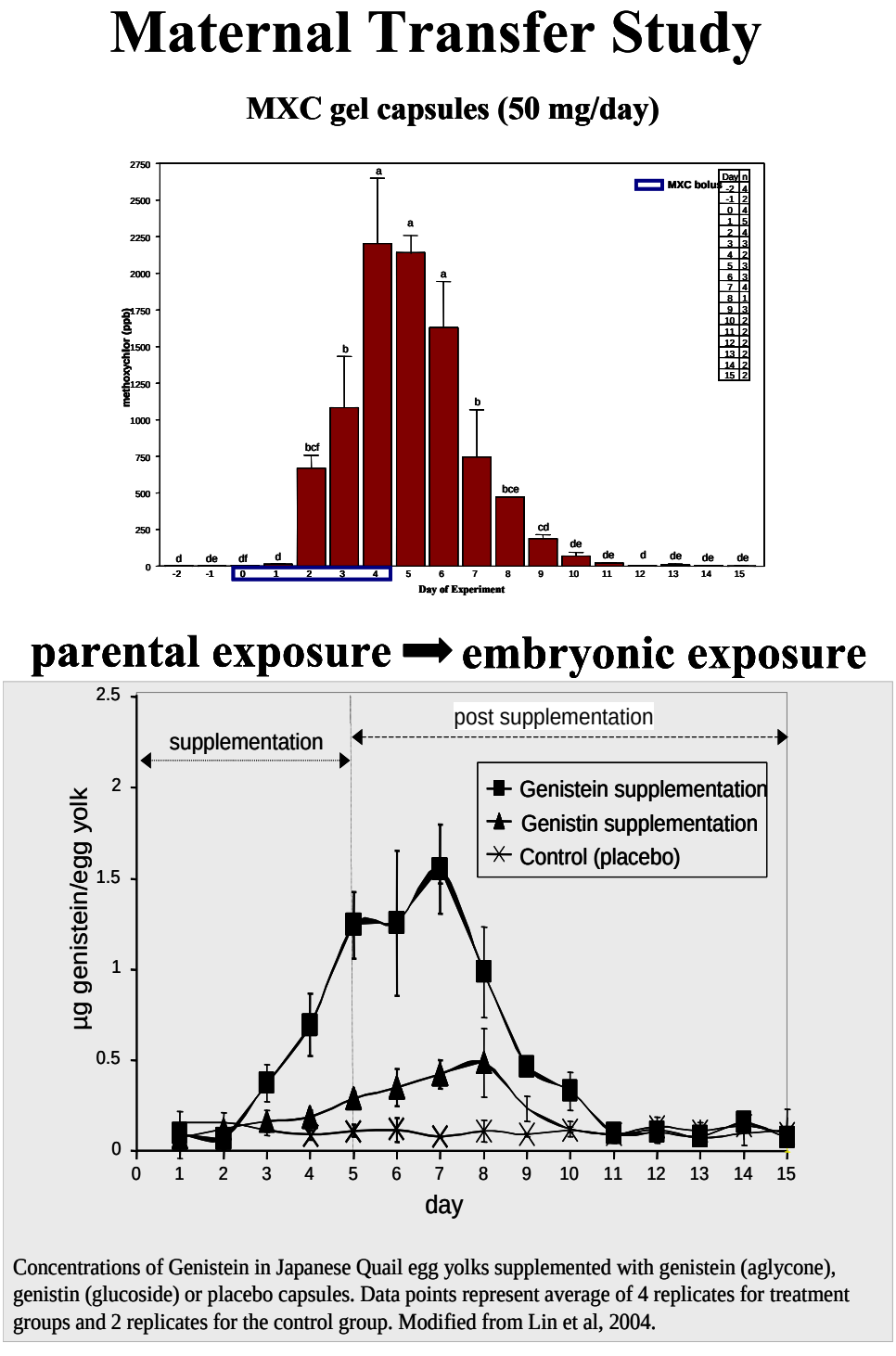
Ottinger, M.A. and F.S. vom Saal. (2002). Impact of environmental endocrine disruptors on sexual differentiation in birds and mammals; in Hormones and Behavior in Higher Vertebrates. Editors: Pfaff, D., A. Arnold, A. Etgen, and R. Rubin. Academic Press. Volume **4**, 325-383.

Rattner, B. A., Pearson, J. L., Golden, N. H., Cohen, J. B., Erwin, R. M., and Ottinger, M. A. (2000). Contaminant exposure and effects in a terrestrial vertebrates database: Trends and data gaps for Atlantic coast estuaries. Environ. Monitoring Assessment **63**, 131-142.

Rissman, E. F., Ascenzi, M., Johnson, P., Adkins-Regan, E. 1984. Effect of embryonic treatment with oestradiol benzoate on reproductive morphology, ovulation, and oviposition and plasma LH concentrations in female quail (*Coturnix coturnix japonica*). *J. Reprod. Fert.* **71**, 411-417.

Stanton, B., DeWitt, J., Henshel, D., Watkins, S., Lasley, B. (2003). Fatty acid metabolism in neonatal chickens (*Gallus domesticus*) treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or 3,3',4,4,',5-pentachlorobiphenyl (PCB-126) in ovo. Comp. Biochem. Physiol. Part C **136**, 73-84.

Figures 1a and b: Transfer of methoxychlor (a) and soy phytoestrogens into the eggs yolks of quail eggs.



Concentrations of Genistein in Japanese Quail egg yolks supplemented with genistein (aglycone), genistin (glucoside) or placebo capsules. Data points represent average of 4 replicates for treatment groups and 2 replicates for the control group. Modified from Lin et al, 2004.

Figures 2 a: Test design for the Avian Species Comparison Two-Generation Study.

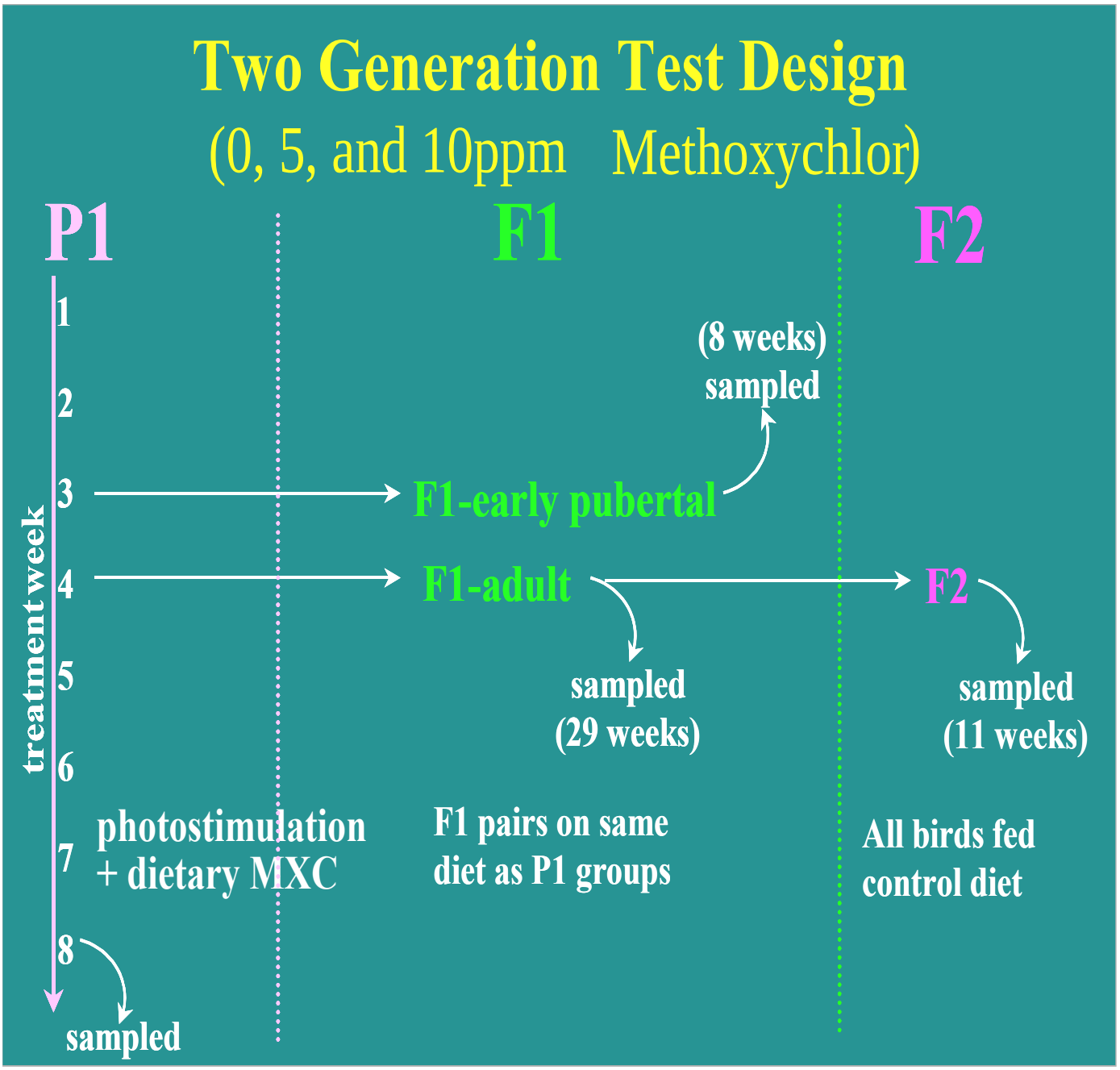


Figure 2b: Experimental schedule for the Avian Species Comparison Study

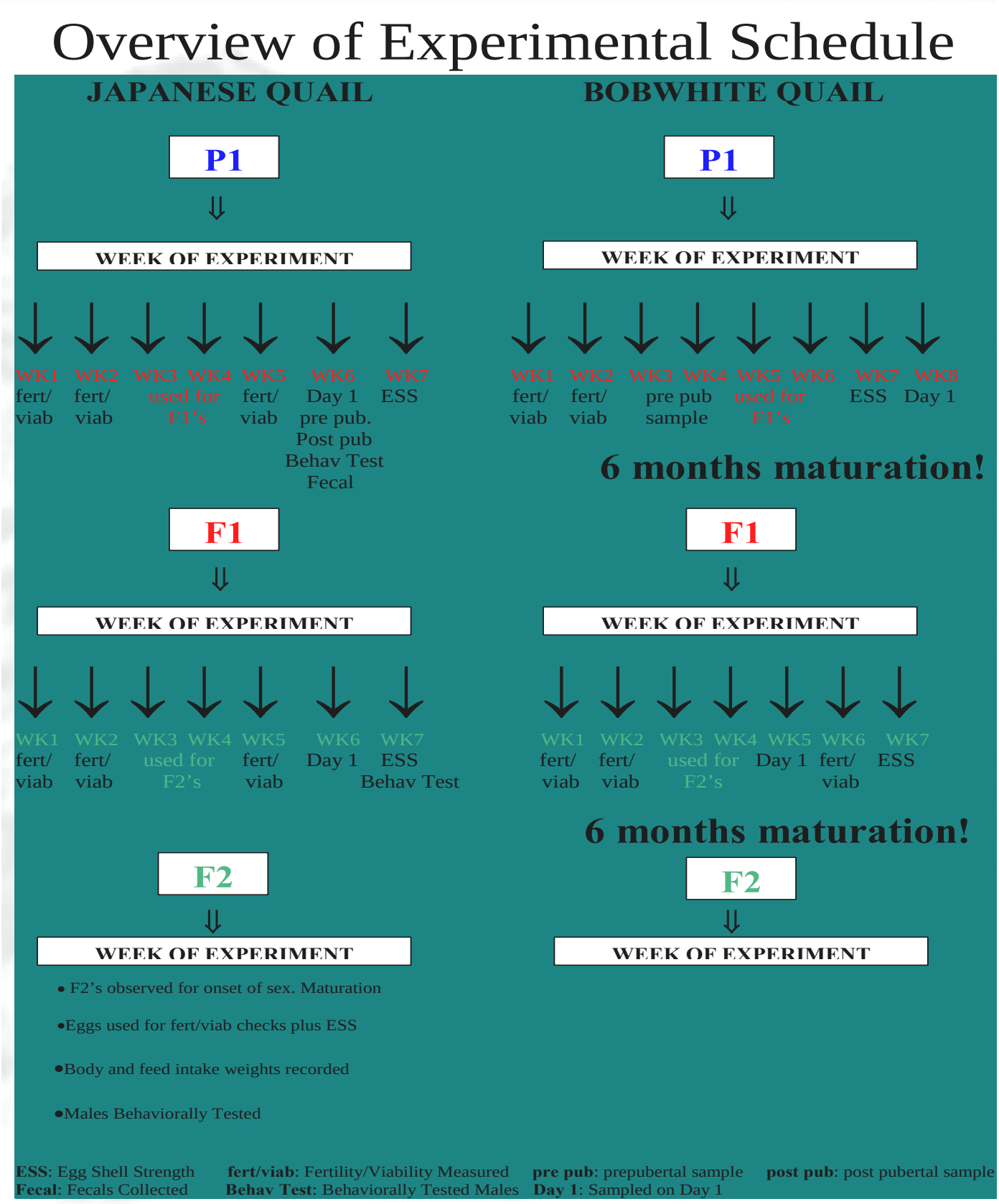


Figure 3a: Feed intake for the P1 and F1 generations for Japanese quail and bobwhite quail

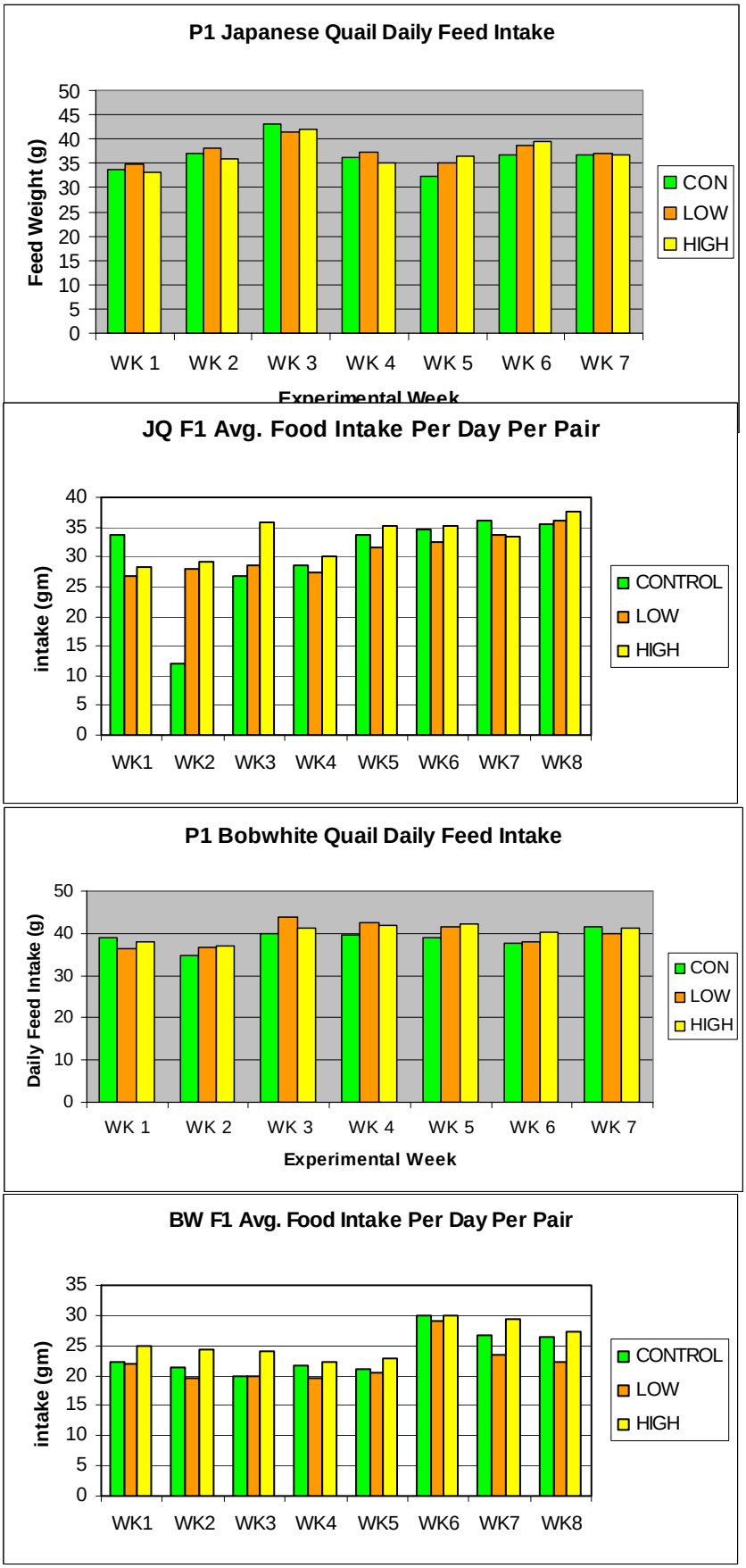
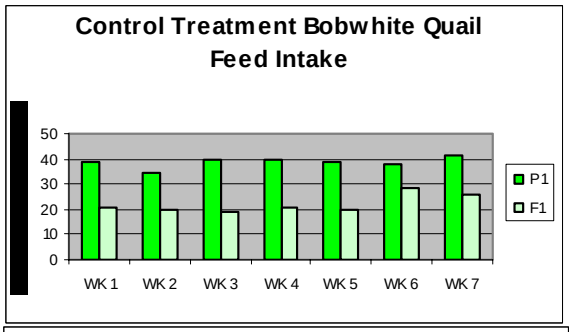
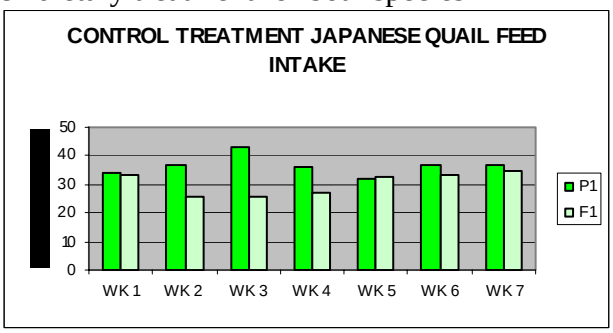
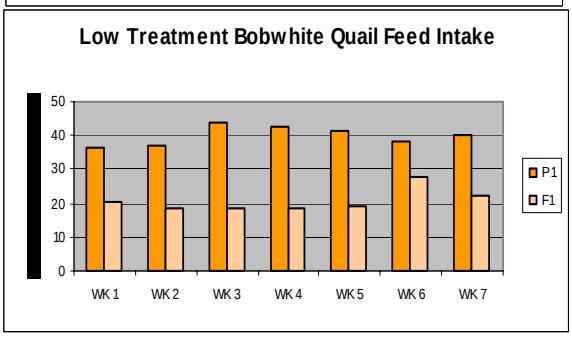
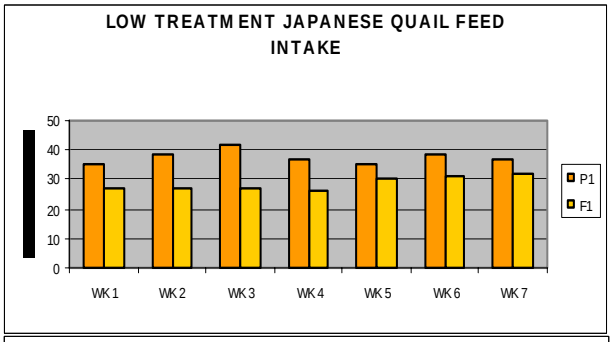


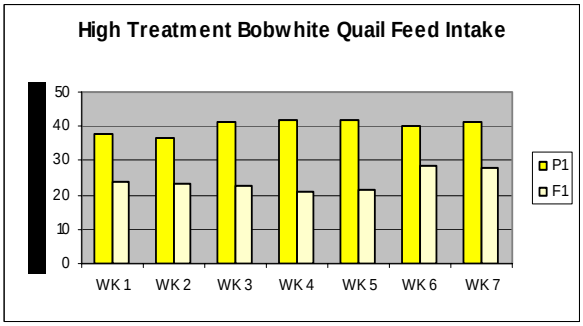
Figure 3b: Feed intake comparing the P1 and F1 on each dietary treatment for both species











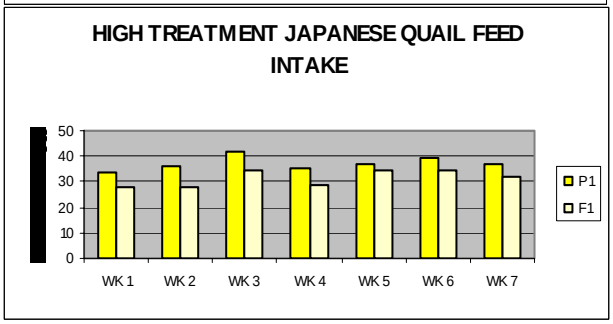
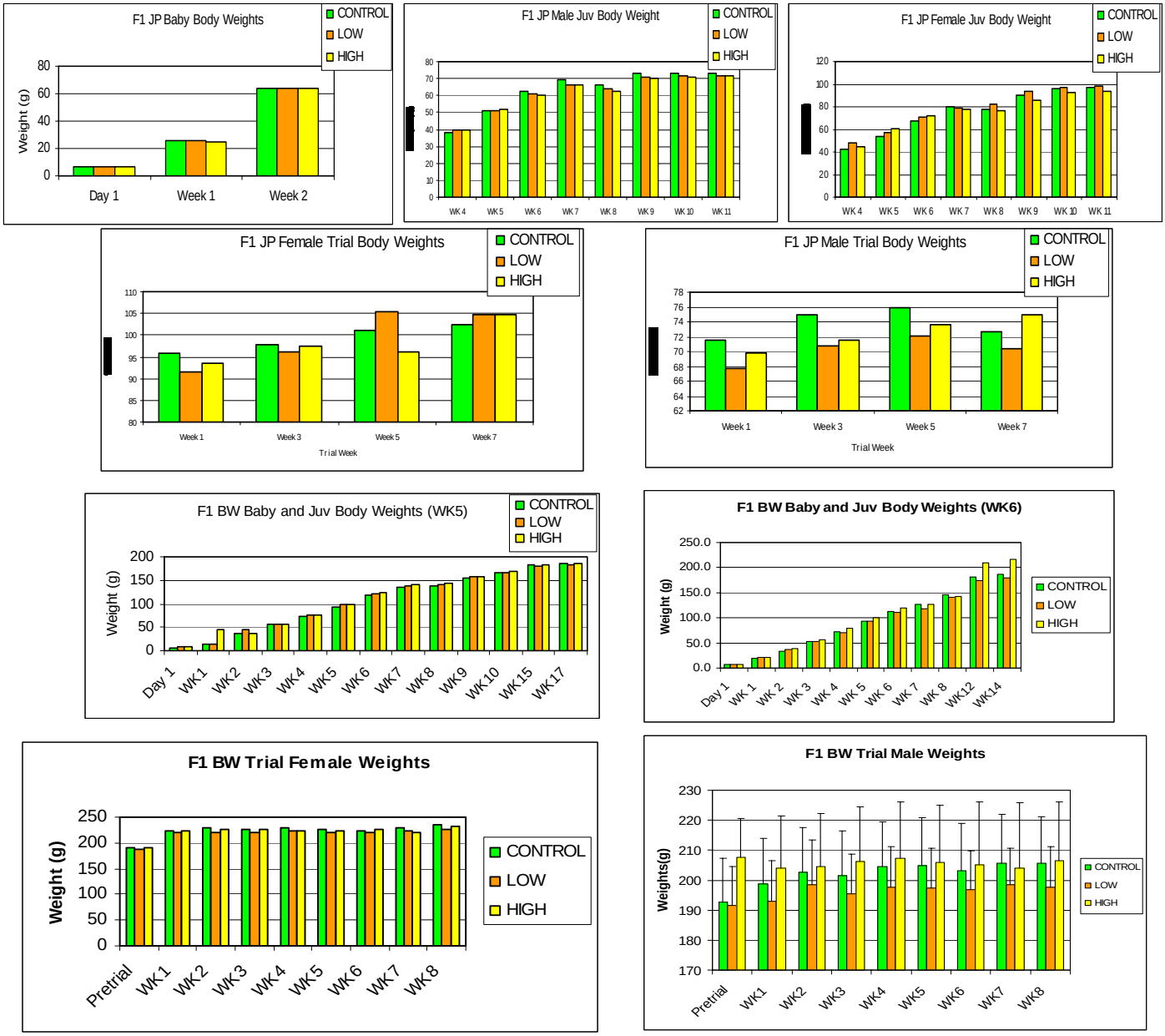
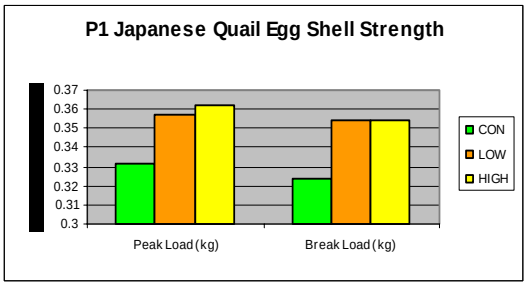
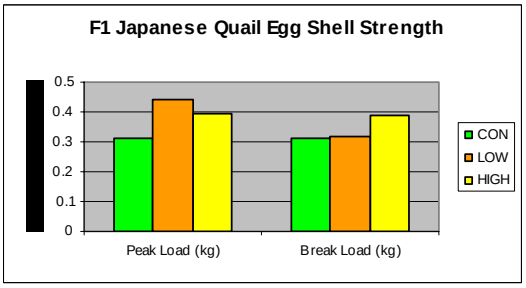


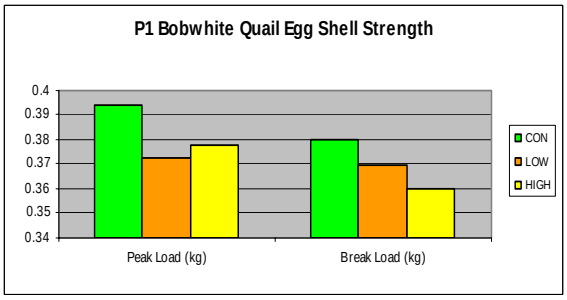
Figure 4: No significant differences were detected in body weight, either at hatch or between generations in either species.

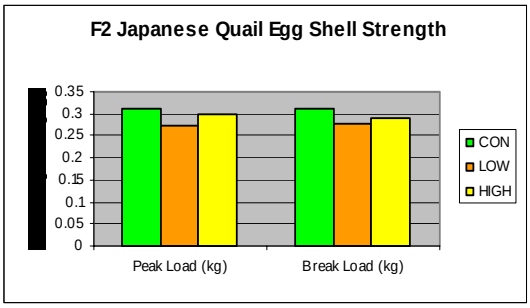




No differences were observed in egg production, or egg shell quality.







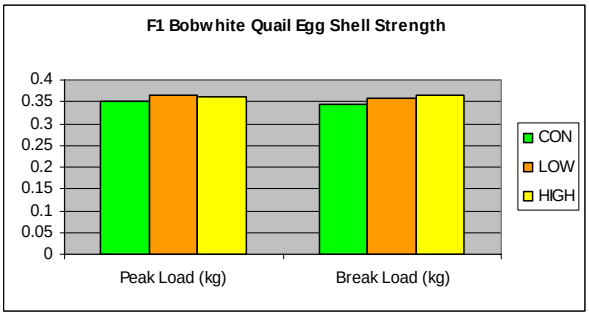


Figure 5: Egg shell strength did not show significant differences with dietary MXC in the P1 or F1 females. Peak load and break load (determined by Instrom) was potentially interesting.

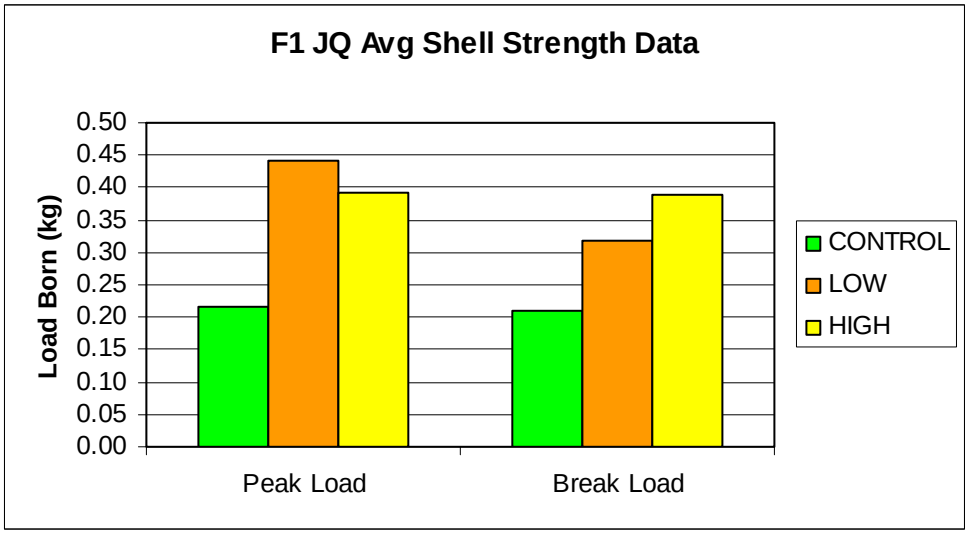


Figure 6: Sexual maturation in female quail.

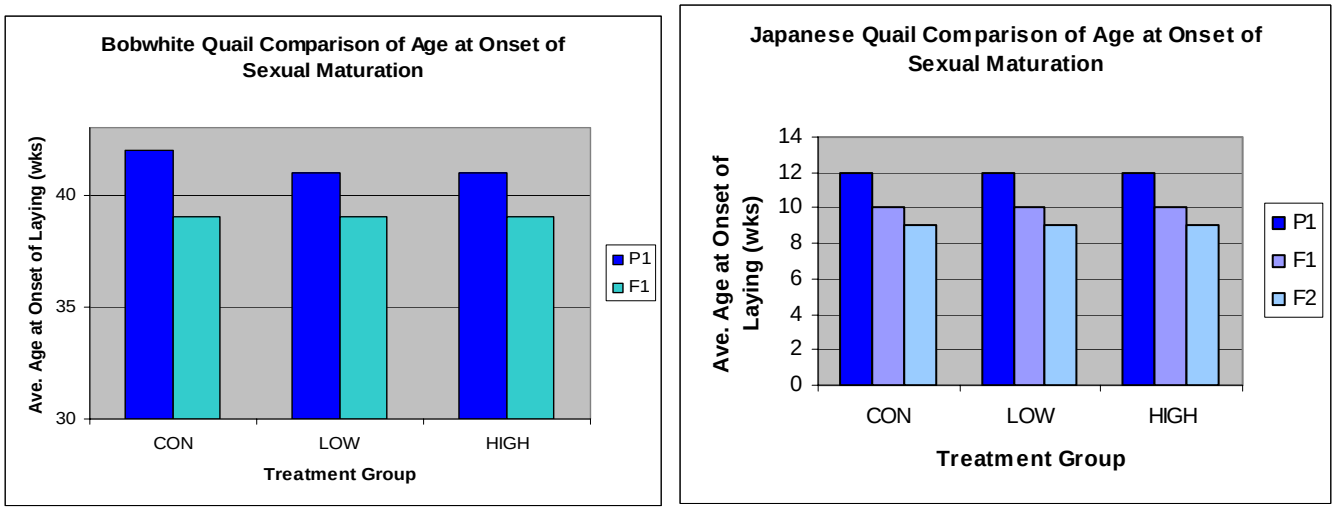


Figure 7: Egg production in bobwhite and Japanese quail.

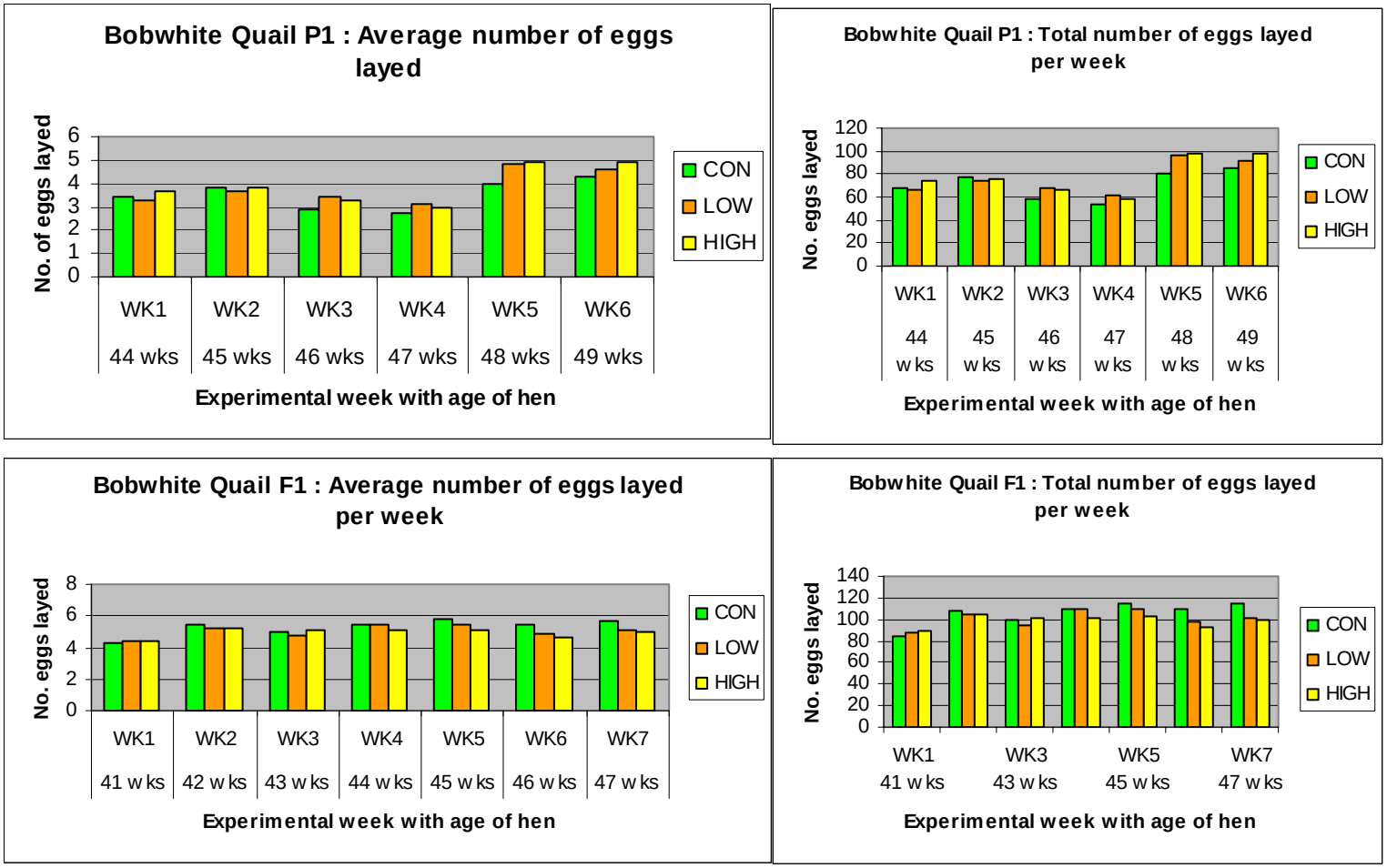


Figure 8: Egg Production in Japanese quail hens over the two generations.

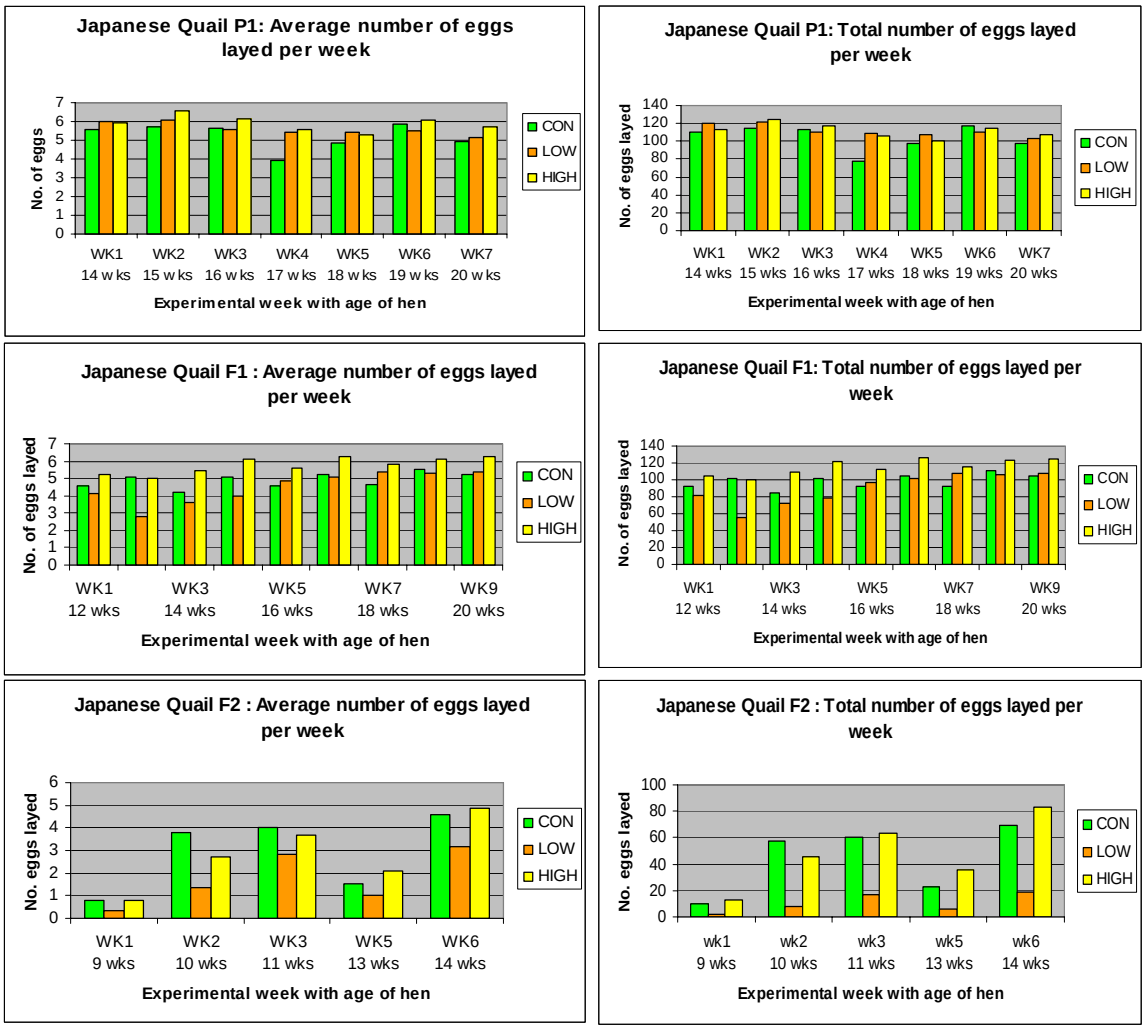
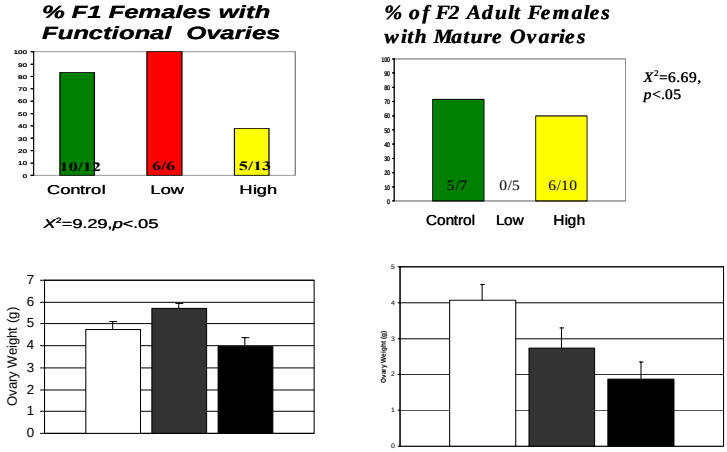
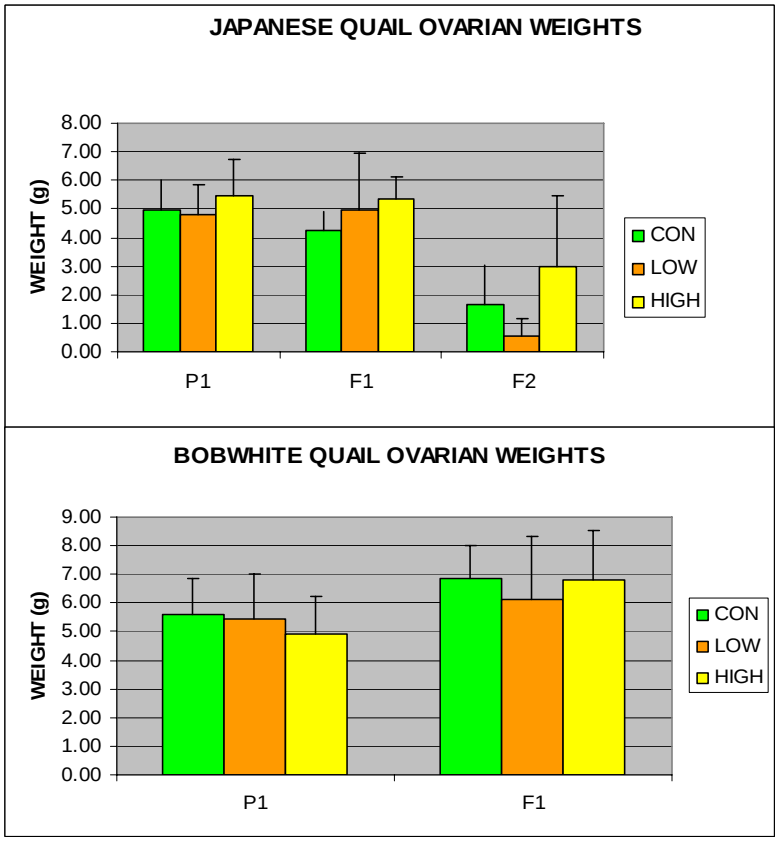


Figure 9: **Japanese Quail: Ovarian Development and Maturation**



F1 hens in the high MXC group and both MXC groups in the F2 hens appeared to mature later; adults did not differ.

Figure 10: Ovarian weights in adult Japanese and bobwhite quail hens



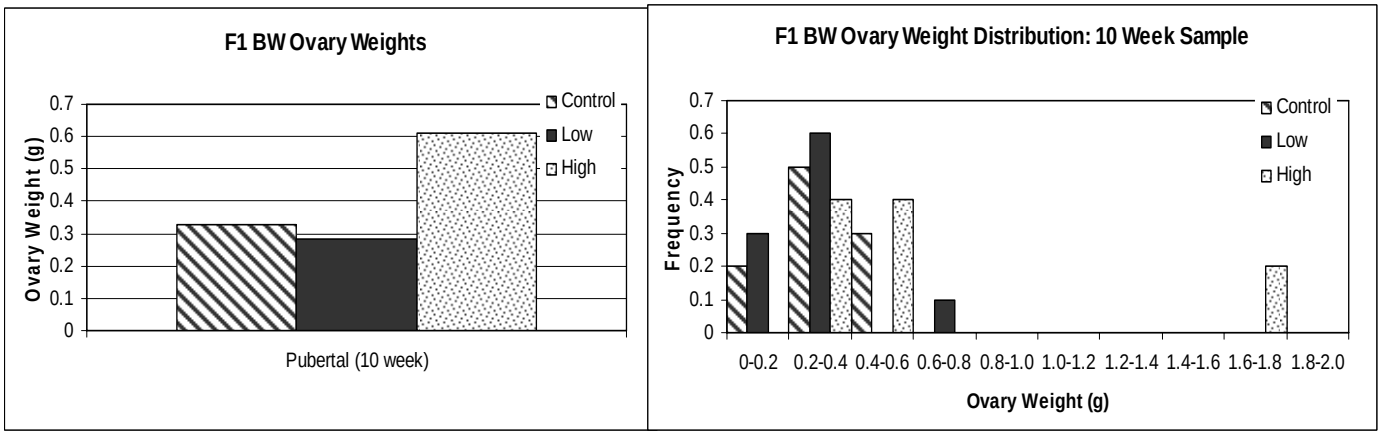
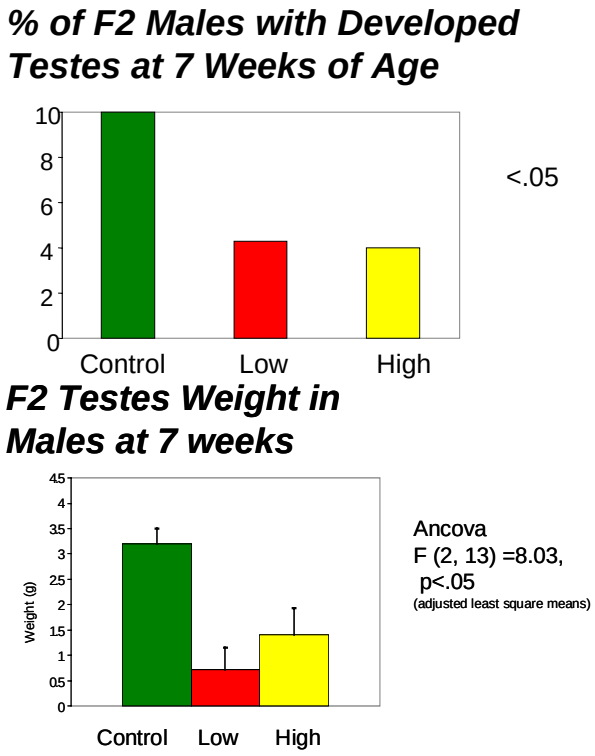
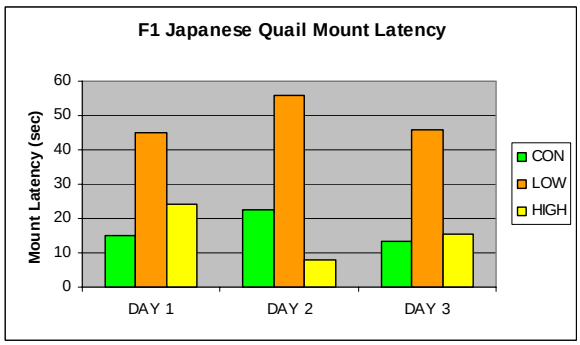
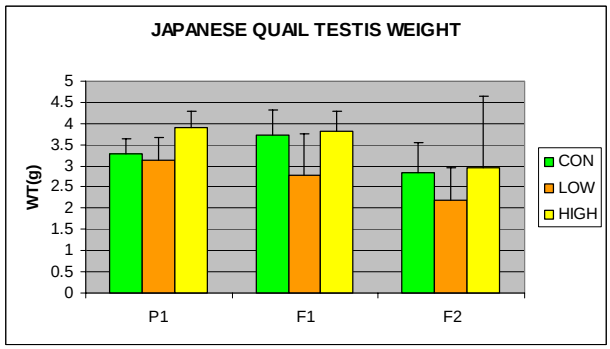
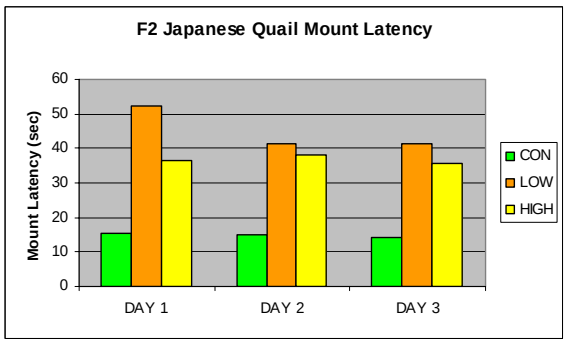


Figure 11: Testes weight in Japanese quail









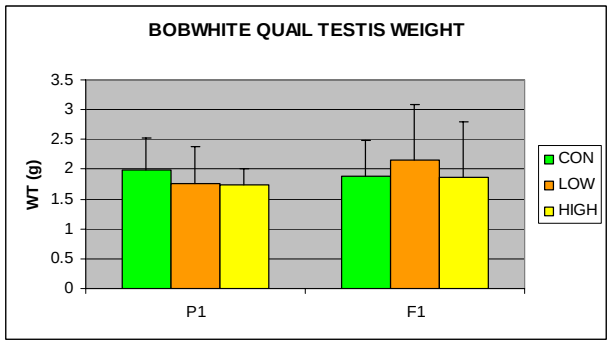
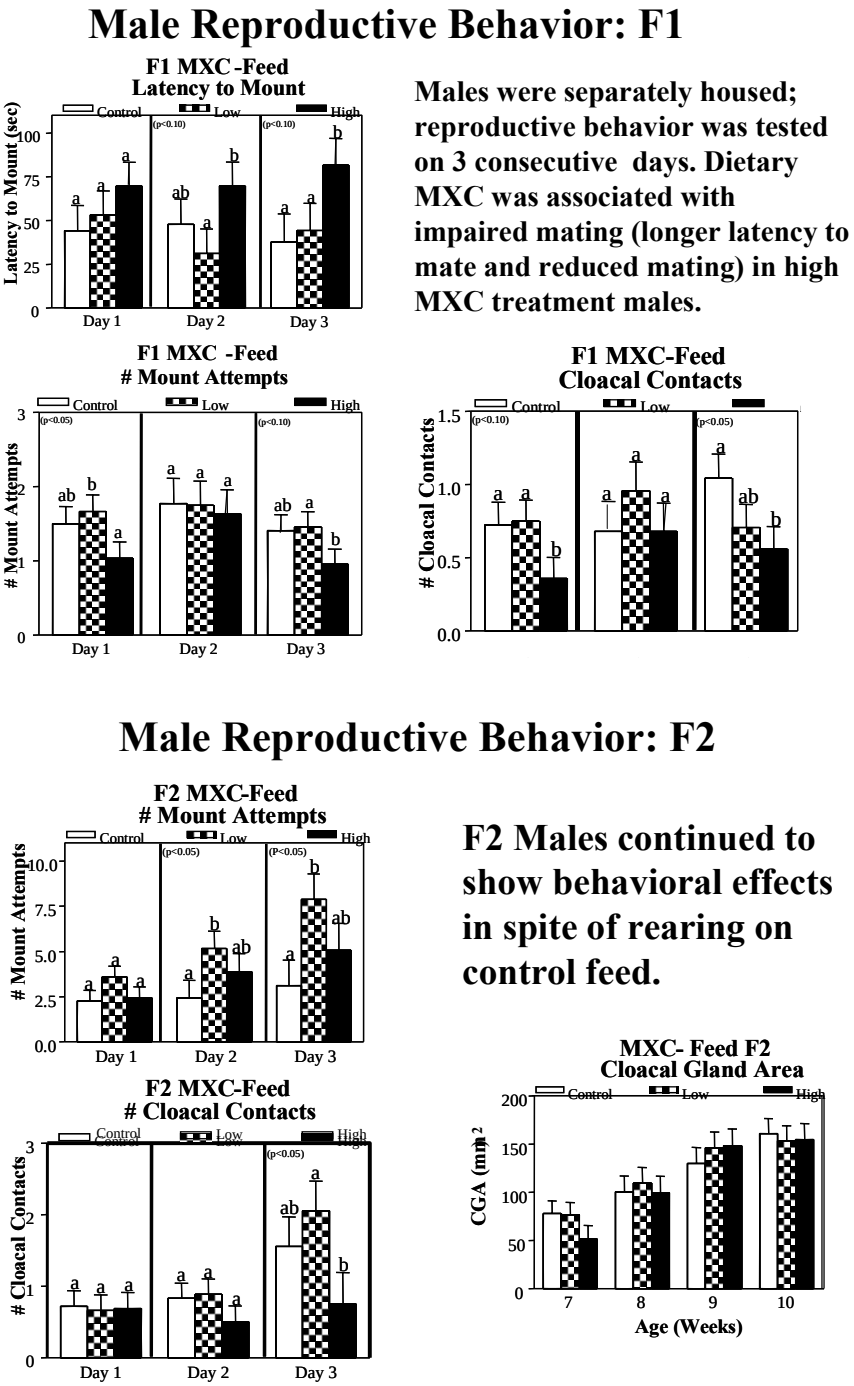
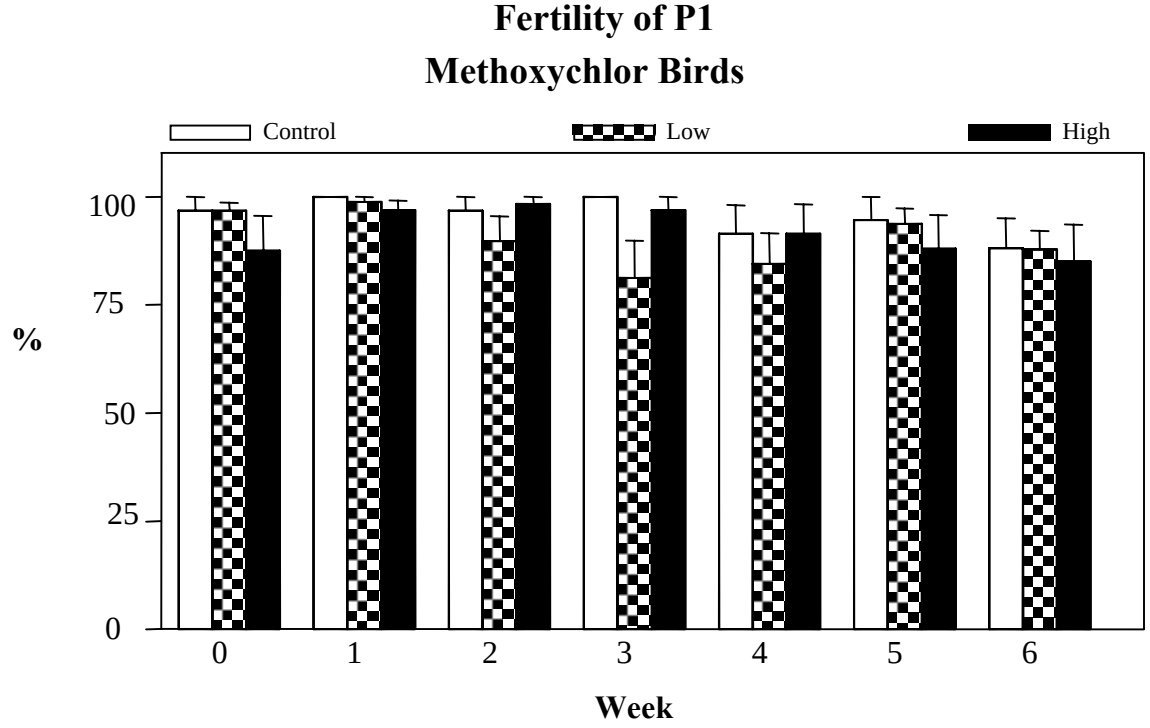
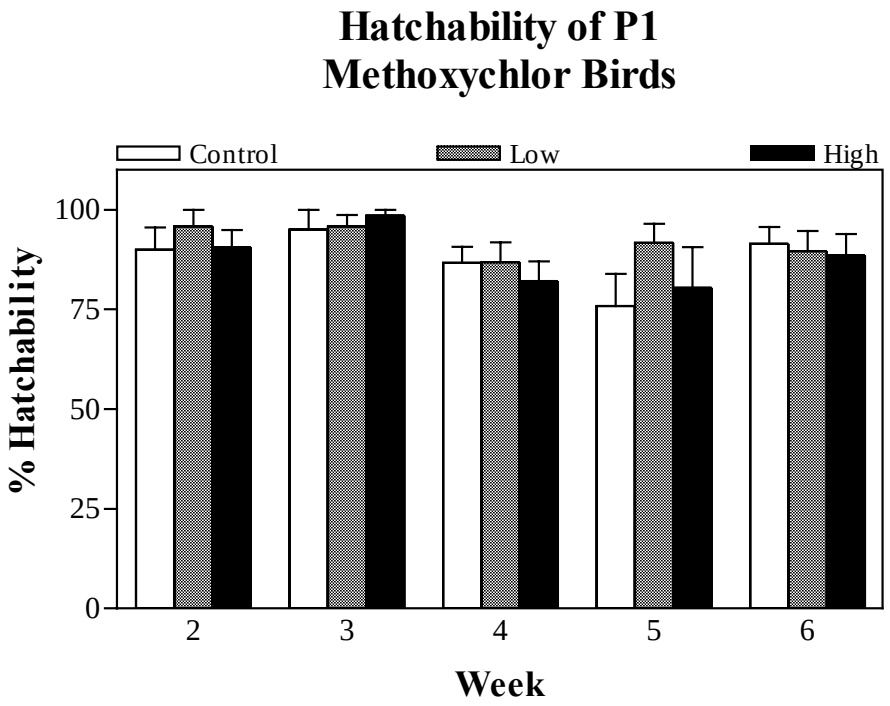


Figure 12: Male reproductive behavior in Japanese quail.

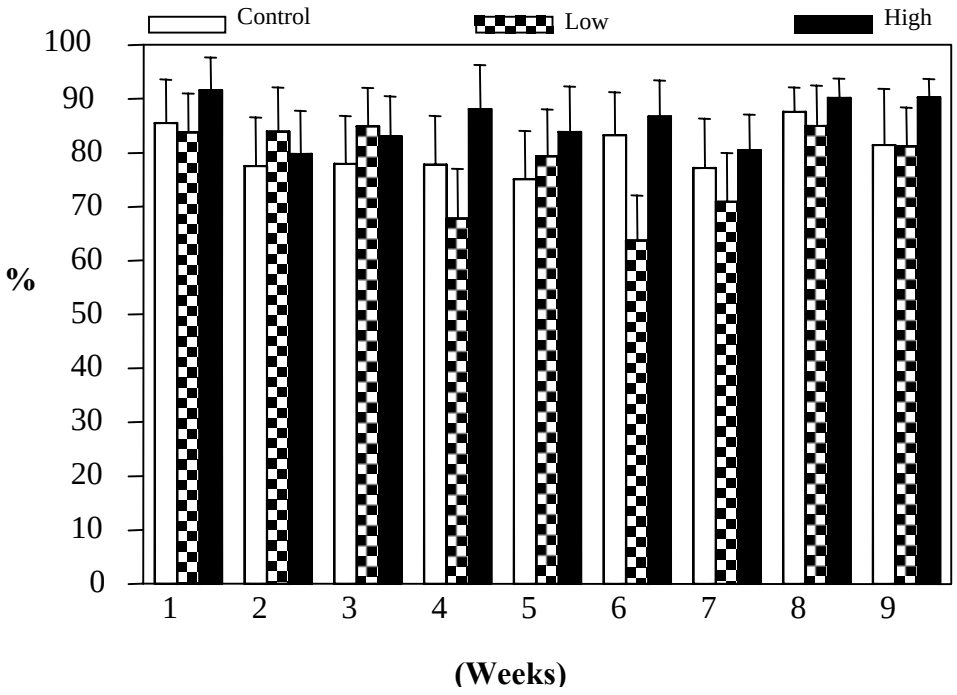


Figures 13 a, b, c, and d: Fertility and hatchability of P1 and F1 MXC Japanese quail





F1 MXC % Fertility



F1 MXC-Feed
% Hatchability

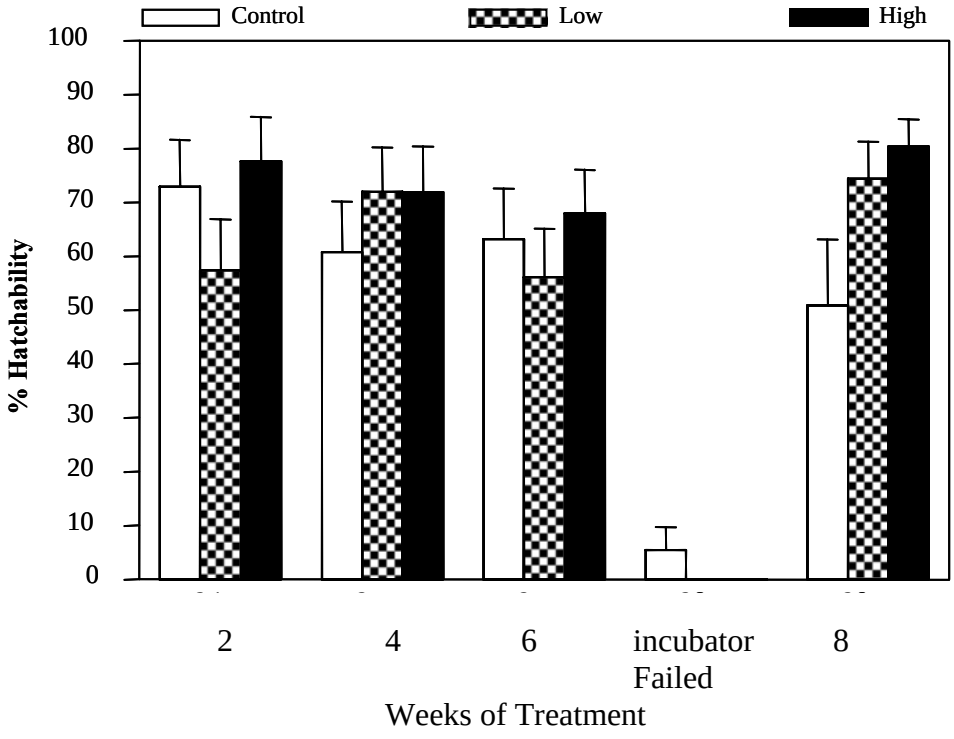
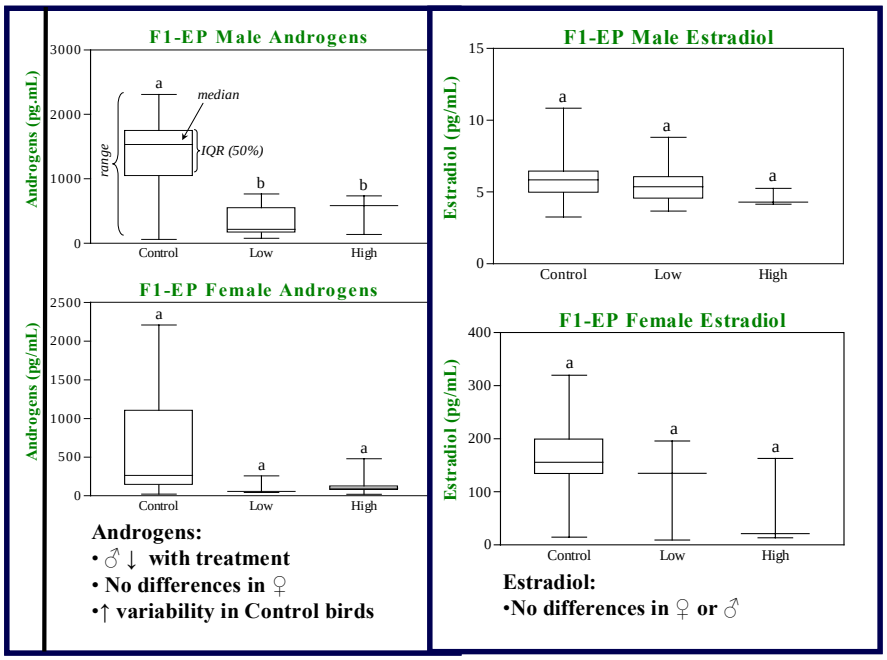
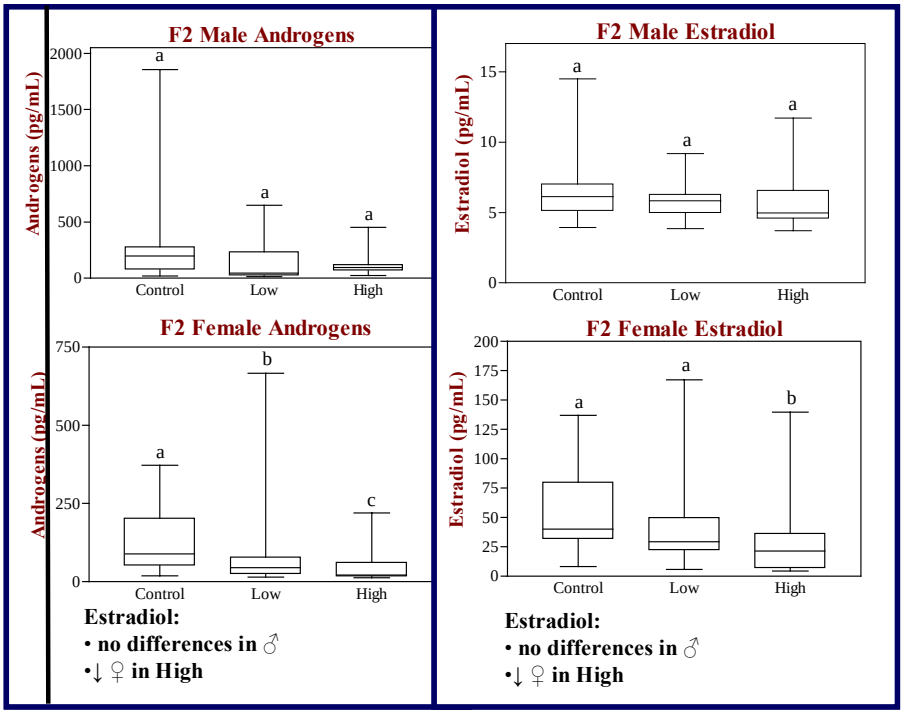


Figure 14: Plasma steroid hormones shown as box diagrams to illustrate the wide variation found between individuals, especially during sexual maturation.

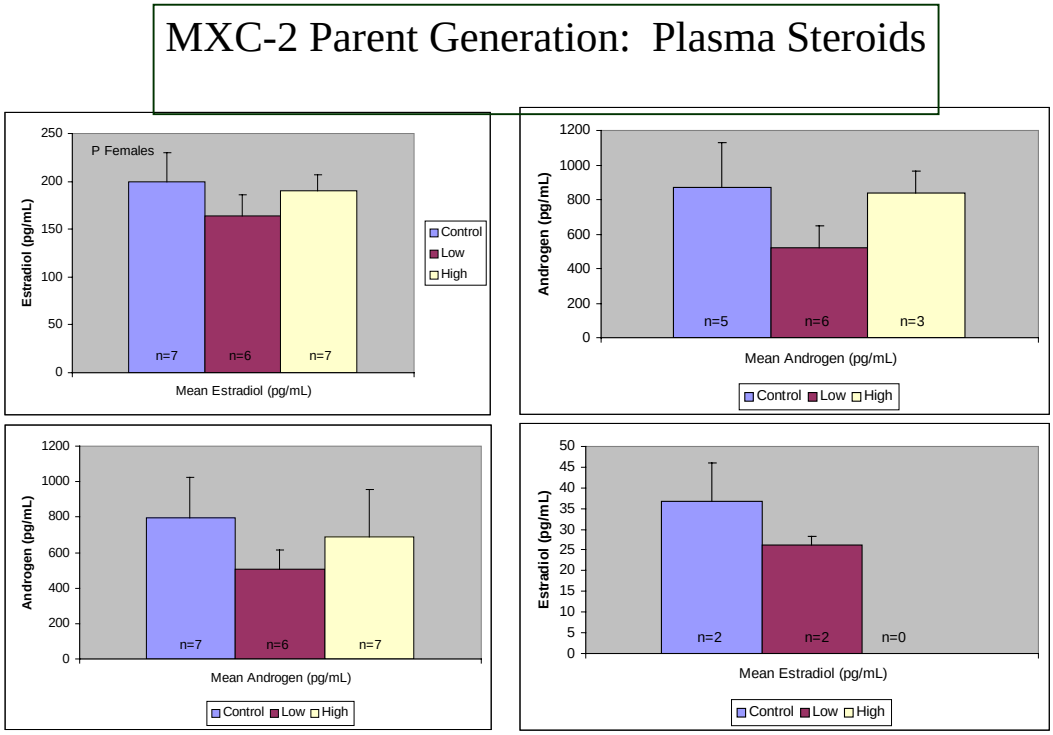


Plasma androgen was reduced in young males; variability was high during maturation in controls due to individual differences in onset of maturation.



Adult females had reduced plasma estradiol as well as individual variability in plasma steroids.

Figure 15: Averaged plasma steroid hormones and fecal hormone measurements from the P1 generation for Japanese quail



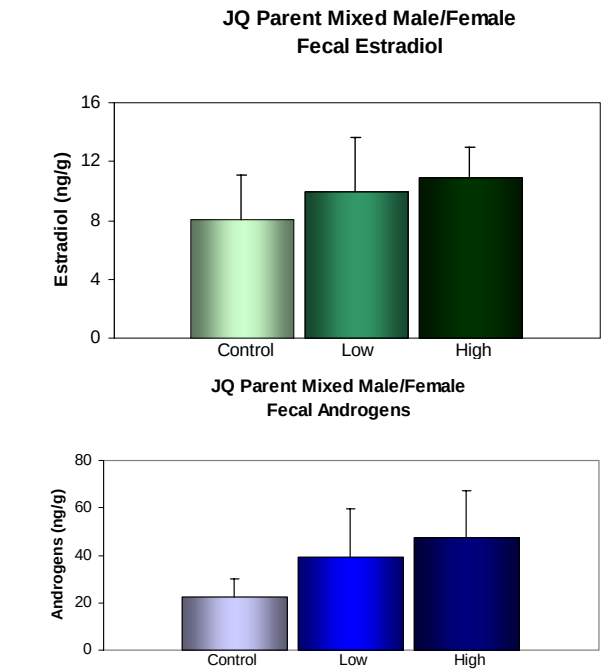
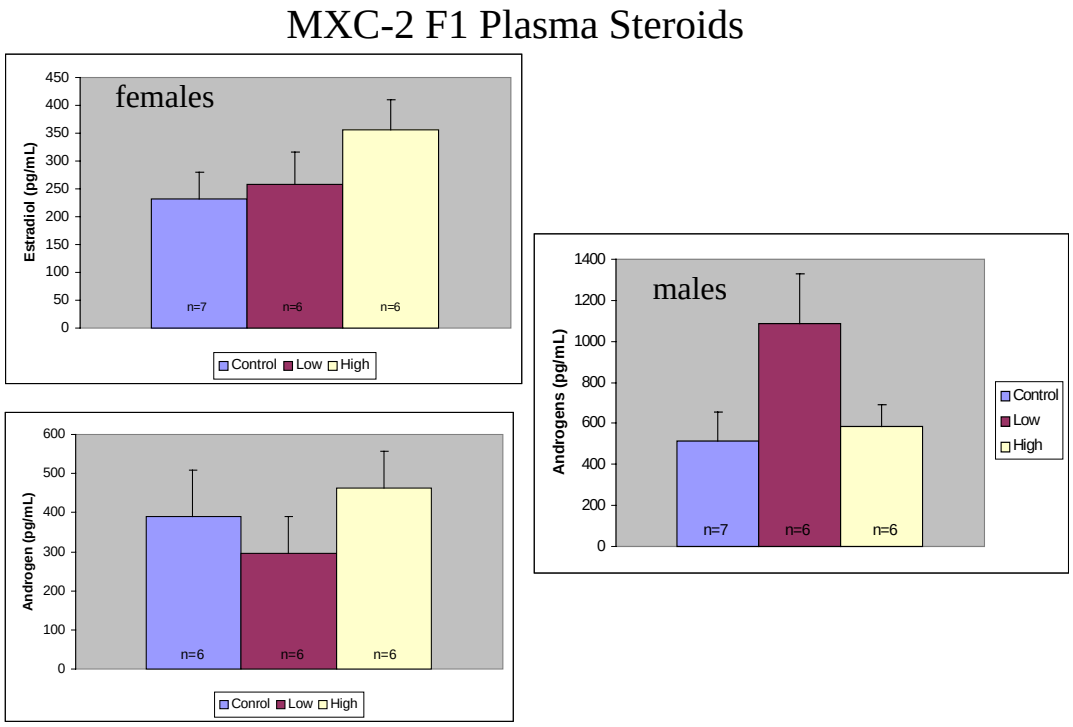


Figure 16: Plasma and fecal steroid hormones in the F1 Japanese quail generation.



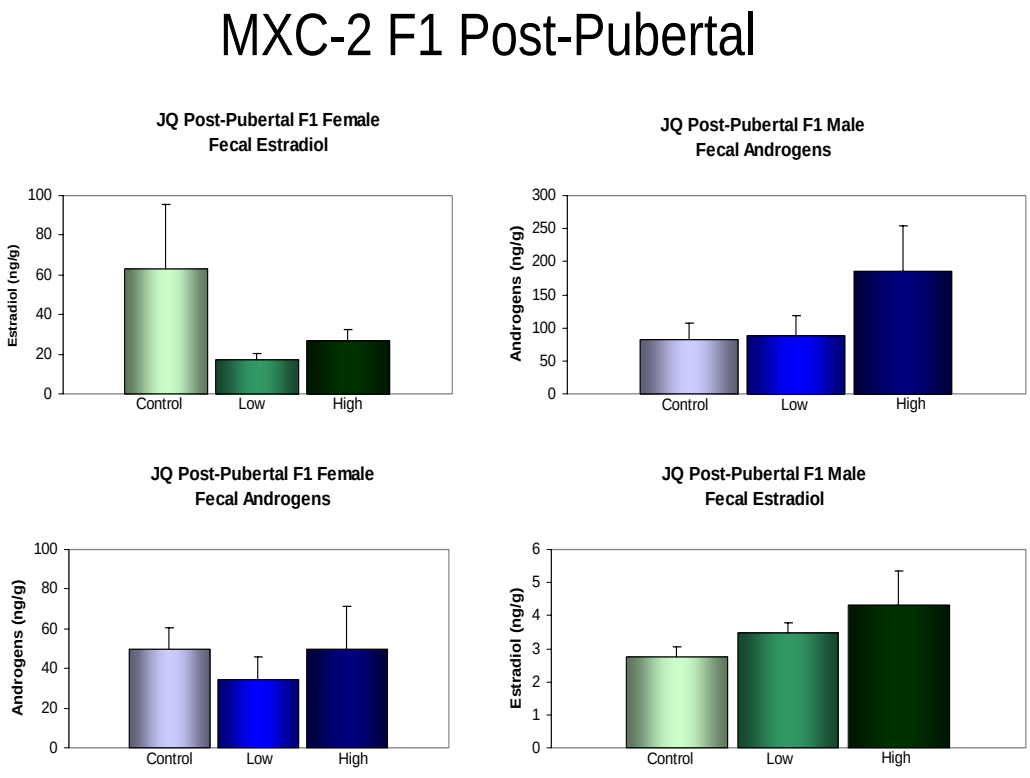
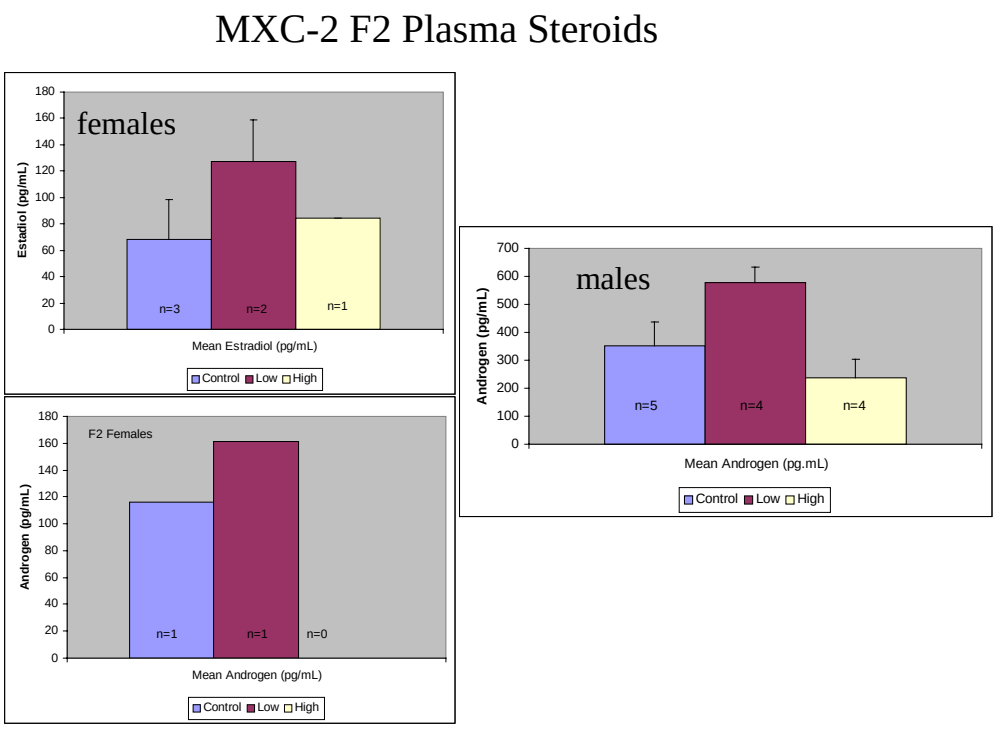
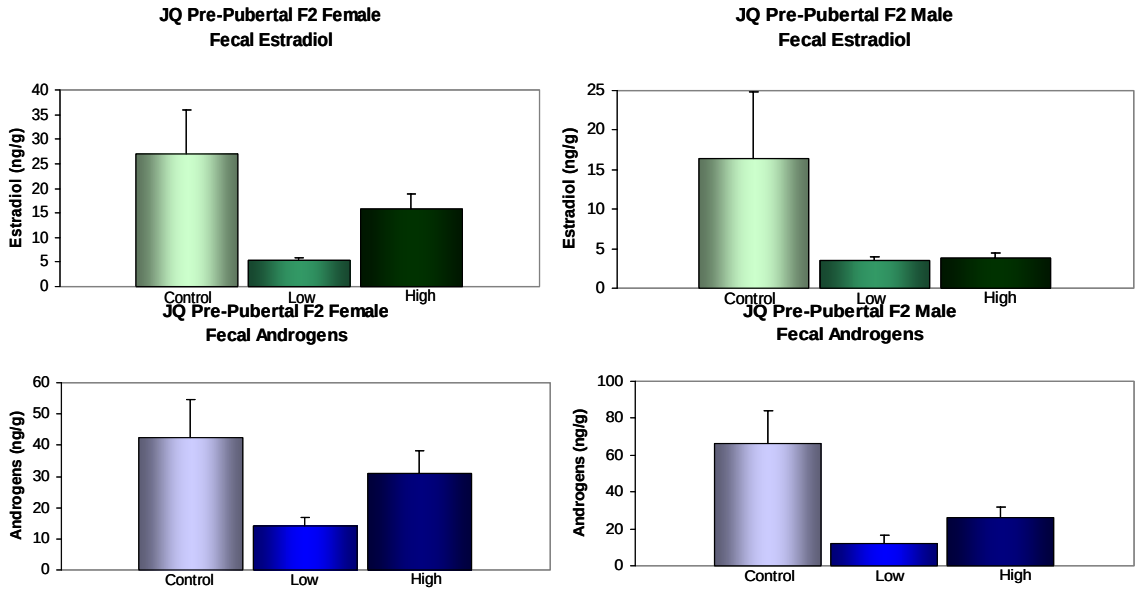
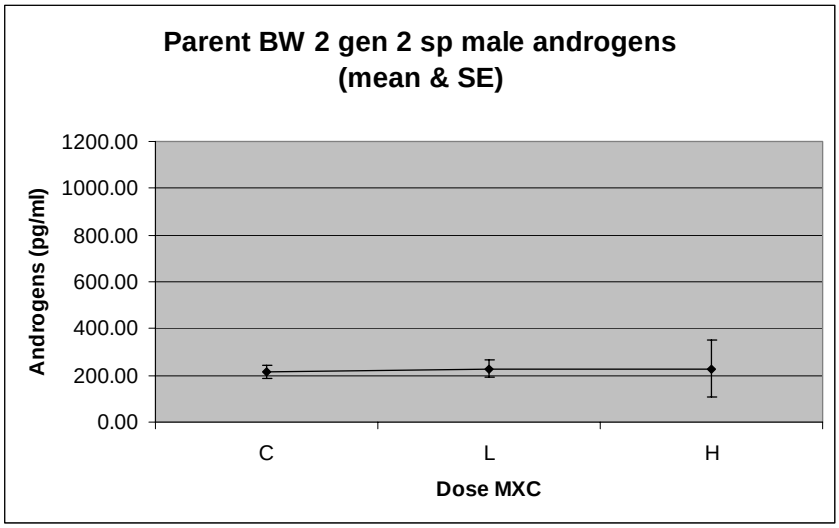


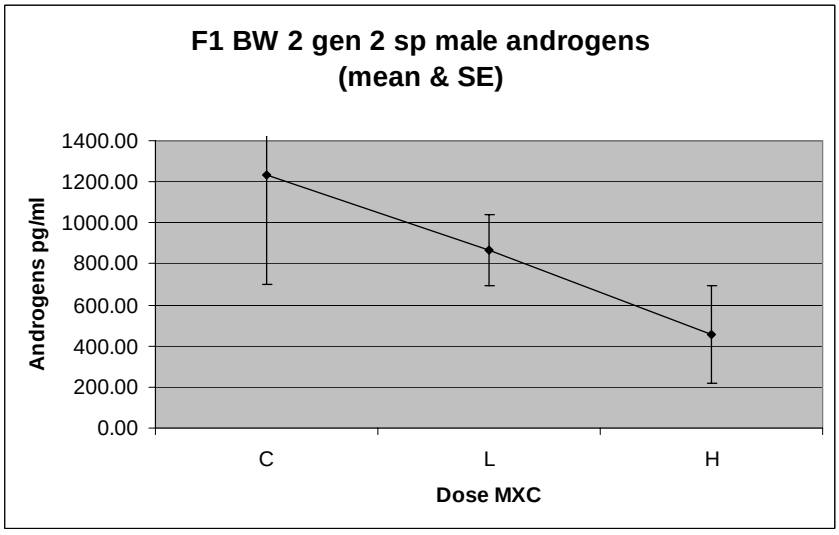
Figure 17: Plasma and fecal steroids for the F2 generation

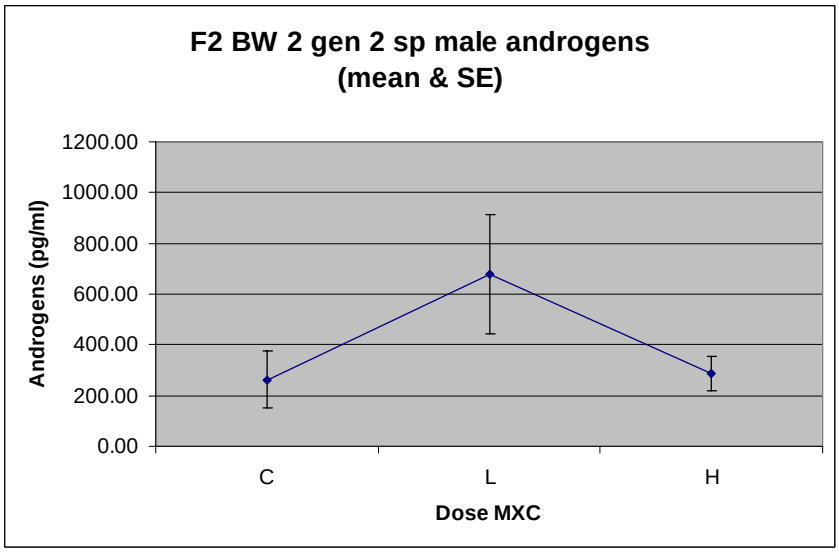


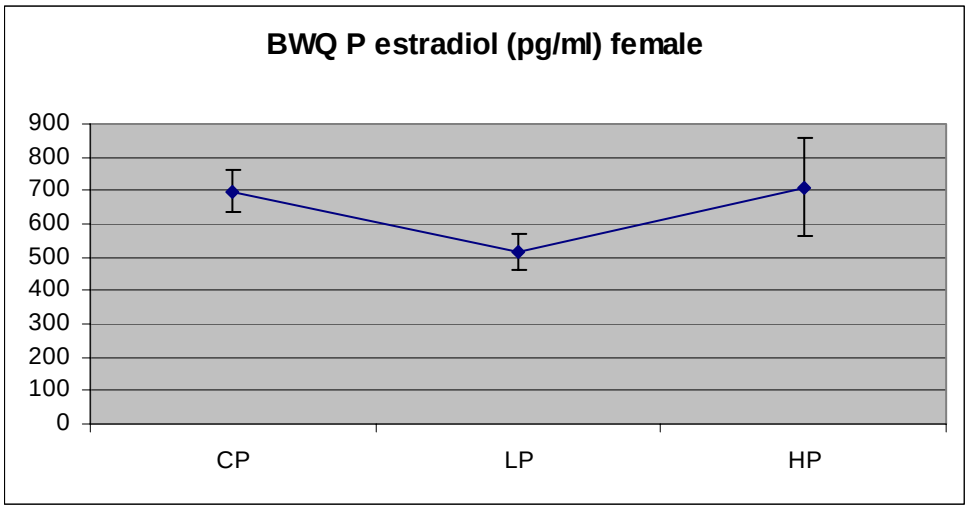


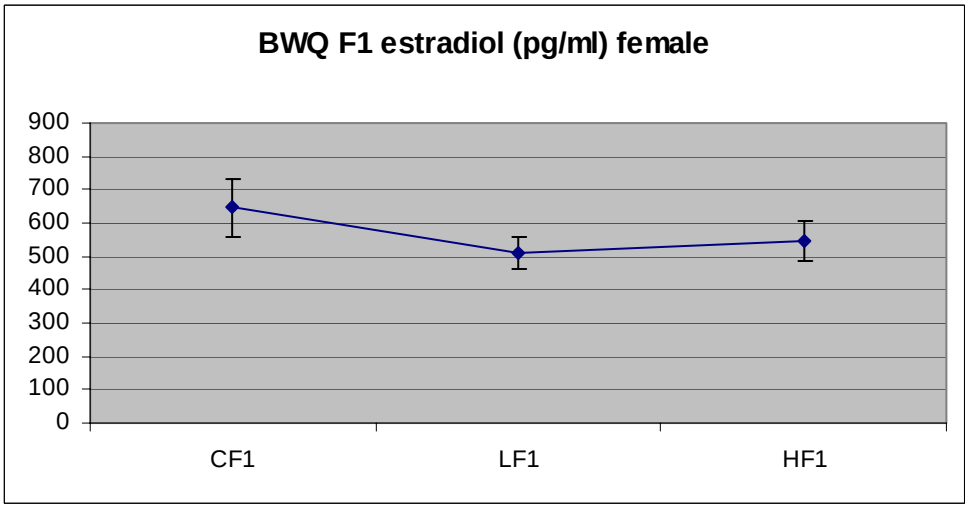
Figures 18 a, b, and c; d, e, and f: Plasma androgens and estrogen in bobwhite quail.

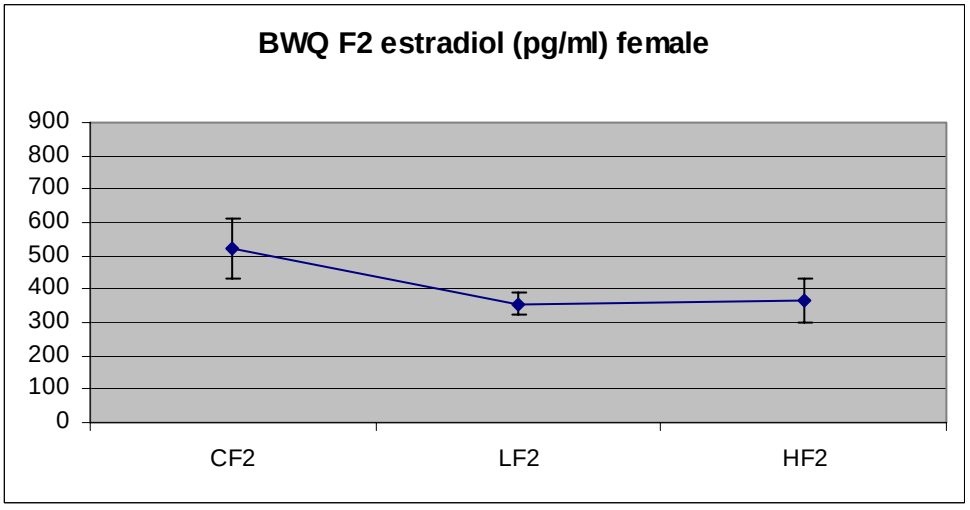







BWQ P estradiol (pg/ml) female
900
800
700
600
500
400
300
200
100
0
CP
LP
HP


BWQ F1 estradiol (pg/ml) female
900
800
700
600
500
400
300
200
100
0
CF1
LF1
HF1


BWQ F2 estradiol (pg/ml) female
900
800
700
600
500
400
300
200
100
0
CF2
LF2
HF2

c

Figure 19: Monoamine content in the hypothalamus in hatchling and maturing Japanese quail.

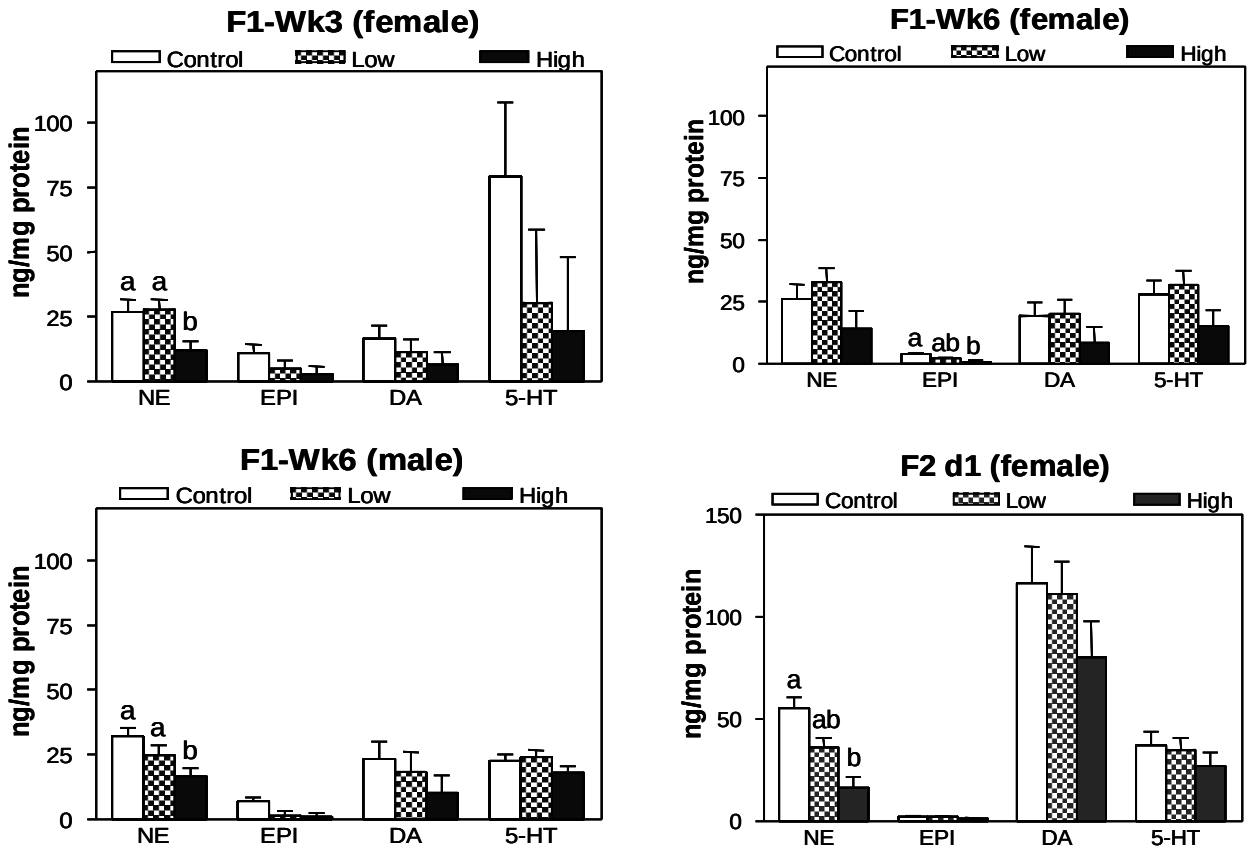
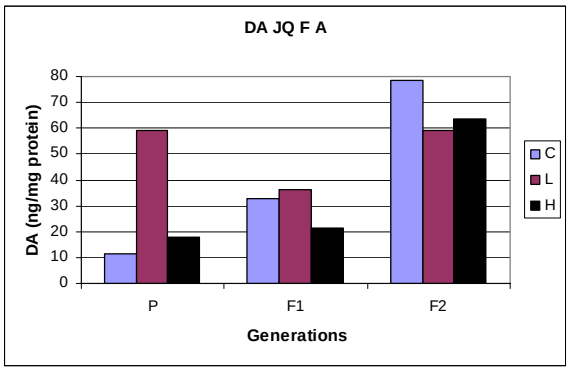
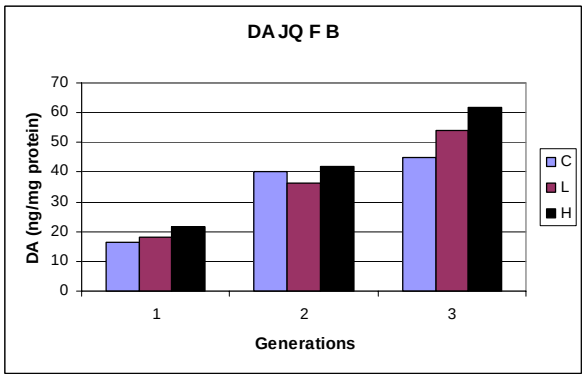
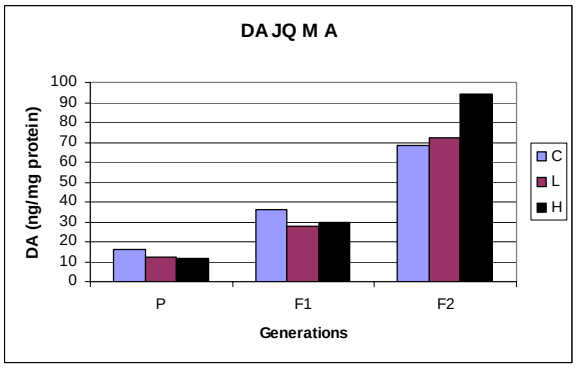
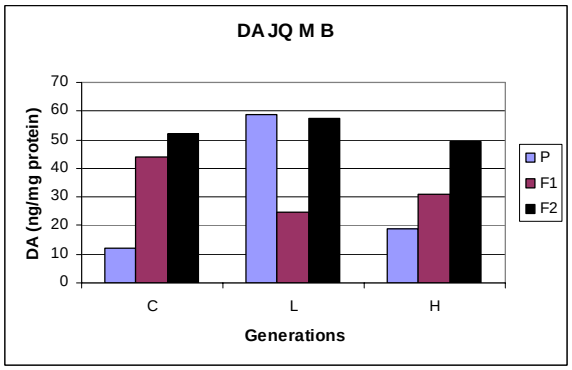


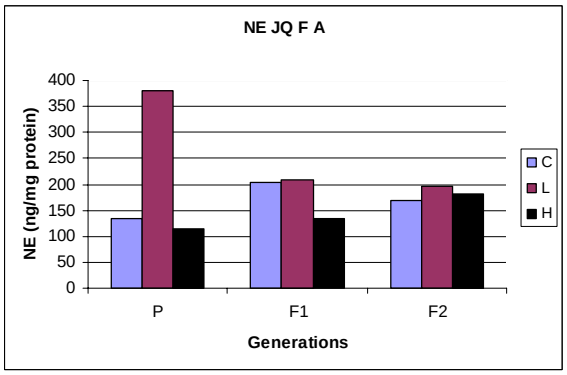
Figure 20: Monoamine levels in adult Japanese quail (M=male; F=female; A=anterior hypothalamus; B=posterior hypothalamus).

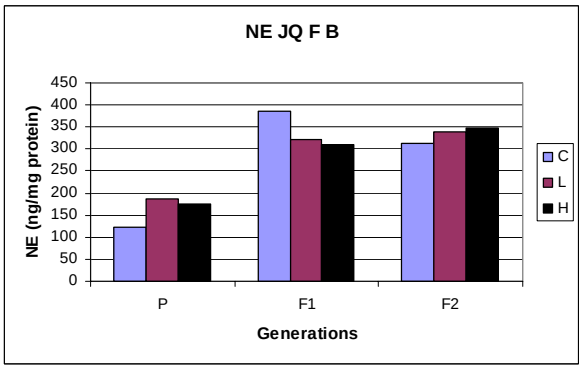


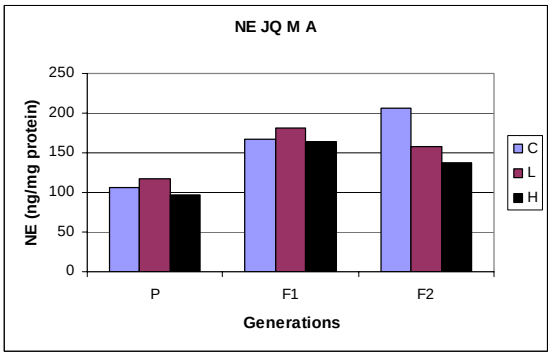












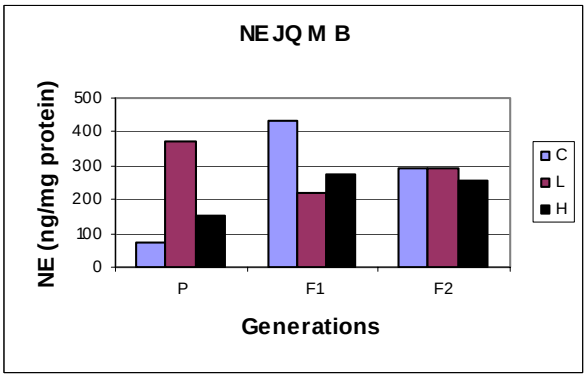
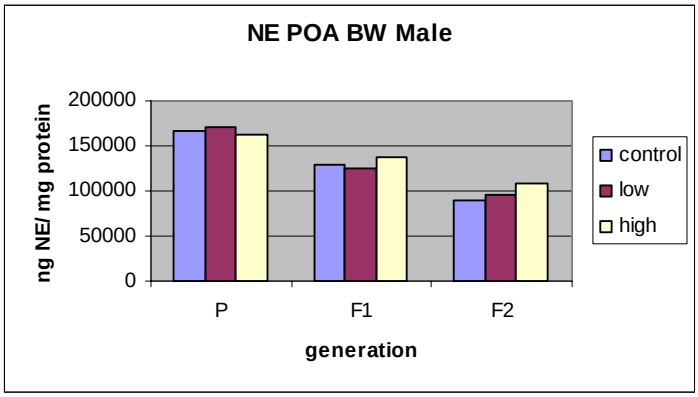
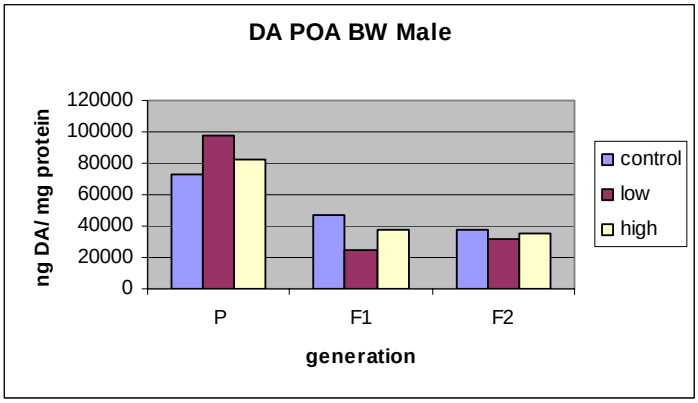
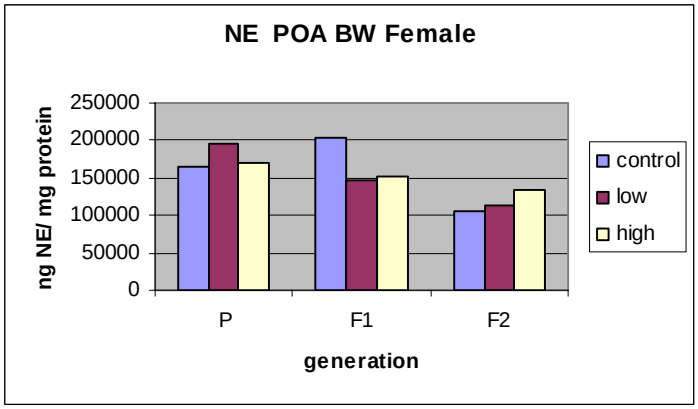


Figure 21: Monoamines in the bobwhite quail.







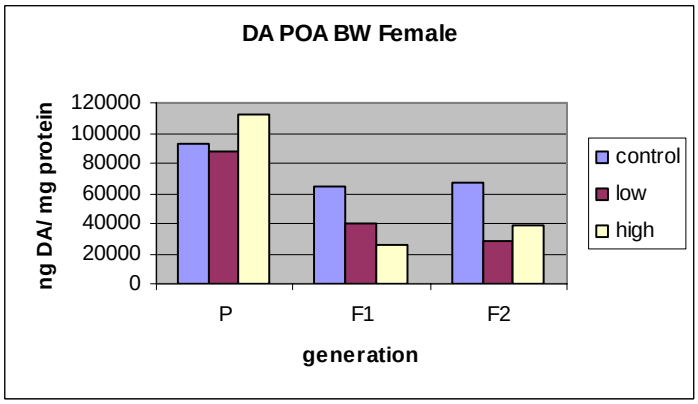
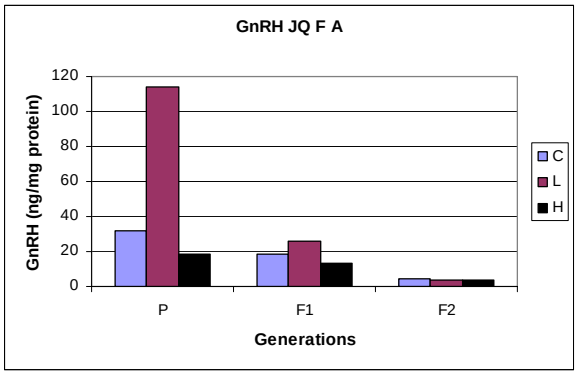
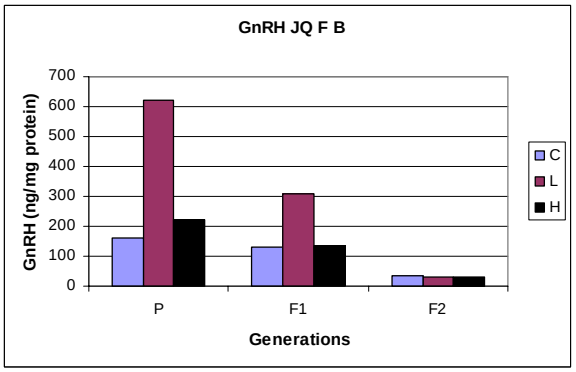
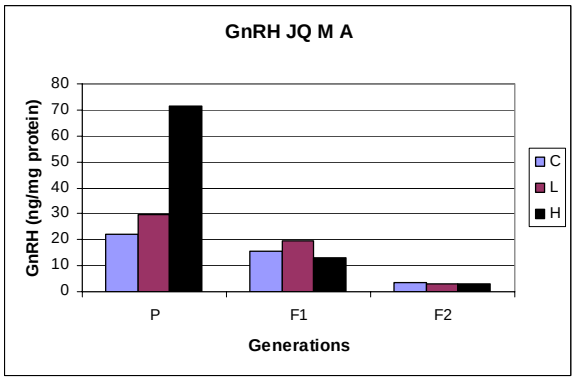
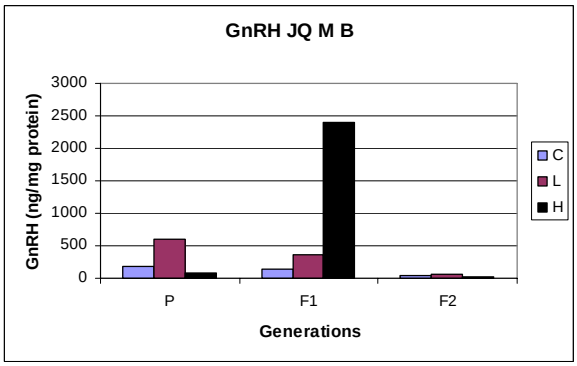


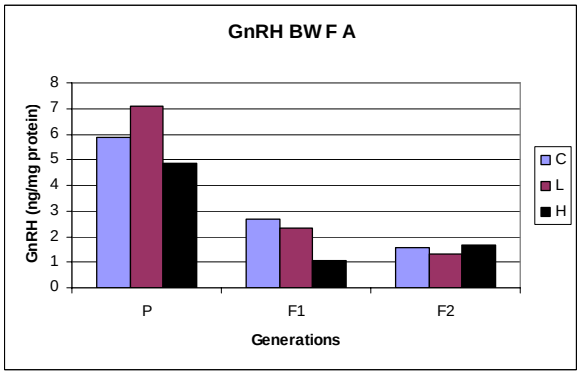
Figure 22: GnRH-I in microdissected hypothalamus.











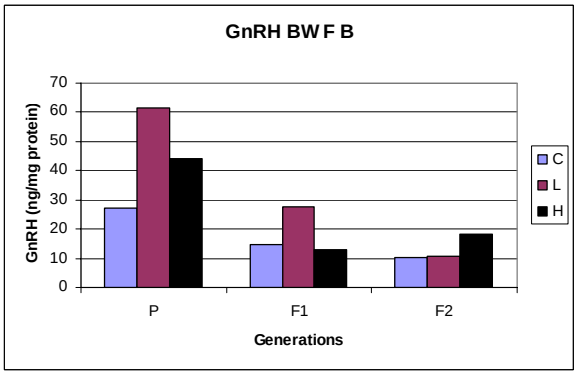
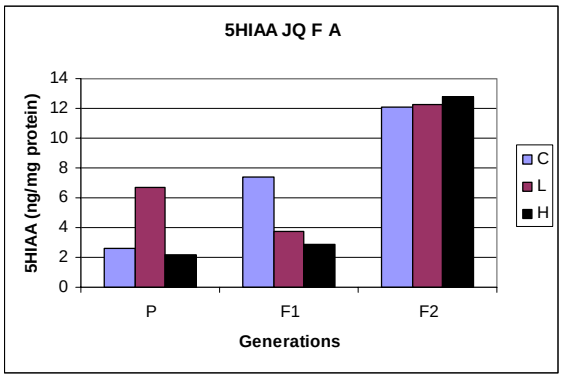
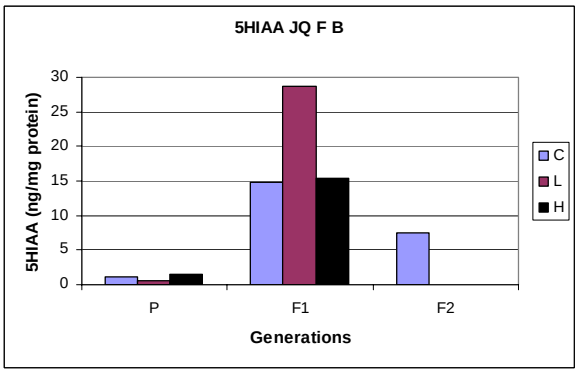
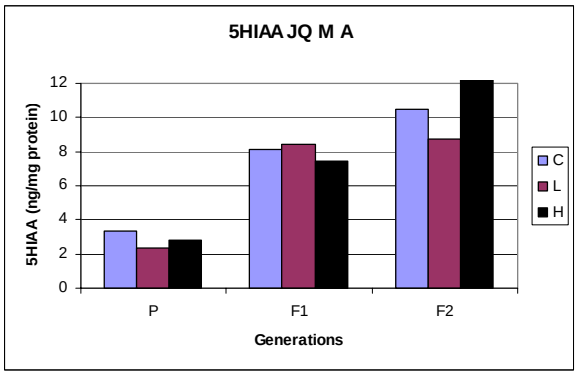
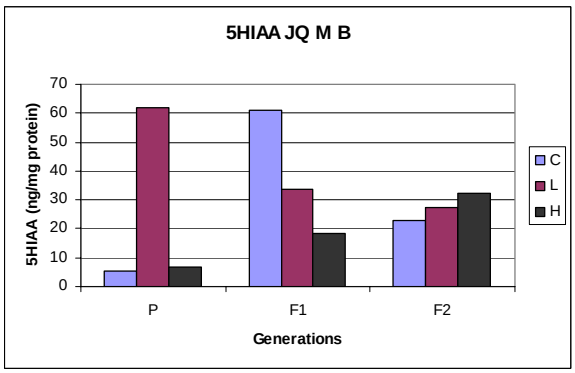


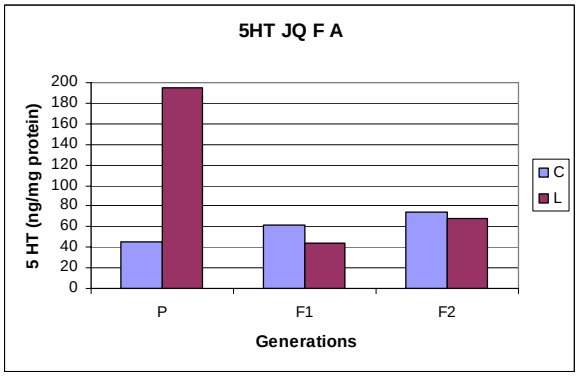
Figure 23: Serotonin (5-HT) and the serotonin metabolite (5-HIAA) in Japanese quail hypothalamus.

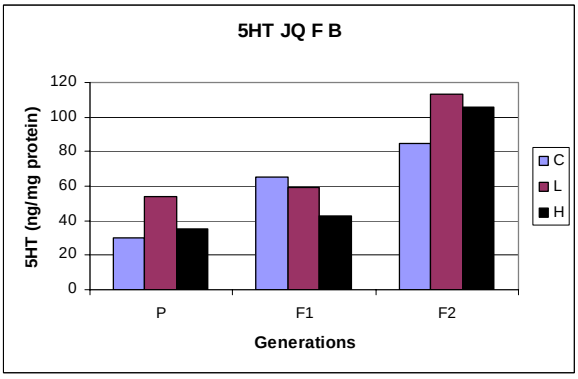


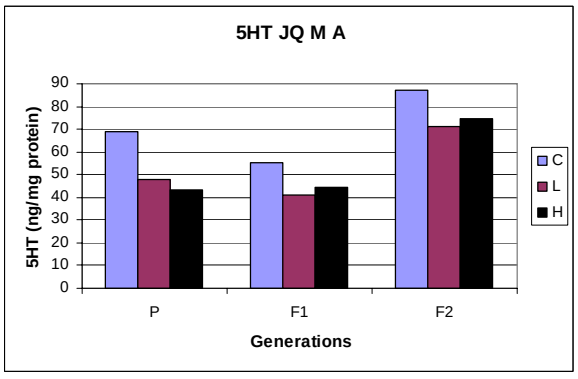












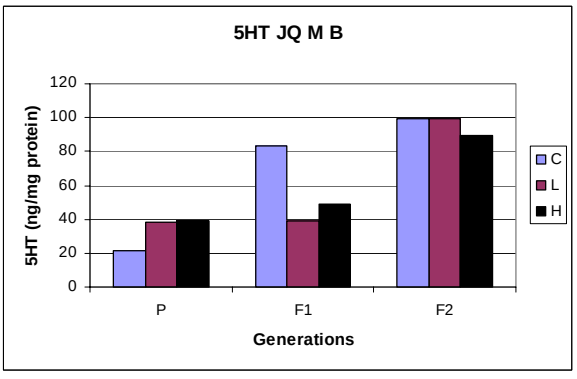


Figure 24: GnRH-I and catecholamines in Japanese quail.

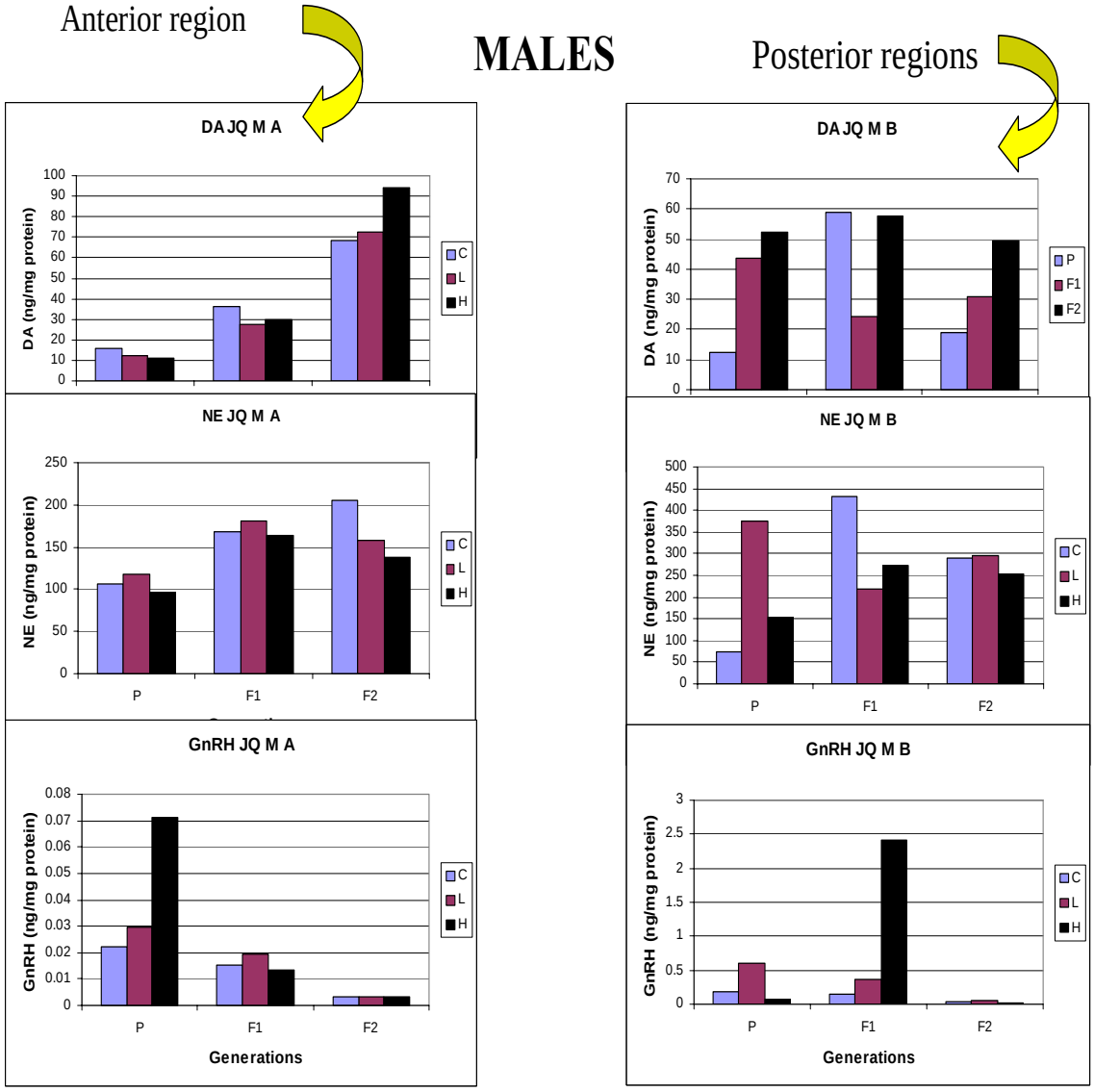
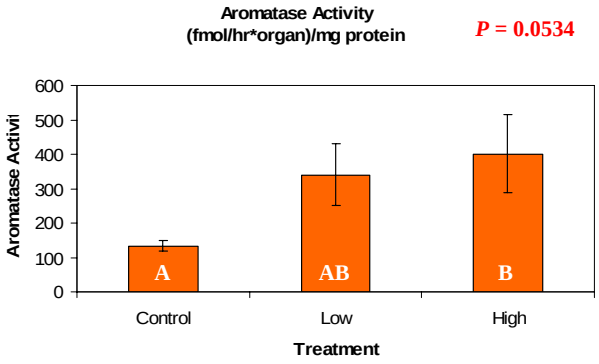


Figure 25:

Hypothalamic Aromatase Activity in Japanese Quail (F1)
Exposed to Dietary Methoxychlor (5 and 10ppm)



Treatment	N	Gender	Aromatase Activity ± SE
0	10	F	124.3 ± 19.1
5ppm	13	F	414.0 ± 158.7
10ppm	15	F	396.0 ± 155.0
0	10	M	142.7 ± 27.4
5ppm	12	M	260.5 ± 81.6
10ppm	11	M	406.7 ± 173.2

Figure . Aromatase Activity per Treatment and Gender

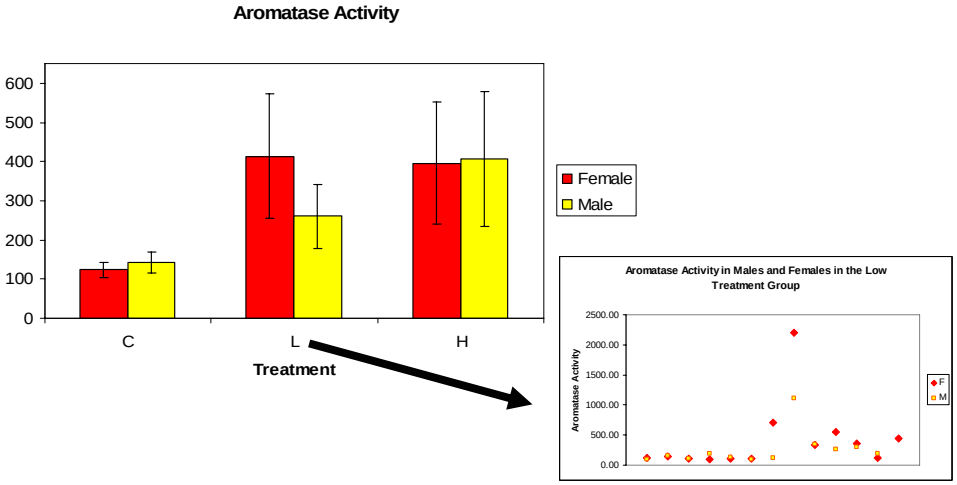


Figure 26: Acknowledgements! This research could not have been conducted without the amazing support by my technician, postdoctoral fellows, graduate students and undergraduate students who did the daily measures, checked the animals and the incubators, labeled tubes, and did lots and lots of assays!! Special thanks goes to Julie Hazelton, whose outstanding organizational skills were invaluable in the daunting organization of this ambitious project!