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Histological Effects of Low Level Inorganic Lead on the Pituitary and Ovary of Rainbow Trout (Oncorhynchus mykiss)

Ruth N. Hull

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

April 1994

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ISBN 0-315-90939-0

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ABSTRACT

Histological Effects of Low Level Inorganic Lead on the Pituitary and Ovary of Rainbow Trout (Oncorhynchus mykiss)

Ruth N. Hull

Sexually-maturing female rainbow trout were exposed to sublethal concentrations of 10 and 25 μg/L inorganic lead (Pb) during endogenous and exogenous vitellogenesis. The endogenous vitellogenesis experiments were conducted for 12 days and the late exogenous vitellogenesis experiment had sampling conducted at Days 12 and 20. Fish were maintained under a 12 hour light/12 hour dark light regime, in water of 12 to 14°C and hardness of 121 to 128 mg/L.

Lead had no impact on reproductive function, as measured by GSI, maximum oocyte diameter, oocyte stage frequency, or the number of pituitary basophils during endogenous vitellogenesis (February/March). The experiment conducted in April occurred at a period of transition in the reproductive cycle; some of the fish had entered exogenous vitellogenesis, while others were still in the endogenous vitellogenesis phase. This phase, at the beginning of exogenous vitellogenesis, may be a sensitive time for Pb exposure of fish. There were trends observed in the GSI, oocyte diameter and stage data which indicated Pb may be having an adverse impact on oocyte development. No effects were observed on pituitary basophils.

There was evidence that Pb impaired reproductive parameters during late exogenous vitellogenesis (September). Oocyte diameter and stage data suggested that early stage oocytes experienced delayed or halted development. In addition, although late stage oocytes were able to complete maturation, this process was delayed. The presence of more early stage and atretic oocytes in Pb-exposed fish suggests that Pb may affect fecundity.

The consistently large number of pituitary basophils in fish exposed to 25 μg/L Pb during late vitellogenesis suggests that gonadotropin (GtH) may not be released from the basophils, or may be released in smaller quantities. Decreased GtH levels could lower the production of steroids (estrogen, estradiol) by the ovary which could subsequently lower the circulating calcium levels in the blood and lower the synthesis of vitellogenin (Vg) by the liver and the uptake of Vg by early stage oocytes.

This study is the first to examine reproductive effects of water-borne Pb at these low concentrations. It has contributed to the understanding of the impact of Pb on rainbow trout reproduction at different periods of the vitellogenesis cycle. These effects may be related to changes in the endocrine mechanism acting through the hypothalamus-pituitary-ovarian axis.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Sylvia Ruby for her support and guidance, and my committee members Dr. Perry Anderson and Dr. Daphne Fairbairn for their ideas and direction. Comments provided by the above professors as well as Dr. Alice Hontela (UQAM) have improved this manuscript. Thanks also go to Zsofia Zsabone for training me in histological technique.

I would like to thank my friends and fellow graduate students, especially John Vidmar and Marco Rigotti for their friendship, appreciation of good coffee, and crossword prowess. Special thanks go to Patricia Jaroslawski for her good company in the laboratory.

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INTRODUCTION

Metals differ from other toxic substances in that they are neither created nor destroyed by humans. Metals are redistributed naturally in the environment by both geological and biological cycles. However, human industrial activity may greatly shorten the residence time of metals in ore, form new compounds, and greatly enhance worldwide distribution (Goyer, 1986). An example of the impact human activity can cause is seen in the 200-fold increase in lead (Pb) content of Greenland ice beginning with a natural level from about 800 B.C. and a gradual rise in lead content of ice through the evolution of the industrial age, followed by a rise in lead corresponding to the period when lead was added to gasoline in the 1920s (Ng and Patterson, 1981 cited in Goyer, 1986).

A recent study by Rosman *et al.* (1993) traced the relative contributions of the U.S.A. and Canada/Eurasia as sources of the Greenland lead, using the isotopic signatures of the atmospheric aerosols from these regions. Eurasia and Canada had similar isotopic signatures and hence could not be considered separately. This study found that the marked decrease in Pb in the Greenland ice after 1976 could be attributed to a decrease in the amount of U.S.A. Pb, whereas Eurasia/Canada maintained a lower but constant output.

Lead is found throughout Canada's rivers and lakes and in the groundwater. The Canadian water quality guidelines recommend maximum levels of between 1 and 7 μg/L total lead for waters of various hardness (Table 1) (Environment Canada,

Table 1: Recommended Guidelines for Total Lead for Waters of Different Hardness (Environment Canada, 1987).

Hardness (mg/L as $CaCO_3$)	Concentration of Lead (µg/L)
0-60 (soft)	1
60-120 (medium)	2
120-180 (hard)	4
>180 (very hard)	7

1987). However, typical freshwater concentrations in Canada are between 1 and 12 µg/L, with some concentrations reaching 30 µg/L (RSC, 1986).

Inputs of lead into the aquatic environment include both natural and anthropogenic sources. There are estimates that between 152 000 and 162 000 tonnes of lead per year enter the world's aquatic ecosystems by weathering of rocks and soils (Demayo et al., 1982). However, anthropogenic inputs have been large. In 1976, it was estimated that between 47 and 93% of the total input of Pb into Lakes Ontario, Erie and Huron was of anthropogenic origin (NRC, 1977). The burning of coal, oil and gasoline, cement manufacturing, the metallurgical industry, waste incineration and the manufacture of iron and steel all contribute to the human input of Pb into the environment. Presently, the primary uses of lead are for batteries, paint pigments, and gasoline additives. However, the major source of environmental pollution by lead is the combustion of gasoline additives (Environment Canada, 1987).

Although the lead compounds in gasoline are organic, inorganic Pb is a more important form of lead in the environment. Lead antiknock compounds are produced in the form of the organic compounds tetramethyl lead (TML) and tetraethyl lead (TEL), with TEL constituting a greater percentage of the blend. TML and TEL are destroyed by the internal combustion process in the car motor, and the Pb emitted is largely in the form of inorganic chlorides and bromides (NRC, 1977). Alkyl Pb compounds from gasoline

evaporation account for 5 - 10 % of the total airborne lead (NRC, 1977). However, the estimated atmospheric half-life of organic lead vapour is less than 12 hours, and organic lead compounds are decomposed in the presence of light. Hence these emissions are not considered very important in the general environment. Presently alkyl Pb additives are being phased out, and replacements are being studied (RSC, 1986).

Chemical reactions in the atmosphere convert primary Pb emissions from automobiles to a large variety of Pb species. The major species include $PbCO_3$, PbO_x, $(PbO)_2PbCO_3$, $PbSO_4$ and $2PbBrCl \cdot NH_4Cl$ (Demayo et al., 1982). Lead released into the atmosphere reaches the land and surface water rapidly by direct sedimentation and rainfall. The residence time of Pb in the atmosphere is estimated at 10 days (RSC, 1986). When not complexed with organic ligands, the main species of Pb in water are Pb^{2+} and $PbCl^+$ (Demayo et al., 1982). Lead nitrate ($Pb(NO_3)_2$) was chosen as the toxicant for the present study because it dissolves readily in water, forming Pb^{2+}. Most inorganic salts are sparingly soluble (e.g., PbF_2, $PbCl_2$) or virtually insoluble (e.g., $PbSO_4$, $PbCrO_4$) in water; the notable exceptions are lead nitrate and lead acetate ($Pb(OCOCH_3)_2$) (U.S. EPA, 1977). Pb species other than Pb^{2+} will be present in the test water. See Davies et al. (1976) for a discussion of Pb species in soft and hard water.

Effects of lead on humans have been studied more extensively than effects on nonhuman organisms. In humans, anemia has been recognized as a toxic effect of Pb for

decades, and even centuries (Waldron, 1966). Pb in the bloodstream has been shown to cause toxicity in children, including lower intelligence and behavioural dysfunction (Marlowe, 1985). In older adults, Pb exposure raises blood pressure (Cramér and Dahlberg, 1966), with attendant increased risks of heart attacks, strokes and death (Driscoll et al., 1992). Lead neurotoxicity has been reviewed by Winder and Kitchen (1984). The influence of leaded gasoline on human blood Pb levels has been documented in the U.S.A. (U.S. EPA, 1985) and Mexico (Driscoll et al., 1992). For more information on the effects of Pb on humans, the reader is directed to U.S. EPA (1977; 1985), any one of several books on toxicity of heavy metals in the environment, and medical journals.

Demayo et al. (1982) have reviewed the subject of Pb toxicity to nonhuman organisms. There have been several studies on the toxic effects of Pb to fish. Many studies have used concentrations of Pb (1-33 mg/L) which would only be found in grossly contaminated lakes and rivers, such as those receiving mining discharge (Davies et al., 1976; Haux et al., 1986; Katti and Sathyanesan, 1986; Ruparelia et al., 1989; Tulasi et al., 1989).

Several studies have considered the biochemical alterations in fish caused by Pb exposure. The biochemical changes measured include changes in ALA-D (δ-aminolevulinic acid dehydratase) activity (Hodson, 1976; Hodson et al., 1978; Johansson-Sjöbeck and Larsson, 1979; Haux et al., 1986),

changes in reproductive hormone levels (Thomas, 1988; 1990), and blood parameters such as glucose, iron, and protein (Hodson et al., 1978; Haux et al., 1986; Tewari et al., 1987; Ruparelia et al., 1989).

Little research has been done concerning the histological effects of Pb on fish. The studies that have been done have considered high lead levels (mg/L) or exposed the fish via food intake. Katti and Sathyanesan (1986) considered changes in neurosecretory material of the pituitary at 5 ppm Pb. Thomas (1988) performed a qualitative examination of the ovaries of Atlantic croaker exposed to Pb in food. However, uptake of Pb from food has been shown to be only a minor route of exposure for fish. Pb uptake from water is the major exposure route (Hodson et al., 1978; Demayo et al., 1982). One study which argues the importance of uptake from food is that by Dallinger and Kautzky (1985). It assumed that uptake from food was an important source of Pb for rainbow trout in their study, because Pb concentrations in the rivers studied were low, and hence the trout must be receiving their exposure from food. However, Dallinger and Kautzky (1985) measured levels of lead in the water that were between 5 and 50 μg/L. These levels are high enough to cause lead accumulation in the tissues of rainbow trout, especially over a lengthy time period (Holcombe et al., 1976; Hodson et al., 1978). Thus, the argument for the importance of Pb uptake from food is questionable. Because the majority of evidence suggests that uptake of Pb from water is more important than from food, fish

were exposed to Pb dissolved in water in the current study.

Although reproductive effects of lead on mammals have been studied, few studies have concentrated on fish. Hormonal control of trout reproduction is reviewed by Scott and Sumpter (1983a). The reproductive endocrine system is very complex, involving secretions from the brain, pituitary, gonads and liver (Figure 1). A wide variety of chemicals interfere with reproduction in vertebrates, and therefore the endocrine system may be a site of toxic action. A chemical effect at any level of the brain-pituitary-gonad-liver axis may cause dysfunction at other levels.

The present study considered the histological effects of sublethal levels of dissolved inorganic lead (10 and 25 μg/L) on the pituitary and ovary of two-year old sexually-maturing female rainbow trout. Experiments were performed during two stages of the female rainbow trout reproductive cycle, early and late vitellogenesis. Quantitative changes in pituitary granular basophils were considered along with changes observed in the ovary. The cells of interest in the pituitary were the granular basophils located primarily in the proximal pars distalis, which are believed to be the gonadotropic cells which secrete gonadotropin (GtH) (van Oordt and Peute, 1983). The gonadosomatic index (GSI), frequency of oocytes in various stages of development including atresia (degradation), and the diameter of the five largest oocytes were calculated for control and Pb-exposed fish.

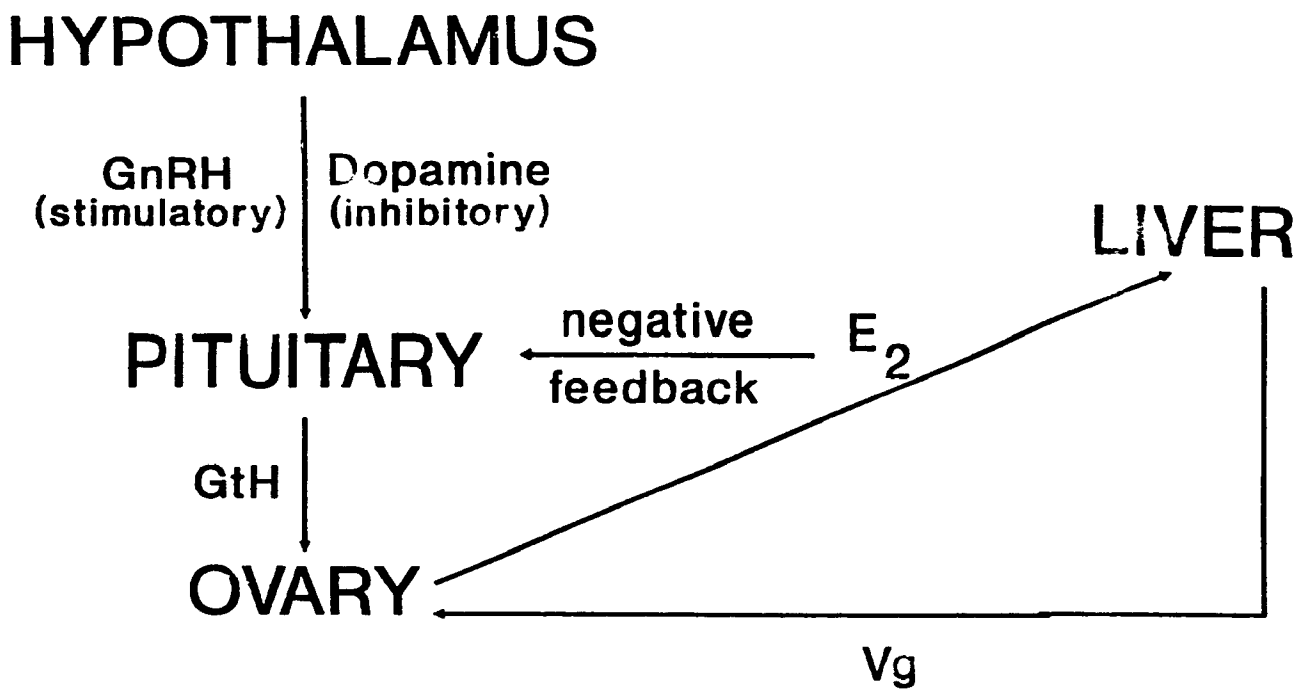


Figure 1: Possible locations along the hypothalamus-pituitary-gonad-liver axis where lead could exert a toxic effect (modified from Thomas, 1990). GnRH=gonadotropin releasing hormone, GtH=gonadotropin, E_2=estrogen and estradiol, Vg=vitellogenin.

MATERIALS AND METHODS

1) Materials

Test Organism

Two year-old sexually maturing female rainbow trout (Oncorhynchus mykiss) were purchased from La Pisciculture du Lac William, St. Ferdinand, Quebec. The fish used in the September 1990 experiment were purchased in August 1990 and placed directly into the exposure tanks and allowed to acclimate for four weeks. The fish used in the February/March and April 1991 experiments were purchased in November 1990, and kept in holding tanks (12 and 17 weeks, respectively) until two weeks prior to the experiment. Therefore, these fish were acclimated to the exposure tanks for two weeks. These fish were fall spawners, and hence were going through late vitellogenesis during September 1990 and early vitellogenesis during February/March and April 1991.

While in the holding tanks, the stock was fed Nutribec floating trout chow ad libitum three times per week. Water temperature in the holding tanks was approximately 13 °C, and the photoperiod regime was 12 hours light and 12 hours dark. Two weeks prior to the experiment, fish were transferred to the 250 L experimental tanks. During an experiment, the fish were fed daily at approximately 1 % of the body weight with the same Nutribec trout food. The mean body weight (g) of the Day 0 control fish for each experiment (± standard error) were as follows: in the February/March experiment, 570.1 ± 54.7; in the April experiment, 674.1 ± 71.4; and in the September experiment, 989.4 ± 41.1.

Apparatus

The laboratory is supplied with City of Montreal water which is dechlorinated by an activated charcoal filter. Water was supplied to the tanks in a continuous flow-through system. The inlet water was delivered to a headtank where it was aerated with compressed air through air stones. Water passed from the headtank to each of the experimental tanks through polyvinyl chloride (PVC) pipes to a flowmeter (Monostat Co.). The water flowrate was adjusted to 2 L/min., providing 99 % replacement in approximately 9 hours (Sprague, 1973). Water was drained from each tank through a PVC standpipe.

Illumination of the tanks was by fluorescent lights. An automatic time-switch controlled the 12 hour light and 12 hour dark photoperiod.

Lead nitrate (Aldrich 99+ % pure) solution was delivered to the tanks from Mariotte bottles (Leduc, 1966) shelved above the tanks. The lead solution was mixed with the inflowing water in a funnel attached to the tank lid. This delivery and mixing system allowed achievement of the desired toxicant concentrations of approximately 10 and 25 μg/L.

Physical and Chemical Characteristics of Water

Chemical characteristics of the water are as follows for September, 1990, February/March 1991, and April 1991, respectively: total hardness 125, 128, and 121 mg/L; alkalinity 87, 90, and 86 mg/L ($CaCO_3$); total organic carbon 173, 256, and 283 mg/L; pH 7.92, 7.88, and 7.83 (City of

Montreal Public Works Department).

Temperature and oxygen were measured twice each week. During the September, February/March and April experiments, temperature was maintained at 12.6 ± 0.3°C, 12.3 ± 0.3°C and 13.6 ± 0.6 °C, respectively. The oxygen was maintained above 84 % saturation.

2) Methods

Experimental Design

Three experiments were performed. The first experiment was conducted in September 1990, during late vitellogenesis. The experiment was repeated in February/March 1991 during early vitellogenesis. Due to a variation in the water flow of the 10 µg/L apparatus in March, this portion of the experiment was repeated in April 1991.

Two tanks containing ten fish each were used for each Pb concentration (0, 10 µg/L, 25 µg/L). Average concentration (µg/L) in the tanks (± standard error) were: in the February/March experiment, 25.2 ± 1.8; in the April experiment, 10.3 ± 0.9; and in September, 10.6 ± 1.1 and 25.8 ± 1.4. No mortality of fish was expected or observed at these concentrations, because these concentrations are representative of low level, chronic exposures. Fish were exposed for 12 and 20 days during the September experiment. In the February/March and April experiments, the exposure was only for 12 days because there were not enough female fish to continue the experiment to Day 20.

The lengths of exposure were chosen for several reasons. First, the author wanted to investigate effects at specific stages of the reproductive cycle, and hence the exposure duration could not be too long. Secondly, ALA-D depression has been observed in rainbow trout exposed to between 10 and 75 $\mu g/L$ Pb^{2+} for between 14 and 30 days (Eisler, 1988). It would be interesting to determine if other impacts occur at low Pb exposure with short durations. Lastly, future comparisons of impacts could be done with the fish in the present study and those of other studies conducted in this laboratory with cyanide. These studies are typically of 12 or 20 days duration.

Physiological Sampling and Histological Tissue Preparation

Sampling was done on Day 0, 12 and 20 during the September 1990 experiment, and on Day 0 and 12 during the February/March and April 1991 experiments. Trout were anesthetized in MS222 (tricaine methane sulfonate). Total body weight was recorded after removing excess water with paper towels. Blood was collected from the caudal blood vessels with a 5cc disposable syringe. Syringes were first rinsed with a 25 % sodium citrate solution, which acts as an anticoagulant. Collected blood was placed in a 1.5 ml Eppendorf tube and placed on ice until sampling was complete. Blood was refrigerated for later Pb analysis.

After the blood was collected, the trout were sacrificed and the pituitary gland was removed within 1 minute and placed

in Hollande fixative (Humason, 1967) for 48 hours. The gonads were removed, weighed, and an approximately 1 cm^2 mid-ovarian sample was placed in Bouin's fixative (Humason, 1967) for 24 hours. The liver was removed and weighed. The remaining gonad was placed in 20 ml polyethylene vials, frozen in liquid nitrogen, and stored at -20°C for later lead analysis. Following fixation of the pituitary and gonad, tissues were washed of excess fixative, dehydrated in alcohol, embedded in paraffin, and serially sectioned at 9 μm. The Cameron and Steele method (1959) was used to stain the pituitaries, and the ovaries were stained in hematoxylin and eosin (Humason, 1967).

Histology

The gonadosomatic index (GSI) of control and Pb-treated fish were compared to determine the status of gonadal development during the course of the experiments. The GSI was calculated as follows: $GSI = \frac{\text{wet gonad weight}}{\text{wet body weight}} \times 100$

The diameter of the five largest control and treated oocytes was measured with an eyepiece micrometer following a method adapted from Mathur and Ramsey (1974). Measurements for ovoid oocytes were standardized by taking the square root of the length multiplied by the width of the five largest oocytes present (Braekevelt and McMillan, 1967).

The effect of Pb on the frequency of individual stages of oocyte development was quantified. The number of oocytes in

each stage of development was recorded for both control and Pb-treated fish in the February/March and April experiments. The September data were evaluated qualitatively because most control oocytes were in one stage (Stage 6), whereas treated fish contained oocytes present in earlier stages and with atresia. This biologically significant observation was made without quantification or statistical analysis. Oocytes were classified according to cytoplasmic and nuclear features and development of egg membranes and their associated follicular cells (Lesniak and Ruby, 1982). Oocytes contained within the boundaries of an ocular grid or touching the upper and left-hand boundaries were included in the count. Four grid areas were counted per ovary section and three sections were counted, ten sections apart. Counting was performed at 250 X magnification. The percent frequency of each stage was calculated.

Counting of basophils in the pituitary was performed following a method modified from Nozaki et al. (1990b). The pituitary stalk was located in a section. Extreme lateral regions of the pituitary were not used for counting purposes due to the absence on basophils in these areas. All granular basophils were counted in this section and in the tenth and twentieth section preceding and following this section, to ensure cells were counted only once. In total, five sections were counted for each fish.

Statistics

Statistically significant results were difficult to obtain because of small sample sizes. The experiments were designed with the assumption that most of the fish would be females. For the September experiment, this assumption was valid. For the February/March and April experiments, females accounted for only 57% and 60% of the population, respectively. Sample sizes were further reduced because fish which were obviously in a physiologically different stage (e.g., the GSI was different from the remainder of the sample by an order of magnitude) were omitted from further study. An error in the staining procedure after the spring experiments caused several pituitaries to be lost. That problem was corrected subsequently in the laboratory.

All data were subject to a two-way analysis of variance (ANOVA). Significance was based on $p<0.05$. When differences were found, two tests were performed, a Tukey test and a Dunnett's test. Multiple comparison tests for means have the same underlying assumptions as does the analysis of variance (ANOVA): population normality and homogeneity of variance. Although the Tukey test appears to be robust with respect to departures from these assumptions (Keselman, 1976 cited in Zar, 1984), the robustness is not well-known. The Tukey test considers differences between all group means. A Dunnett's test was done also to compare the treatments (all groups other than the Day 0 control) with a single control group (the Day 0 control).

Fortunately, the analysis of variance (ANOVA) is robust,

operating well even with considerable heterogeneity of variances as long as all sample sizes (n_i) are equal or nearly equal (Glass et al., 1972 cited in Zar, 1984). If the n_i are quite different, then the probability of a Type I error will differ markedly from α, to a degree dependent on the magnitude of the heterogeneity (Box, 1954 cited in Zar, 1984); if larger variances are associated with the larger sample sizes, the probability of a Type I error will be less than α, and if they are associated with the smaller samples, this probability will be greater than α (Kohr and Games, 1974 cited in Zar, 1984). Many tests of variance homogeneity have been proposed but they are all adversely affected by non-normality, or are very low in power, or have other serious drawbacks (Zar, 1984). Because of the poor performance of tests for variance homogeneity, and the robustness of ANOVA for multisample testing among means, it is not recommended that the former be performed as a test of the underlying assumptions of the latter (Zar, 1984).

It is difficult to test goodness of fit of a normal distribution when sample sizes are very small. It is an advantage that the ANOVA also is robust with respect to the assumption of the population's normality. The validity of the analysis is affected only slightly by even considerable deviations from normality, especially as n increases (Zar, 1984).

Because of small sample sizes in this study, several statistical tests were used. The minimum detectable

difference between population means can be estimated from the formula (Zar, 1984):

$$\delta = \sqrt{\frac{2ks^2\phi^2}{n}}$$

where: δ is the minimum detectable difference

k is the number of populations

s^2 is an estimate of the variance σ^2=MSE

ϕ^2 is obtained from Zar (1984) Figure B1, requiring v_1=groups degrees of freedom (df) and v_2=error df=k(n-1)

n is the sample size from the ANOVA

Power is specified at 0.90

Significance level α is specified at 0.05

Also, the power of an ANOVA after it has been performed can be estimated by the formula (Zar, 1984):

$$\phi = \sqrt{\frac{(k-1)\ (\text{groups MS} - s^2)}{ks^2}}$$

The power will tell us the probability of rejecting the null hypothesis (which is that the population means are equal) when it is in fact false and should be rejected. For example, with a power of 0.30, there is a 30% chance that we have correctly rejected the null hypothesis (and therefore there is a difference). Determining the power of a performed test is useful also if the null hypothesis is not rejected (Zar, 1984). A power of 0.30 then would indicate that there was a 70% chance of having committed a Type II error, which is to accept the null hypothesis when it is false.

Thus, Zar (1984) concludes that ANOVA may be depended upon unless the data deviate severely from the underlying assumptions. It is possible to test nonparametrically for intergroup differences where k>2 (the number of populations is greater than 2). This is done by the Kruskal-Wallis test, often called an analysis of variance by ranks. This analysis may be applied when the k samples do not come from normal populations and/or when the k population variances are heterogeneous (Zar, 1984). This test was done on all data with the exception of the individual oocyte stage data.

Linear regression was performed on the fish body weight, GSI and basophil data. The regressions were done to determine if one could predict the number of pituitary basophils based on the body weight, or the number of basophils based on the GSI data.

Analyses of Lead in Water and Tissues

The concentration of lead in the exposure tanks was measured twice each week using a Perkin-Elmer 503 graphite furnace atomic absorption spectrophotometer (AA). The wavelength measured was 283 nm, and the charring and atomizing temperatures were 700 °C and 2300 °C, respectively. Tank water concentrations were compared to standards of 10, 25, and 50 µg/L.

Lead levels in blood were measured using the method of Hodson et al. (1977). A 50 µL aliquot of blood was placed in a 1.5 ml Eppindorf tube and frozen in liquid nitrogen. 100 µl

of 10 % Triton X-100 and 400 μL of formic acid were added and mixed to digest the blood. A 20 μL aliquot was taken for graphite furnace AA analysis. Results were compared with Pb standards in Triton X-100 and formic acid.

Lead levels in the ovary and liver were measured using the method of Aysola et al. (1988). Ten mL of each of HNO_3 and H_2SO_4 were added to 0.25 g of tissue in a 250 mL Erlynmeyer flask. The flask was placed in a sealed microwave-safe container. A beaker containing 50 mL water was placed in the microwave along with the container to protect the microwave from damage. The microwave was set at a power setting of 70 watts. Microwaves were applied in pulses of 10 seconds, with 180 seconds between pulses. This cycle was continued for a total of 19 minutes, providing 6 cycles of heating. Samples were diluted to 100 mL with distilled water. 20 μL aliquots were measured using graphite furnace AA, and compared with standards of 25, 50 and 100 μg/L. Standards were made with the same concentrations of HNO_3 and H_2SO_4 as were the samples.

Pb in each standard and sample was measured twice, and the average taken. Linear regression was used to plot the standard curve. The mean $\pm$ standard error values were determined for each control and Pb-treated group.

RESULTS

Physiological Stages of Ovarian Development

For the purpose of this study, the annual reproductive cycle of the female rainbow trout has been divided into three main physiological stages (Bohemen et al., 1981): previtellogenesis, endogenous vitellogenesis, and exogenous vitellogenesis. Vitellogenesis in fish has been reviewed extensively (Guraya, 1986; Ng and Idler, 1983; Scott and Sumpter, 1983b).

During previtellogenesis, the reproductive system appears to be quiescent. During endogenous vitellogenesis, the oocyte cytoplasm appears basophilic when stained with hematoxylin and eosin, and hence is in the RNA and protein synthesizing phase.

Exogenous vitellogenesis is characterized physiologically by high blood levels of estrogen, estradiol and vitellogenin (Vg). Vg is a phospholipoglycoprotein produced and secreted by the liver, transported in the blood, and sequestered by the developing oocytes where it is stored as yolk (de Vlaming, 1983). During exogenous vitellogenesis, oocytes undergo secondary yolk deposition, increase rapidly in size (from 1% or less of body weight up to 20% or more according to Tyler et al., 1991), and the cytoplasm stains acidophilic with hematoxylin and eosin. After the oocytes are fully mature, they are released and stored in the body cavity in preparation for spawning.

Gonadosomatic Index

The GSI data are summarized for the February/March,

April, and September experiments in Table 2, and in Appendices 1, 2 and 3, respectively. Summary statistics are given in Table 3 for these three experiments.

The February/March (25 μg/L) and April (10 μg/L) experiments were performed during the period of endogenous vitellogenesis. In the February /March experiment, there was no statistically significant change ($p > 0.05$) in gonadosomatic indices (GSI) over the 12 days in control or Pb-treated fish (Figure 2). The mean GSI ± standard error for Day 0 control, Day 12 control and Day 12 25 μg/L Pb groups were 0.56 ± 0.22, 0.40 ± 0.05 and 0.39 ± 0.05, respectively. The cytoplasm of all oocytes was basophilic when stained with hematoxylin and eosin which indicates that these oocytes were in the RNA synthesizing phase. The April experiment was performed at a transition period in the reproductive cycle. As a result, some of the fish had already entered the exogenous vitellogenesis phase, while others were still in the endogenous vitellogenesis phase. Therefore, in the April experiment, there was no statistically significant difference ($p > 0.05$) between the GSIs over the course of the experiment in control or Pb-treated fish, or between control and Pb-treated females at Day 12 (Figure 3). The mean GSI ± standard error for the Day 0 control, Day 12 control, and Day 12 10 μg/L Pb groups were 0.47 ± 0.10, 0.69 ± 0.07, and 0.59 ± 0.05, respectively. There was a trend indicating growth in the control group, although this trend was not statistically significant ($p > 0.05$) (Table 3). Ovaries of both control and

Table 2: Summary of results: GSI, oocyte diameter, and mean number of pituitary basophils (± SE) for rainbow trout exposed to lead in the early (February/March, April) and late (September) vitellogenesis experiments (Sample size is in parentheses)

Experiment	Day	Lead Conc. (ug/L)	GSI	SE	Oocyte Diameter (um)	SE	Number of Pituitary Basophils	SE
February/ March	0	0	0.56 (4)	0.22	306.8 (4)	40.6	445 (4)	269
	12	0	0.40 (9)	0.05	307.2 (9)	14.1	461 (9)	266
	12	25	0.39 (9)	0.05	330.4 (9)	17.5	279 (7)	153
April	0	0	0.47 (6)	0.10	348.5 (6)	24.7	1093 (5)	325
	12	0	0.69 (7)	0.07	445.7 (7)	26.5	498 (3)	17.3
	12	10	0.59 (9)	0.05	404.6 (9)	34.4	532 (4)	134
September	0	0	1.12[13] (4)	0.08	582.3[abd] (4)	16.8	97 (4)	50
	12	0	2.52[12] (5)	0.31	773.9[bc] (5)	45.5	1911 (4)	830
	12	10	0.92[2] (5)	0.17	546.2[ce] (5)	27.5	1114 (5)	503
	12	25	1.58 (6)	0.30	622.9 (6)	48.4	1828 (7)	376
	20	0	3.20[3] (3)	0.71	768.7[a] (3)	37.5	1622 (3)	527
	20	10	1.69 (4)	0.6?	685.2[e] (4)	49.7	2384 (6)	1052
	20	25	1.50 (4)	0.11	748.8[d] (4)	27.8	2805 (2)	1089

1, 2, 3, a, b, c, d, e denote a significant difference at $p < 0.05$

Table 3: Summary statistics for the GSI data for the February/March, April and September experiments exposing 2-year old rainbow trout to Pb nitrate. For all experiments, k=3 and v_1=2. An ANOVA or Kruskal-Wallis statistic $\leq$ 0.05 is significant.

Experiment	ANOVA (Prob.>F)	Dunnett's / Tukey	Minimum Detectable Difference	Power	Statistical Parameters	Kruskal-Wallis
Feb./March	0.412		0.23	<0.20	n=22, v_2=63	0.972
April	0.155		0.21	0.30	n=22, v_2=63	0.204
Sept: All controls	0.0125	Sign. Diff. betwen Day 0 and Day 12 controls and Day 0 and Day 20 controls	3.08	0.88	n=12, v_2=33	0.019
Sept: Day 0, Day 12 and 20 10 µg/L Pb	0.354		1.13	0.20	n=13, v_2=36	0.403
Sept: Day 0, Day 12 and 20 25 µg/L Pb	0.396		0.73	0.20	n=14, v_2=39	0.314
Sept: All Day 12 treatments	0.005	Sign. Diff. between Day 12 control, 10 µg/L Pb	0.82	0.92	n=16, v_2=45	0.013
Sept: All Day 20 treatments	0.119		1.62	0.35	n=11, v_2=30	0.08

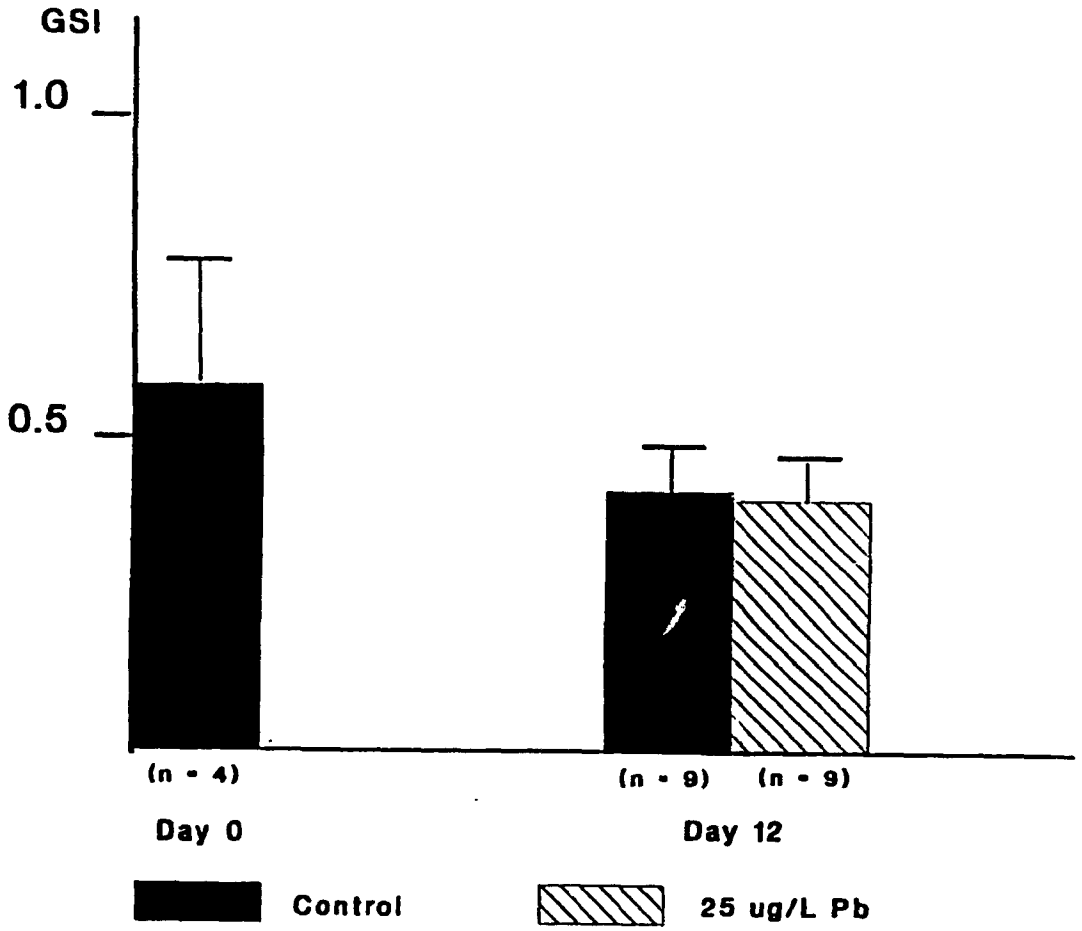


Figure 2: Average GSI (± standard error) for the February/March experiment exposing 2-year old rainbow trout to 25 μg/L lead nitrate over 12 days during early vitellogenesis.

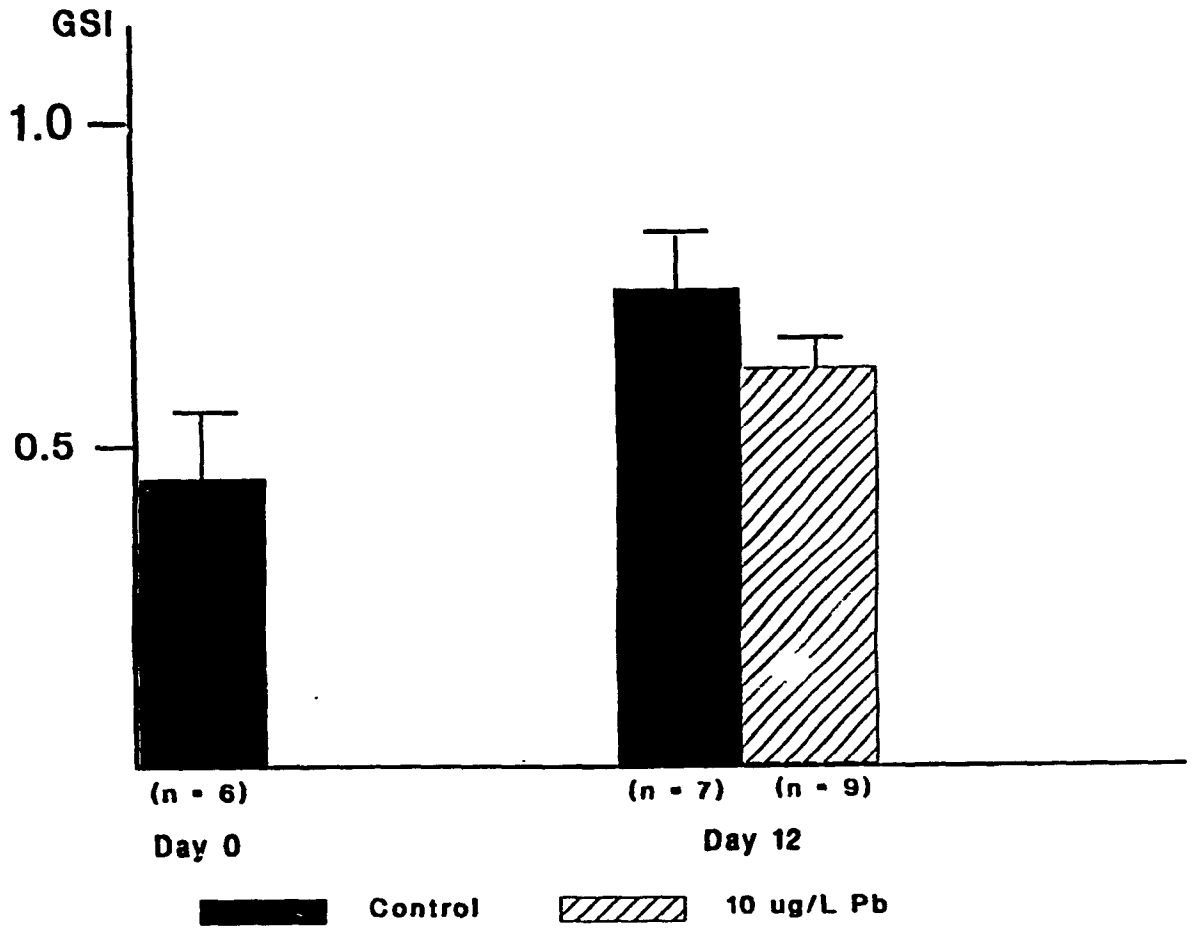
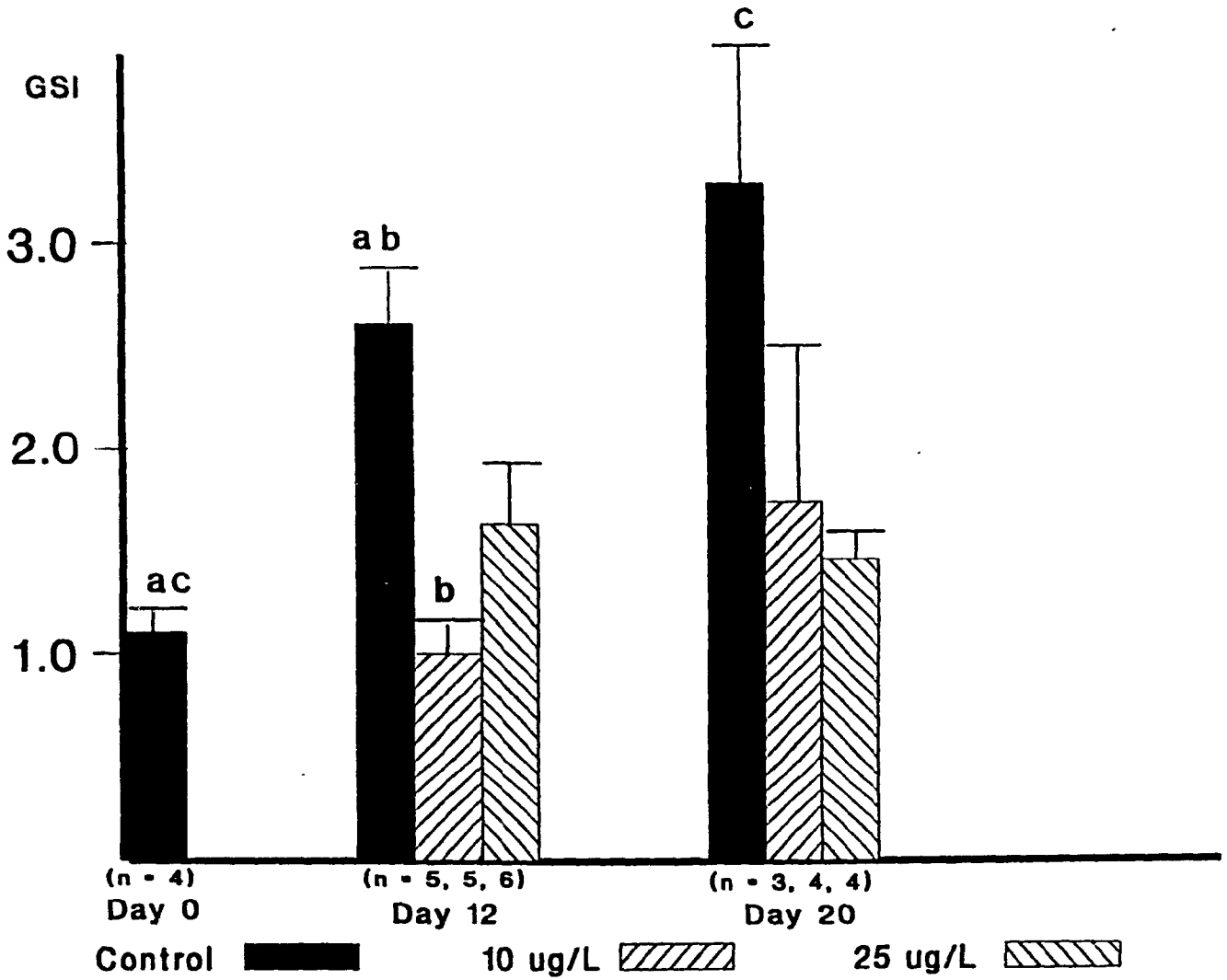


Figure 3: Average GSI (± standard error) for the April experiment exposing 2-year old rainbow trout to 10 μg/L lead nitrate over 12 days during early vitellogenesis.

Pb-treated fish contained oocytes with both acidophilic- and basophilic-staining cytoplasm. This indicates that the oocytes were entering exogenous vitellogenesis and were beginning to sequester vitellogenin (Vg). Vg is synthesized in the liver and transported in the blood to the ovary. Fish with larger GSIs contained oocytes with acidophilic-staining cytoplasm; indicating that oocytes were commencing exogenous vitellogenesis and secondary yolk deposition. Fish with smaller GSIs contained oocytes with basophilic-staining cytoplasm; oocytes were still in a period of RNA synthesis. However, the transition between endogenous and exogenous yolk production is not distinct based soley on staining properties of the oocyte. Secondary yolk deposition (exogenous vitellogenesis) normally occurs in stage 4 oocytes, but may develop at stage 3 (Lesniak and Ruby, 1982).

The September experiment was performed during late vitellogenesis. All control and Pb-treated fish ovaries contained late stage oocytes with acidophilic-staining cytoplasm; all fish were in the exogenous phase of vitellogenesis. The average GSI of control fish increased over the 12 and 20 days of the experiment from 1.12 to 2.42 to 3.27, respectively (Figure 4). The increases from Day 0 to Day 12 and Day 0 to Day 20 are both statistically significant. However, the change in controls from Day 12 to 20 was not statistically significant. The GSI did not change significantly in lead-exposed fish over the 20 days. At Day 12, the GSI of control fish was significantly greater than



a, b, c denote a significant difference at $p < 0.05$

Figure 4: Change in GSI (mean $\pm$ standard error) over 12 and 20 days of 2-year old rainbow trout exposed to 10 and 25 μg/L lead nitrate during late vitellogenesis.

that of the 10 μg/L Pb-treated fish. There was no significant difference in GSI between the two Pb treatments at Day 12. At Day 20, there was no significant difference between the control and treated fish.

Oocyte Diameters

A summary of the oocyte diameters for the February/March, April and September experiments is given in Table 2 and in Appendices 1, 2 and 3, respectively. Summary statistics are provided in Table 4.

In the February/March experiment, there was no significant difference ($p > 0.05$) in oocyte diameter between Day 0 and Day 12 in either control or Pb-treated fish (Figure 5). All of the ovaries contained oocytes in the RNA synthesizing phase. In April, the diameter of the control oocytes did increase over the 12 days (Figure 6), but this difference was not statistically significant (Table 4).

In September, during late vitellogenesis, the control fish oocyte diameter increased from 582.3 ± 16.8 μm at Day 0 to 773.9 ± 45.5 μm at Day 12 (Figure 7), and did not increase in diameter from Day 12 to Day 20. At Day 12, the control fish oocytes were significantly larger than the 10 μg/L Pb-treated fish oocytes but not the 25 μg/L Pb-treated oocytes. There was a significant increase in diameter in the 10 μg/L Pb-treated oocytes between Day 12 and 20. By Day 20, there was no significant difference in oocyte diameter between the control fish (768 ± 37.5 μm) and 10 and 25 μg/L Pb-exposed

Table 4: Summary statistics for the oocyte diameter data for the February/March, April and September experiments exposing 2-year old rainbow trout to Pb nitrate. For all experiments, k=3 and v_1=2. An ANOVA or Kruskal-Wallis statistic $\leq$0.05 is significant.

Experiment	ANOVA (Prob.>F)	Dunnett's / Tukey	Minimum Detectable Difference (μm)	Power	Statistical Parameters	Kruskal-Wallis
Feb./March	0.620		41.7	<0.20	n=22, v_2=63	0.737
April	0.140		91.9	0.30	n=22, v_2=63	0.104
Sept: All controls	0.01	Sign. diff. 1) Day 0 and Day 12 controls, 2) Day 0 and Day 20 controls	116.9	0.91	n=12, v_2=33	0.025
Sept: Day 0, Day 12 and 20 10 μg/L Pb	0.037	Sign. diff. 1) Day 12 and 20 10 μg/L Pb	99.0	0.65	n=13, v_2=36	0.068
Sept: Day 0, Day 12 and 20 25 μg/L Pb	0.047	Sign. diff. between Day 0 and Day 20 25 μg/L Pb	119.4	0.55	n=14, v_2=39	0.112
Sept: All Day 12 treatments	0.009	Sign. diff. between Day 12 control, 10 μg/L Pb	127.0	0.86	n=16, v_2=45	0.015
Sept: All Day 20 treatments	0.356		119.3	0.20	n=11, v_2=30	0.608

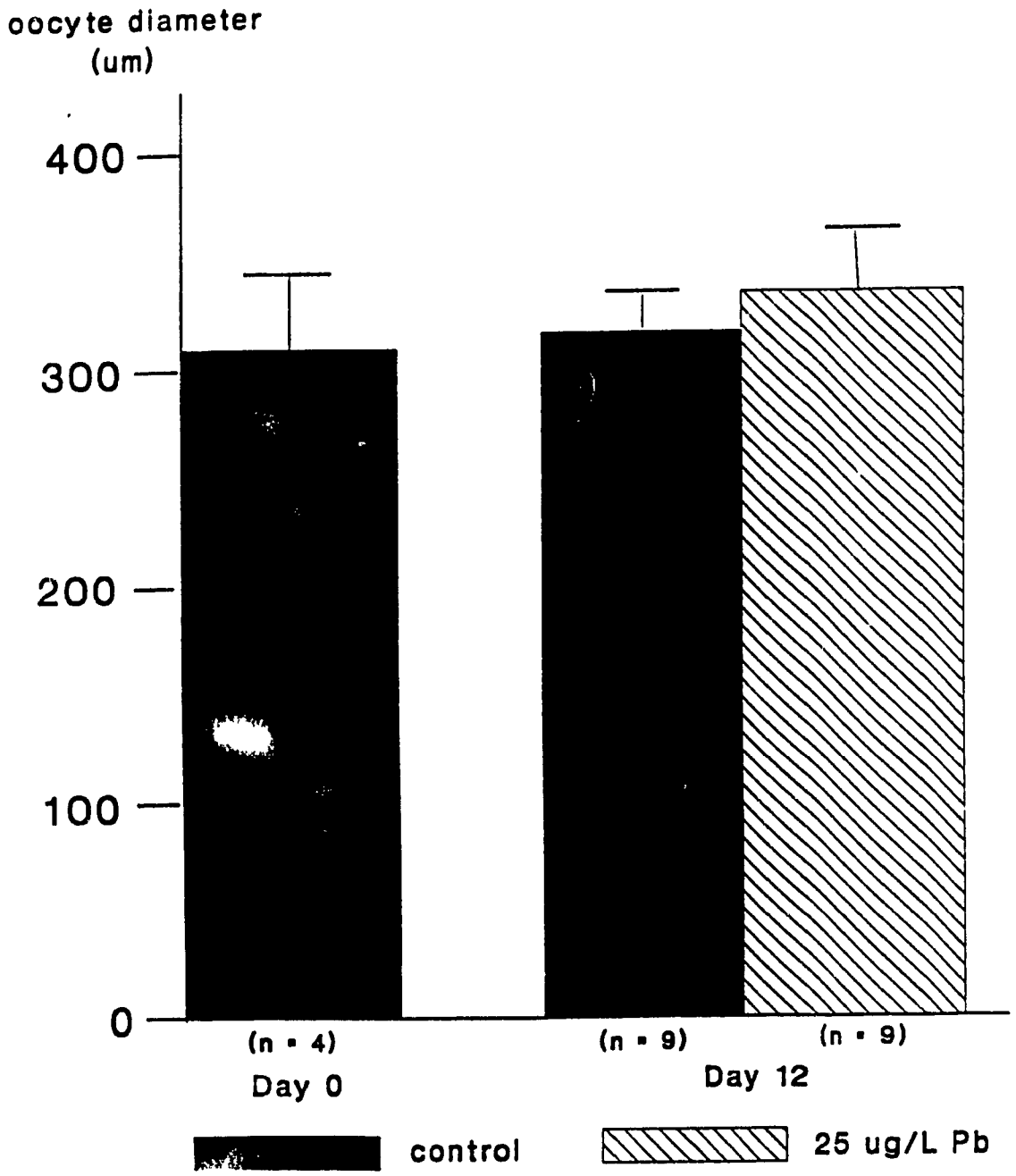


Figure 5: Mean oocyte diameter (± standard error) in February/March experiment exposing 2-year old rainbow trout to 25 μg/L lead nitrate over 12 days during early vitellogenesis.

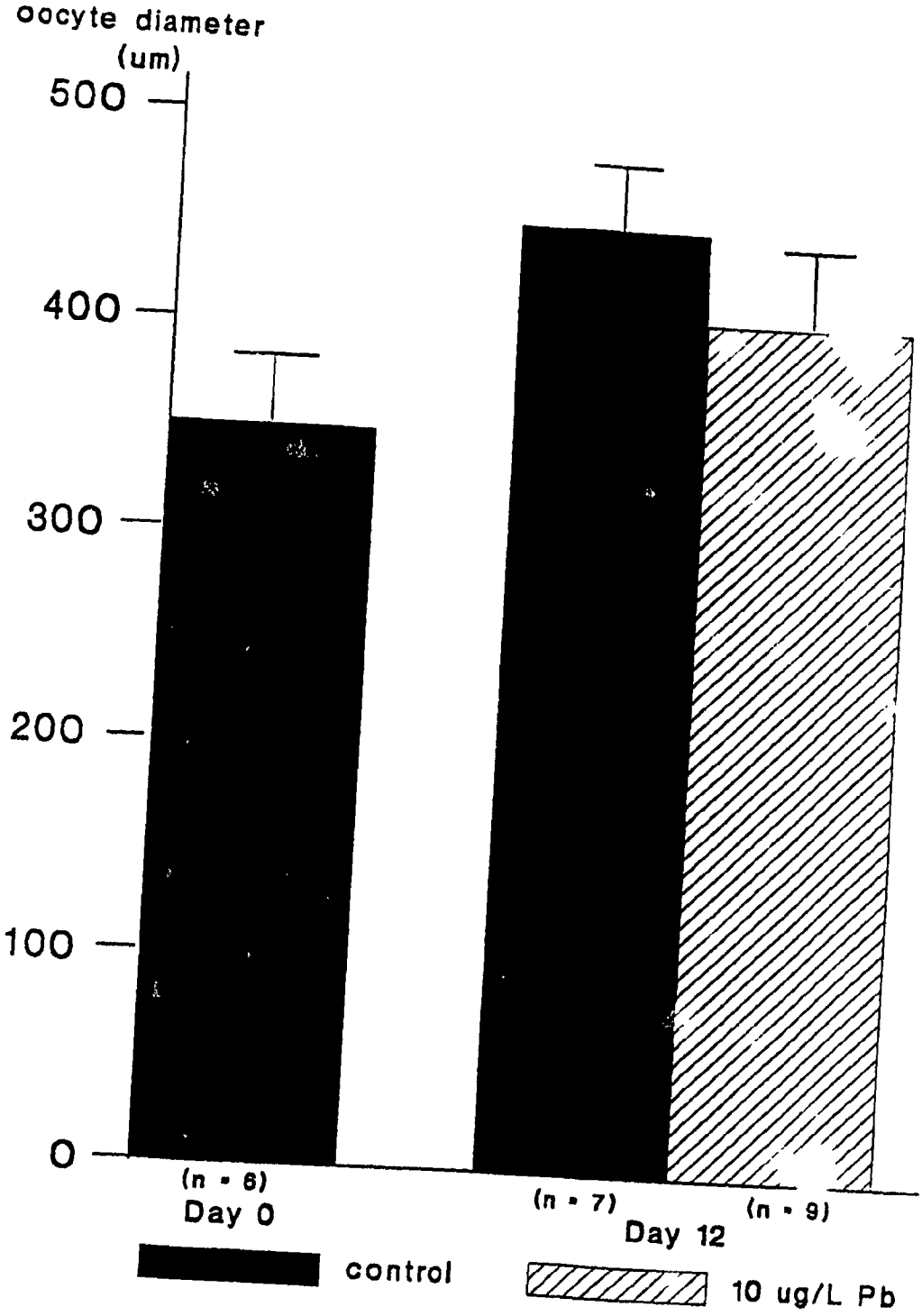
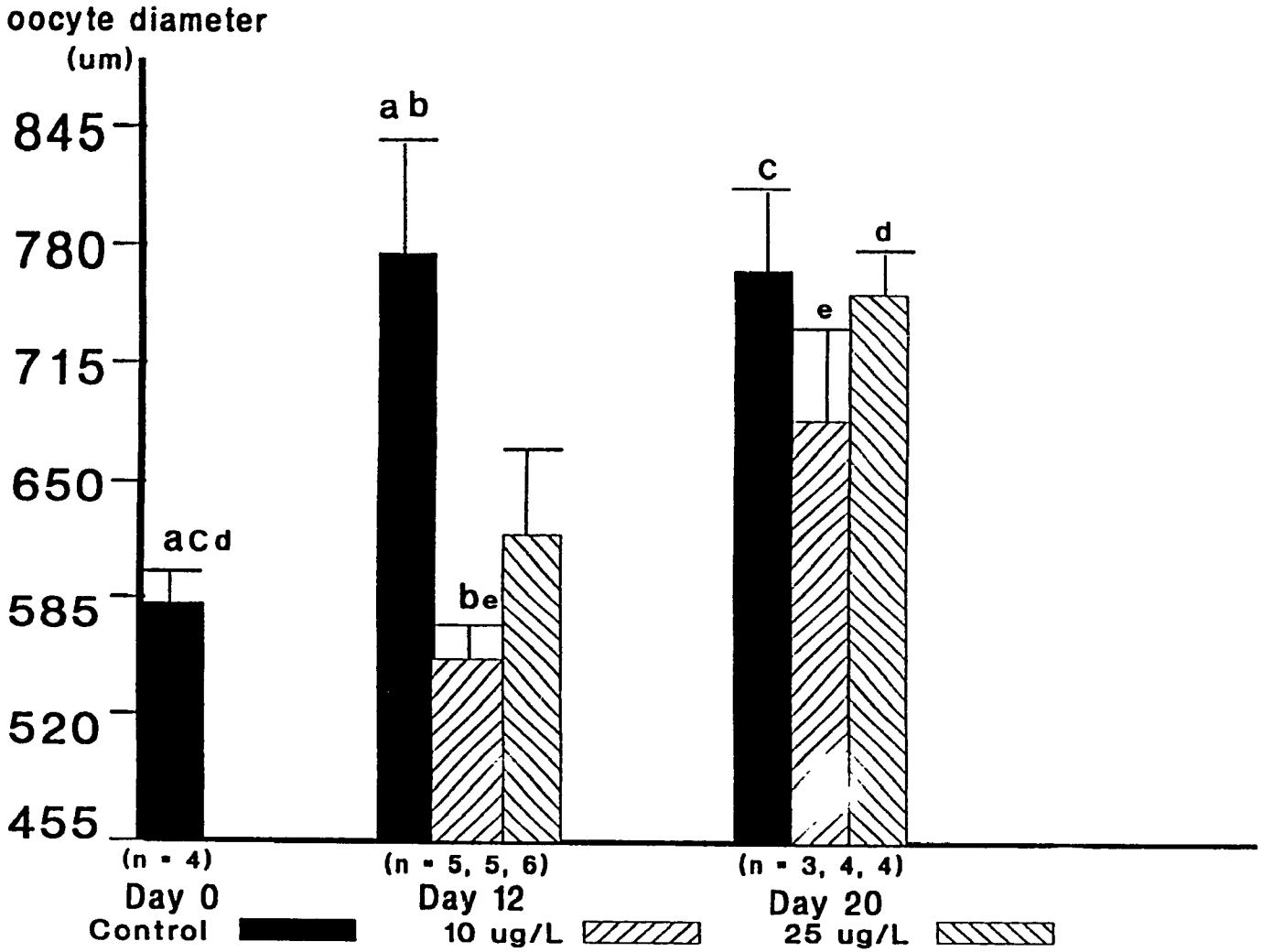


Figure 6: Change in oocyte diameter (mean ± standard error) for the April experiment exposing 2-year old rainbow trout to 10 µg/L lead nitrate over 12 days during early vitellogenesis.



a, b, c, d, e denote a significant difference at $p < 0.05$

Figure 7: Change in oocyte diameter (mean $\pm$ standard error) for the September experiment exposing 2-year old rainbow trout to 10 and 25 μg/L lead nitrate over 12 and 20 days during late vitellogenesis.

fish (685.2 ± 49.7 μm and 748.8 ± 27.8 μm, respectively).

Frequency of Oocyte Stages

Oocytes were classified into six arbitrary stages of development according to a method modified from Lesniak and Ruby (1982). These stages were based upon nuclear and cytoplasmic features, and development of egg membranes and their associated follicular cells (Table 5). Figures 8-13 illustrate each state of oocyte development. A seventh class included any degenerating (atretic) oocytes (Figure 14).

In the February/March experiment, there were no significant differences ($p > 0.05$) in the frequency of any of the seven oocyte stages, between controls at Days 0 and 12, or between controls and Pb-treated fish (Table 6). Table 7 shows the minimum detectable difference in the percentage of oocytes at each stage necessary to detect a significant difference, and the power of the ANOVA.

In April, no stage frequencies were significantly different at $p > 0.05$. However, some trends were observed in the data (Tables 8 and 9). The number of stage 1 and 3 oocytes decreased from Day 0 to Day 12 in the control group. There was a corresponding increase in stage 5 and 6 oocytes in control fish from Day 0 to Day 12. This trend was not observed in the Pb-treated fish. Table 9 shows the minimum differences for each stage needed to detect a significant difference, and the power of the ANOVA.

Table 5: Summary of features defining arbitrary stages of oocyte development (Lesniak and Ruby, 1982)

Stage	Diameter (um)	Nucleus	Nucleoli (number)	Cytoplasm	Membranes	Follicular layer
1	< 20	Round, large pale	none	opaque, lightly basophilic	distinct nuclear indistinct cytoplasmic	incomplete cells
2	20-80	Ellipsoidal eccentric	9 large, few scattered	strongly basophilic	distinct nuclear and cytoplasmic	incomplete squamous layer
3	80-150	Ellipsoidal	21 mixed sizes migrate to periphery	dispersing Balbiani, primary yolk vesicles	completed zona pellucida	thin, complete layer
4	150-260	Ellipsoidal central indented	35 small peripheral	secondary yolk vesicles	thickened zona pellucida	inner layer epithelial, outer thecal cells, both squamous
5	> 260	Irregular, moves to one pole	up to 53, small, follow nuclear outline	coalesced yolk globules, acidophilic	wavy, thick zona pellucida	follicular layer of cuboidal cells
6	> 260	cannot be observed		yolk totally shattered, when sectioned	like stage 5	like stage 5

Figure 8: Stage 1 of oocyte development in the rainbow trout ovary. Note the round, large, pale nucleus (n) with no nucleoli present. (magnification 250 x).

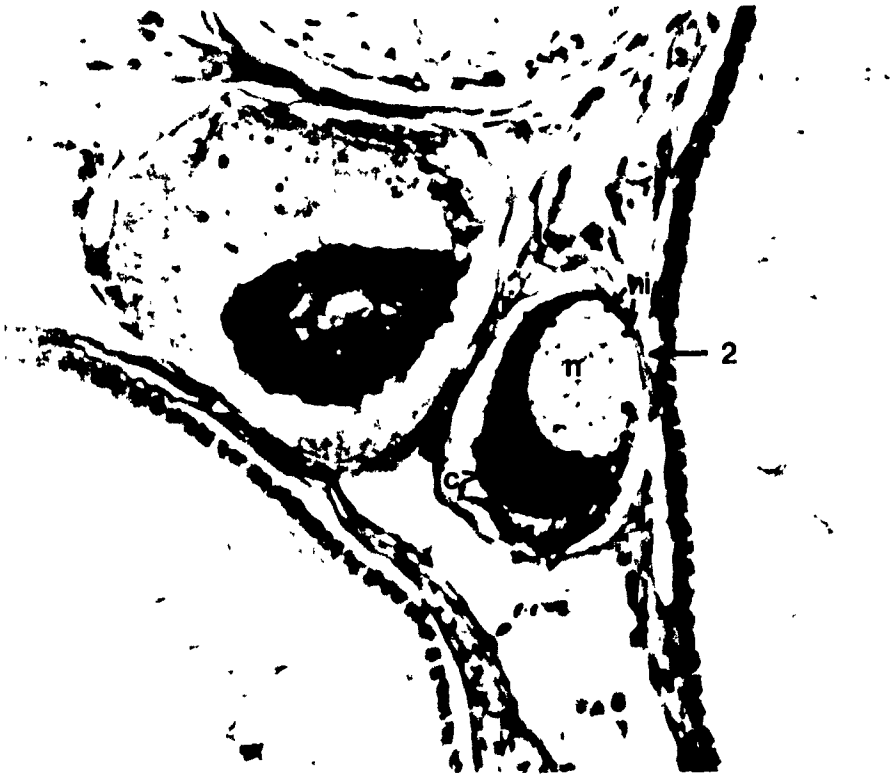


Figure 9: Stage 2 of oocyte development in the rainbow trout ovary. Note the ellipsoidal nucleus (n), the few nucleoli (ni), and the strongly basophilic cytoplasm (c). (magnification 250 x).

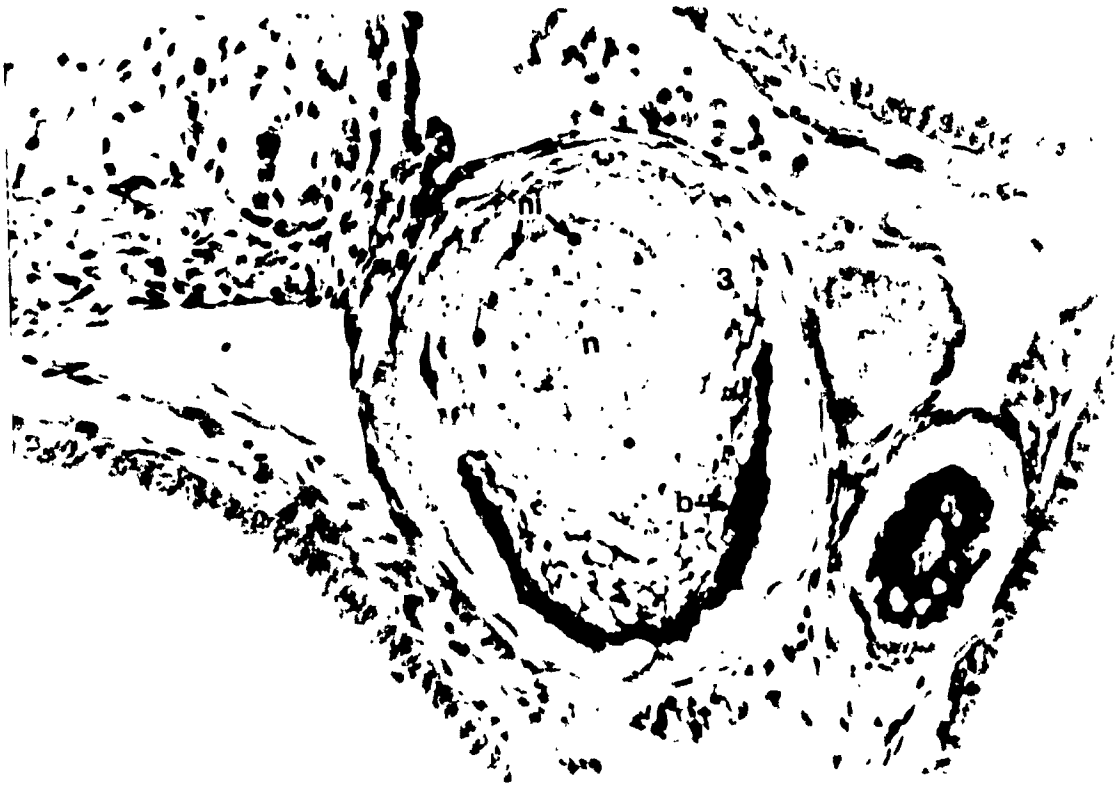


Figure 10: Stage 3 of oocyte development in the rainbow trout ovary. Note the numerous nucleoli (ni) at the periphery of the nucleus (n), and the dispersing Balbiani (b)(magnification 250 x).

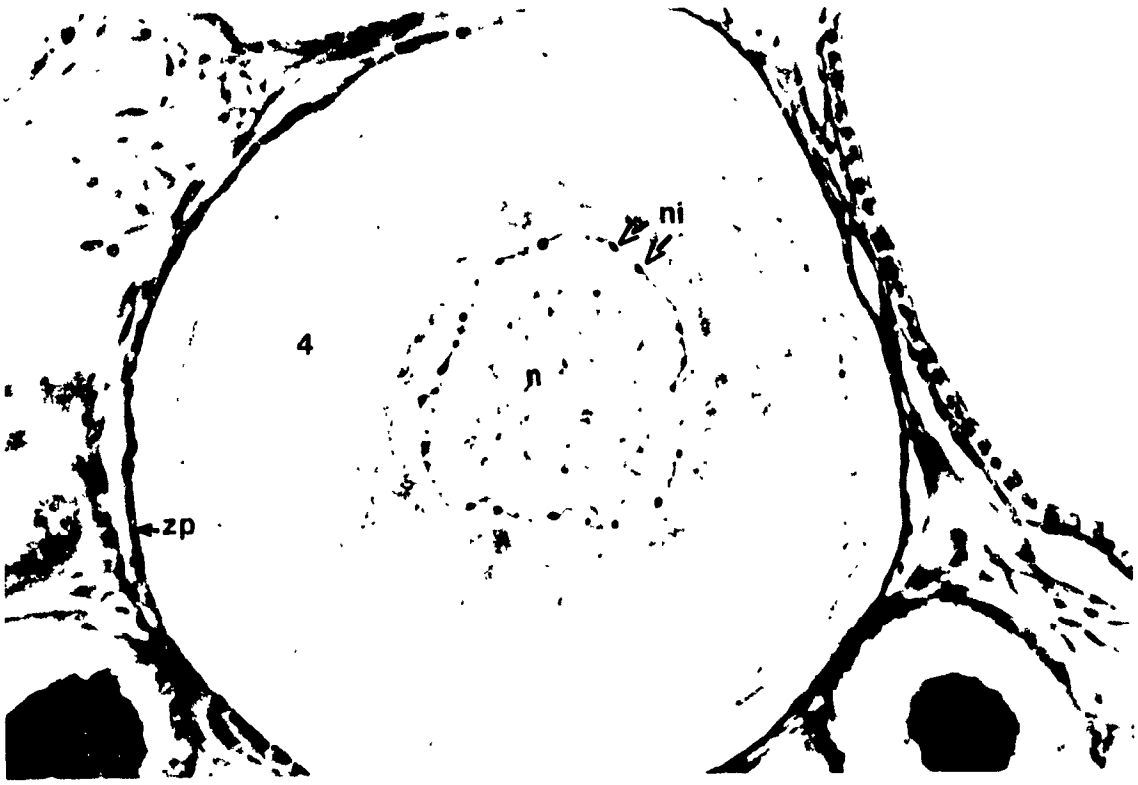


Figure 11: Stage 4 of oocyte development in the rainbow trout ovary. Note the numerous nucleoli (ni) at the periphery of the central, indented nucleus (n), and the thickened zona pellucida (zp). (magnification 250 x).

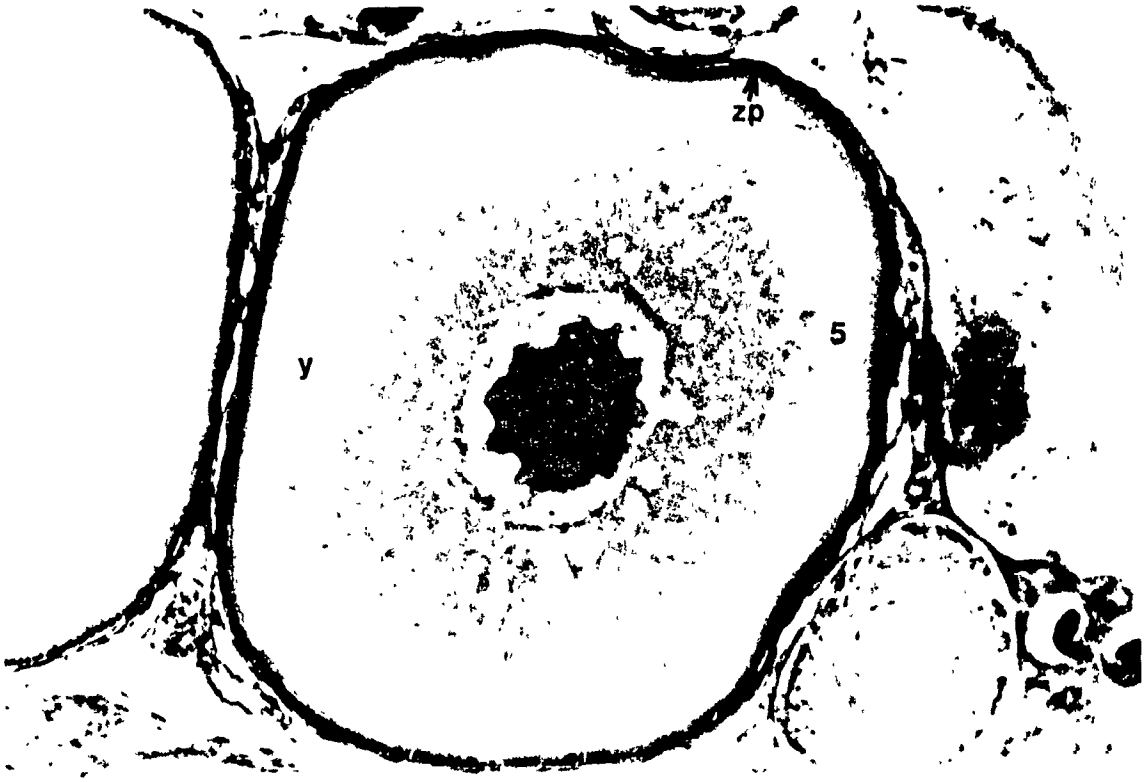


Figure 12: Stage 5 of oocyte development in the rainbow trout ovary. Note the irregular shape of the nucleus (n), the coalesced yolk globules (y) and the thick zona pellucida (zp). (magnification 100 x).

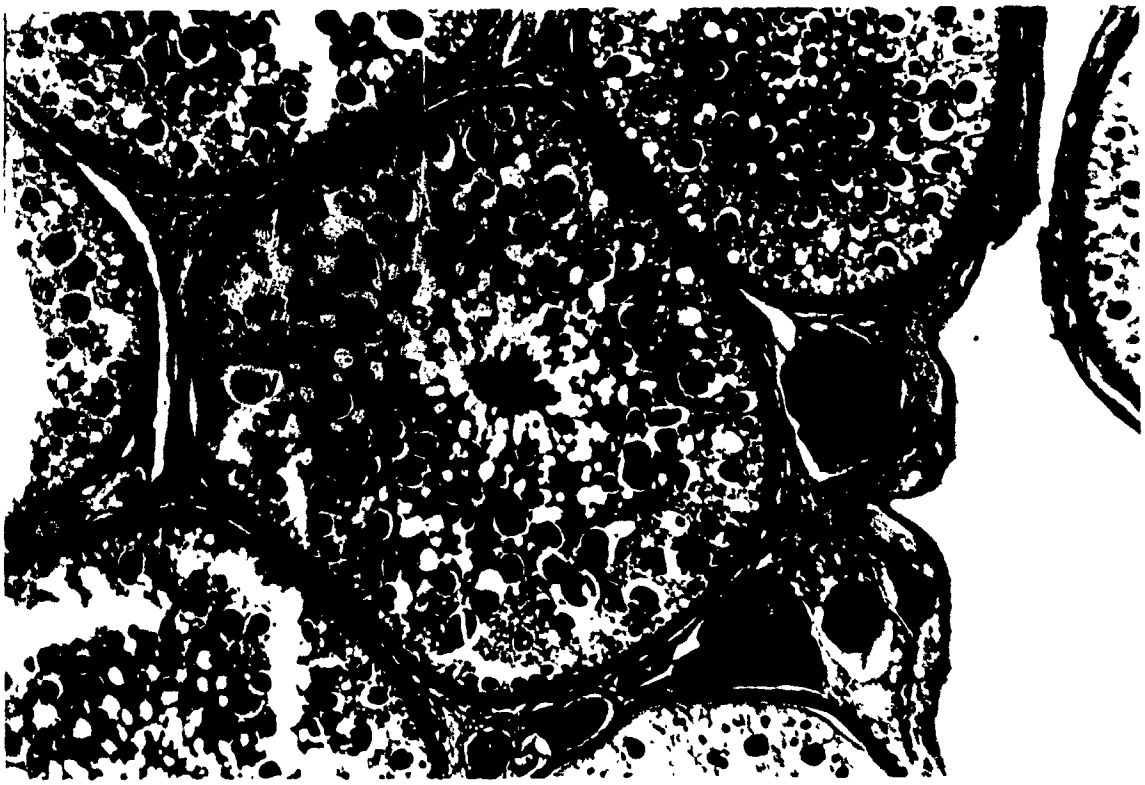


Figure 13: Stage 6 of oocyte development in the rainbow trout ovary. Note the yolk (y) which has shattered from sectioning. A Stage 1 oocyte is identified also to illustrate the size difference of these oocytes. (magnification 40 x).

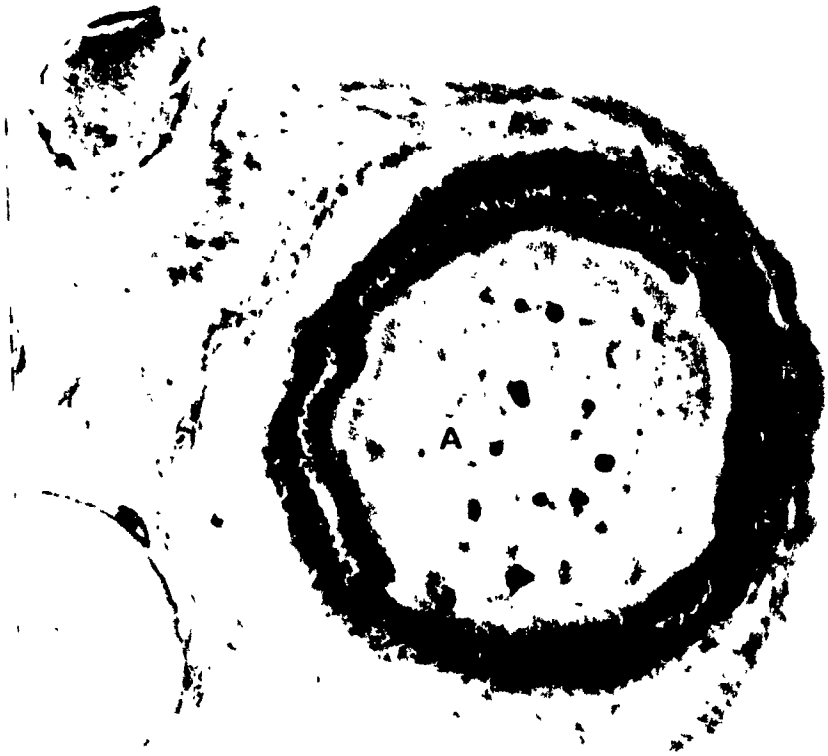


Figure 14: Atretic (A) oocyte in the rainbow trout ovary. (magnification 100 x).

Table 6: Summary of oocyte stage frequency data for the February/March early vitellogenesis experiment (mean percentage of total oocytes counted ± standard error) exposing 2-year old rainbow trout to 25 μg/L lead nitrate for 12 days.

Day	Lead Conc.	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Atretic
0	0	3.5 ± 1.1	25.7 ± 2.7	29.2 ± 2.1	15.5 ± 0.9	7.8 ± 0.9	0.0 ± 0.0	20.0 ± 3.3
12	0	2.8 ± 0.8	23.2 ± 2.0	26.8 ± 2.0	15.5 ± 2.8	11.5 ± 2.7	0.8 ± 0.8	19.3 ± 1.2
12	25	3.3 ± 0.6	31.1 ± 3.6	22.9 ± 1.9	10.2 ± 2.1	11.4 ± 1.9	0.0 ± 0.0	21.1 ± 3.6

Table 7: Summary statistics for individual oocyte stage data for the February/March experiment exposing 2-year old rainbow trout to 25 μg/L Pb nitrate for 12 days. Statistical parameters for the minimum detectable difference and power are: k=3, v_1=2, v_2=57, n=20. An ANOVA Prob>F must be less than or equal to 0.05 for significance.

Experiment	ANOVA (Prob.>F)	Minimum Detectable Difference (%)	Power
Stage 1	0.801	2.5	<0.2
Stage 2	0.130	8.5	0.3
Stage 3	0.160	6.1	0.25
Stage 4	0.257	7.6	0.2
Stage 5	0.608	7.3	<0.2
Stage 6	0.568	1.8	<0.2
Atretic	0.864	7.8	<0.2

Table 8: Summary of oocyte stage frequency data for the April early vitellogenesis experiment (mean percentage of total oocytes counted ± standard error) exposing 2-year old rainbow trout to 10 μg/L lead nitrate for 12 days.

Day	Lead Conc.	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Atretic
0	0	3.9 ± 1.0	22.2 ± 2.1	19.9 ± 3.2	19.5 ± 0.4	19.6 ± 2.8	0.0 ± 0.0	14.8 ± 1.5
12	0	1.2 ± 0.4	16.8 ± 3.4	11.4 ± 1.6	13.7 ± 2.1	26.1 ± 4.5	13.9 ± 8.7	16.9 ± 3.1
12	10	2.4 ± 0.7	20.0 ± 2.8	17.1 ± 2.2	12.0 ± 2.0	19.1 ± 4.9	6.9 ± 3.1	22.4 ± 3.1

Table 9: Summary statistics for individual oocyte stage data for the April experiment exposing 2-year old rainbow trout to 10 µg/L Pb nitrate for 12 days. Statistical parameters for minimum detectable difference and power tests are: k=3, v_1=2, v_2=63, n=22.

Stage	ANOVA (Prob. > F)	Minimum Detectable Difference (%)	Power
Stage 1	0.055	2.1	0.3
Stage 2	0.508	9.0	<0.2
Stage 3	0.055	6.7	0.48
Stage 4	0.055	5.8	0.48
Stage 5	0.485	13.8	<0.2
Stage 6	0.328	17.6	0.2
Atretic	0.217	9.0	0.25

The oocyte stages were grouped into endogenous vitellogenic stages (stages 2,3), exogenous vitellogenic stages (stages 4,5,6), and atretic oocytes. There still were no significant differences in the February/March experiment (Table 10; Figure 15). In the April experiment (Table 10; Figure 16), there was a trend observed in the data, towards a decrease in the stage 2,3 oocytes between Day 0 and Day 12 in the control group. There was a corresponding trend showing an increase in stage 4,5,6 oocytes. These trends were not observed in the Pb-treated groups relative to controls. Table 11 presents the statistical parameters for both the February/March and April experiments for the ANOVA and Kruskal-Wallis test, as well as the tests for minimum detectable difference and power.

The September data were evaluated qualitatively because, for control fish at Day 12 and 20, most oocytes were in stage 6; few earlier stage oocytes were present. The oocytes had completed oogenesis, and soon would be released into the body cavity. These stage 6 oocytes are large, and so few oocytes would have been counted in control fish, relative to Pb-treated fish, thereby potentially affecting the results. There were early stage oocytes and atretic oocytes in the Pb-treated fish. Many Pb-exposed oocytes had an irregular membrane contour, and the zona pellucida was detached from the follicular epithelium (Figure 17).

Table 10: Summary of oocyte stage frequency data (mean % of total oocytes counted ± standard error) for the February/March and April experiments. Oocytes were grouped into endogenous (stages 2,3), exogenous (stages 4,5,6), and atretic stages.

February/March Experiment	Stages 2,3	Stages 4,5,6	Atretic
Day 0 (n=4)	55.0 ± 3.1	23.3 ± 1.5	19.7 ± 3.4
Day 12 Control (n=9)	50.0 ± 2.6	27.7 ± 2.7	18.9 ± 1.3
Day 12 25 µg/L (n=7)	54.0 ± 4.1	21.5 ± 3.3	20.9 ± 3.4

April Experiment	Stages 2,3	Stages 4,5,6	Atretic
Day 0 (n=5)	42.1 ± 3.2	39.1 ± 2.8	14.8 ± 1.5
Day 12 Control (n=7)	28.3 ± 3.3	45.9 ± 4.8	15.6 ± 3.3
Day 12 10 µg/L (n=9)	37.2 ± 4.8	38.0 ± 6.3	22.4 ± 3.1

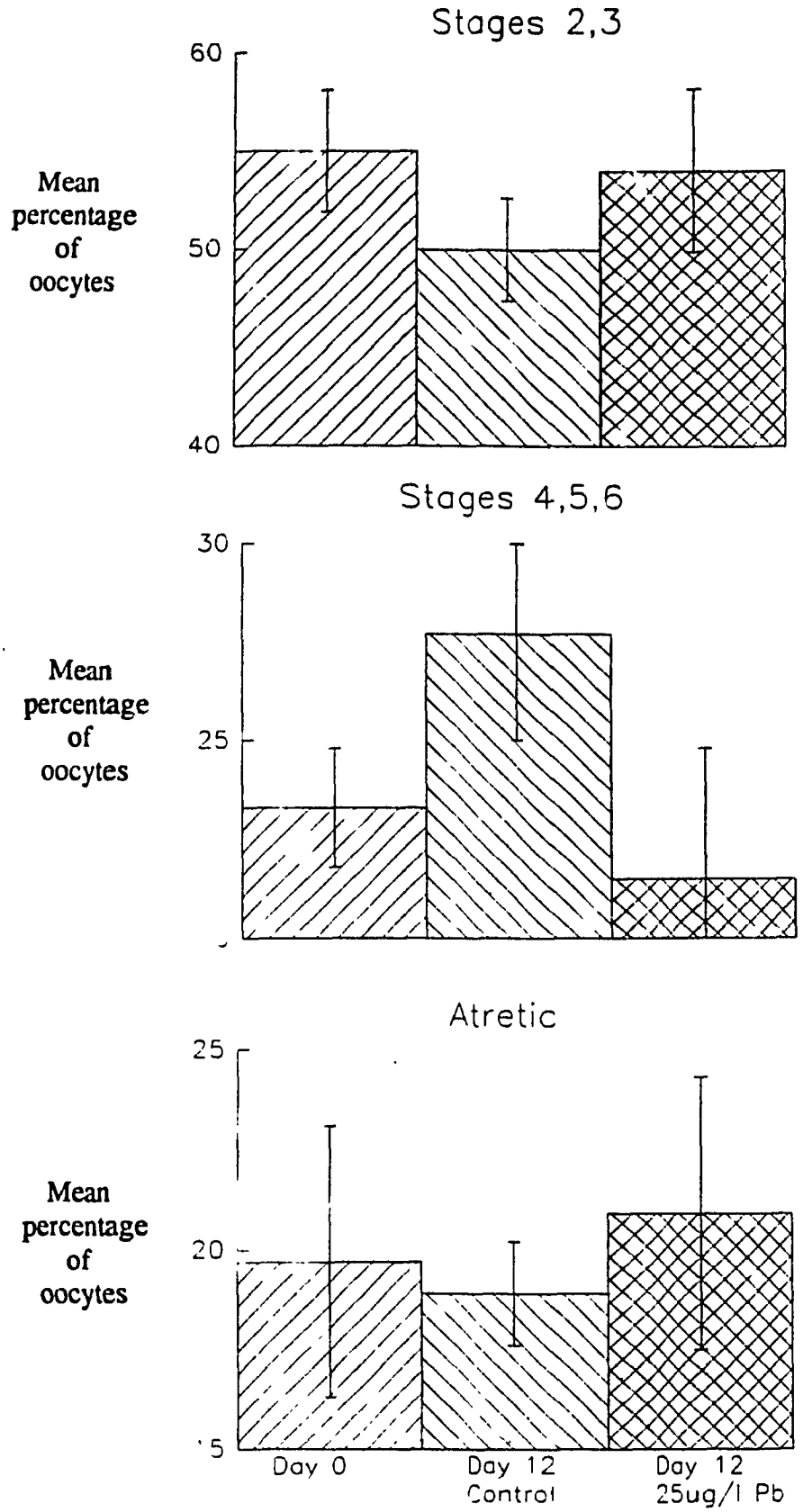


Figure 15: Mean percentage (± standard error) of oocytes in endogenous vitellogenesis (stages 2,3), exogenous vitellogenesis (stages 4,5,6), or that were atretic in the February/March experiment exposing 2-year old rainbow trout to 25 μg/L lead nitrate over 12 days.

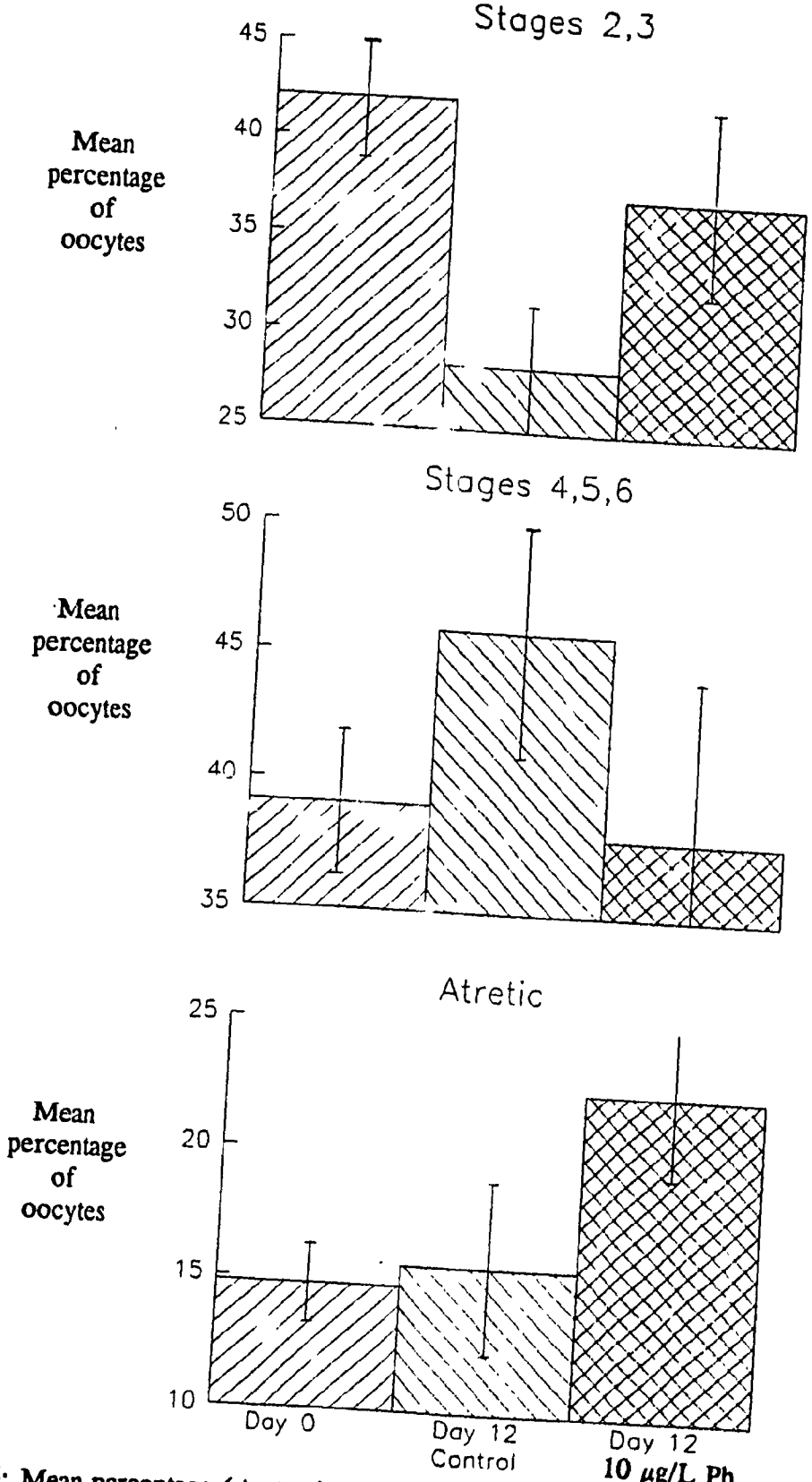


Figure 16: Mean percentage (± standard error) of oocytes in endogenous vitellogenesis (stages 2,3), exogenous vitellogenesis (stages 4,5,6), or that were atretic in the April experiment exposing 2-year old rainbow trout to 10 µg/L lead nitrate over 12 days.

Table 11: Summary statistics for oocyte stage frequency data for the February/March and April experiments. Oocytes were grouped into endogenous (stages 2,3), exogenous (stages 4,5,6), and atretic stages. Statistical parameters for minimum detectable difference and power tests are for February/March k=3, v_1=2, v_2=60, n=21, and for April k=3, v_1=2, v_2=57, n=20. ANOVA and Kruskal-Wallis statistics $\leq$ 0.05 are significant.

Experiment	ANOVA (Prob.>F)	Minimum Detectable Difference	Power	Kruskal-Wallis test
Feb./March stages 2,3	0.54	10.1	<0.20	0.55
Feb./March stages 4,5,6	0.29	8.9	0.25	0.29
Feb./March atretic	0.84	7.6	<0.20	0.99
April stages 2,3	0.11	12.4	0.31	0.09
April stages 4,5,6	0.54	16.5	<0.20	0.20
April atretic	0.17	9.2	0.25	0.122

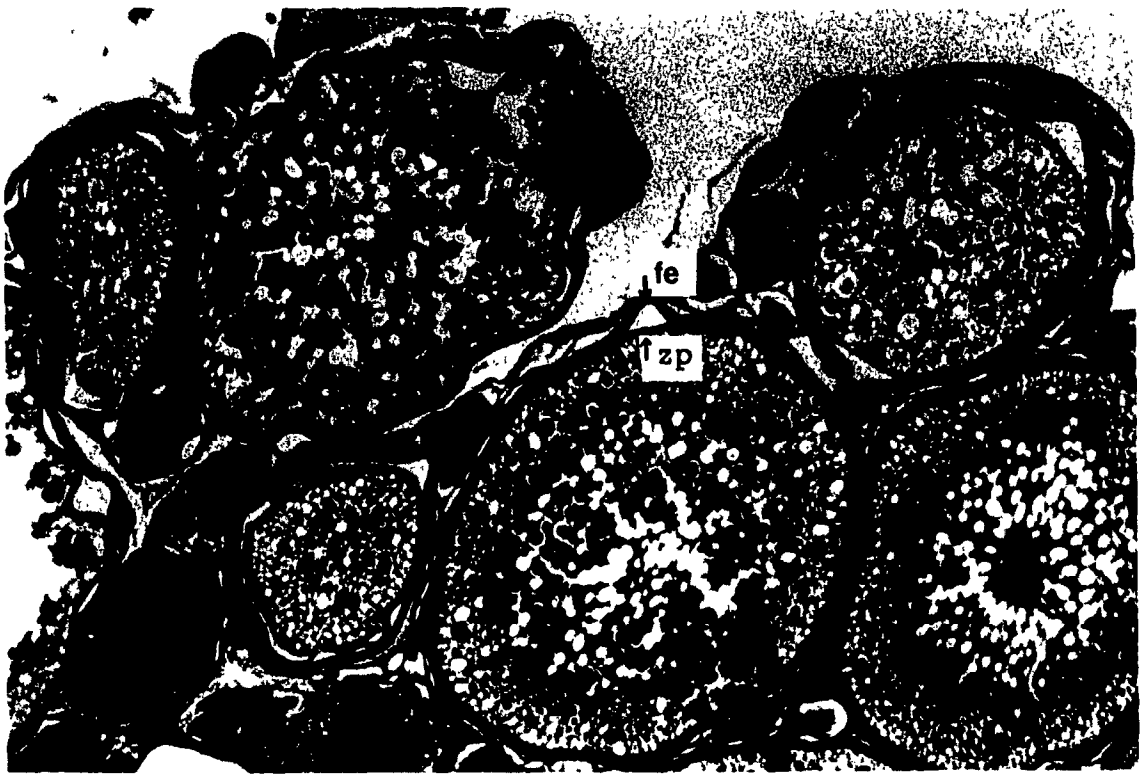


Figure 17: Stage 6 oocytes of sexually-maturing female rainbow trout exposed to lead during late vitellogenesis. Note the irregular membrane contour, and the zona pellucida (zp) detached from the follicular epithelium (fe) (magnification 40 x).

Pituitary Basophils

The pituitary gland (Figure 18) is formed from two primary embryonic origins. The adenohypophysis originates as a dorsal extension from the roof of the buccal cavity, and the neurohypophysis is a ventral protrusion from the floor of the diencephalon of the brain (Andersen and Mitchum, 1974).

The adenohypophysis is differentiated into the proximal and rostral pars distalis, and the pars intermedia. Several functionally specific cell types are located within the rostral pars distalis: the prolactin cells, the adrenocorticotropin hormone cells, and thyroid stimulating hormone cells. The proximal pars distalis contains cells of two types: growth hormone cells and gonadotropin-containing cells. The pars intermedia forms nearly two-thirds the volume of the entire pituitary, and contains melanophore stimulating hormone cells (Andersen and Mitchum, 1974).

The neurohypophysis is composed of axonal nerve fibers which originate from nuclei within the hypothalamus. These nerve fibers form a pituitary stalk (neurohypophyseal tract) into the pituitary gland. The neurohypophyseal tissue extends projections into the adenohypophysis (Andersen and Mitchum, 1974).

The gonadotropin-containing cells have been identified in the present study as the granular basophils (Figure 19). During endogenous vitellogenesis, only GtH I (vitellogenic GtH) cells are present in the rainbow trout pituitary (Nozaki et al., 1990a). The fish in the February/March experiment in

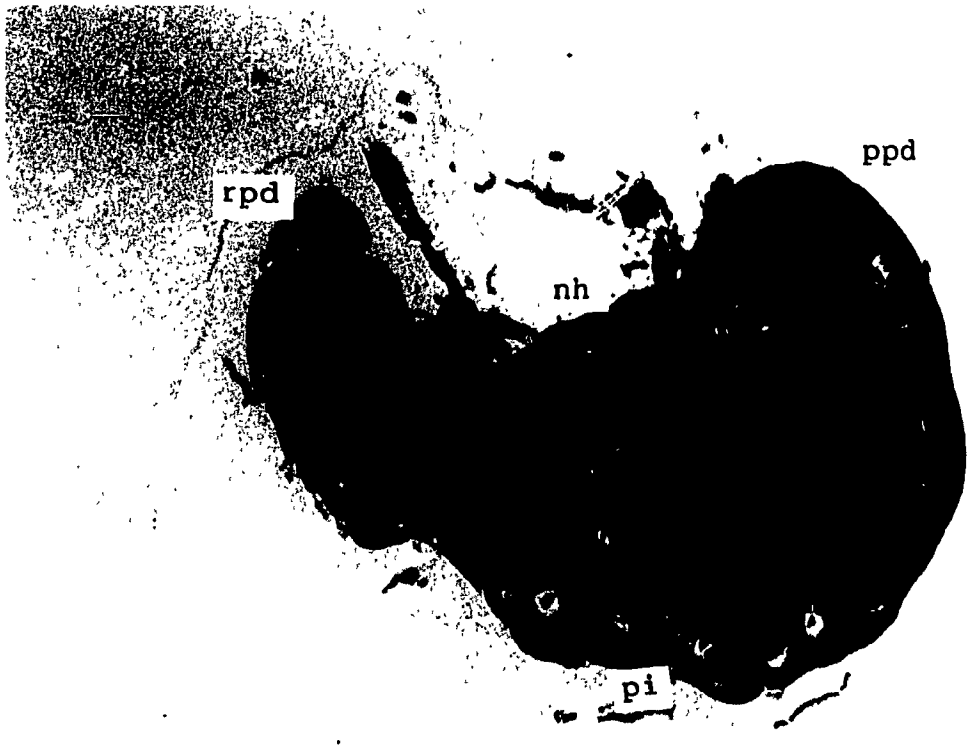


Figure 18: Midsagittal section of the pituitary gland of a sexually-maturing female rainbow trout. The proximal pars distalis (ppd) contains the majority of GtH cells. Note the rostral pars distalis (rpd), pars intermedia (pi) and neurohypophysis (nh) (magnification 40 x).

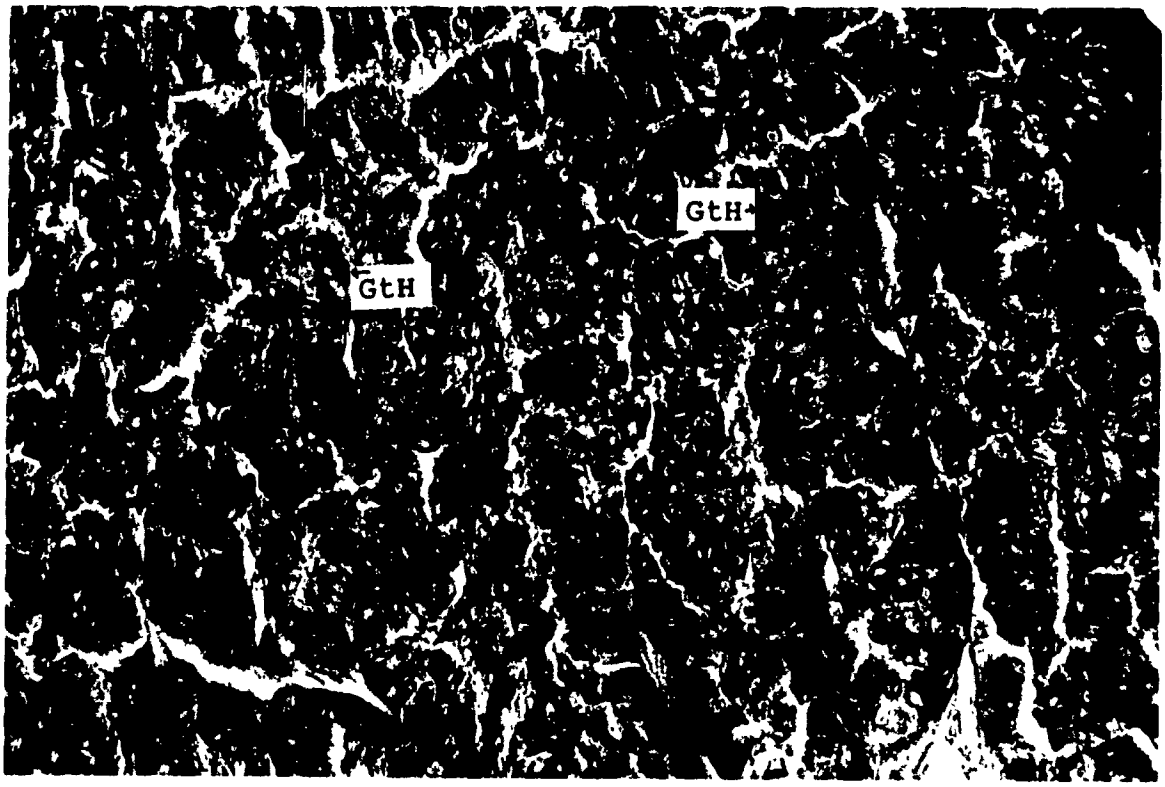


Figure 19: The gonadotropin-containing cells (GtH) are the granular basophils of the proximal pars distalis. The green-staining cells are somatotropin-containing cells. (magnification 250 x).

the present study are in this stage. GtH II (maturational GtH) cells appear coincident with the onset of exogenous vitellogenesis (Nozaki et al., 1990a). This period occurred during the April experiment. Therefore, both GtH I and GtH II cells were present in the pituitary in April. Female trout have an increase in plasma levels of GtH II at the time of oocyte maturation (Suzuki et al., 1988). Histologically, the release of GtH would result in fewer pituitary basophils containing GtH granules at this time. The trout in the present study were fall spawners; final oocyte maturation would have occurred shortly after the September experiment.

The number of dark granular pituitary basophils for the February/March experiment is given in Table 2 and Appendix 4. Sixty per cent of control fish pituitaries contained less than 100 basophils. In pituitaries with more than 100 basophils, the range in the number of basophils was approximately from 600 to 2400. Table 12 presents the statistics that show no statistical difference in the February/March experiment. The minimum detectable difference would be 756 basophils. In the April experiment, the number of basophils in control fish ranged between approximately 200 and 2200; the range was 200 to 800 for Pb-treated fish (Table 2, Appendix 5). Table 12 shows the statistics for this experiment; a difference of 736 basophils would have been needed to observe a statistical difference between the groups.

The number of pituitary basophils for the September experiment is shown in Table 2 and Appendix 6. Summary

statistics are presented in Table 12. Day 0 control fish pituitaries contained less than 250 basophils. In Day 12 control pituitaries there were between 700 and 3600 basophils, and in Day 20 control pituitaries there were between 1000 and 2600 basophils. There were significantly fewer basophils at Day 0 than in Day 12 or 20 control pituitaries (Table 12). The variance in the number of basophils at both Pb concentrations is greater than the variance seen in controls. The range is more than one order of magnitude, between 100 and 3000 basophils for Day 12 10 μg/L Pb, and between 500 and 7000 for Day 20 10 μg/L Pb. At Day 20, 25 μg/L Pb, only two pituitaries were available for counting and these contained 1700 and 3900 basophils. Six of the seven pituitaries of fish from Day 12 25 μg/L Pb contained 1000 to 3500 basophils.

Linear regression was used to determine if there was a correlation between the number of pituitary basophils and either GSI or body weight. Table 13 shows the results, including the r^2 values, the p values and degrees of freedom, for all experiments. All correlations were positive. There was a strong correlation between pituitary basophils and GSI in the April and September experiments, and between pituitary basophils and body weight in the April experiment.

Lead Accumulation in Blood, Ovary, Liver

Mean blood Pb levels ± standard error for rainbow trout exposed to 10 and 25 μg/L Pb nitrate during both early and late vitellogenesis experiments are given in Table 14.

Table 12: Summary statistics for the pituitary basophil data for all experiments exposing 2-year old rainbow trout to Pb nitrate. ANOVA and Kruskal-Wallis statistics $\leq$ 0.05 are significant.

Experiment	ANOVA (Prob.>F)	Minimum Detectable Difference	Power	Statistical Parameters	Kruskal-Wallis test
Feb/March (difference between Day 0 and Day 12 controls, and Day 12 25 µg/L)	0.84	756	0.2	k=3 v_1=2 v_2=33 n=12	0.925
April (difference between Day 0 and Day 12 controls, and Day 12 10 µg/L)	0.21	736	<0.2	k=3 v_1=2 v_2=57 n=20	0.174
September (difference between the three control groups)	0.047	1426	0.63	k=3 v_1=2 v_2=30 n=31	0.029
September (difference between three Day 12 groups)	0.475	1417	<0.2	k=3 v_1=2 v_2=45 n=16	0.259
September (difference between three Day 20 groups)	0.819	3428	<0.2	k=3 v_1=2 v_2=30 n=11	0.635

Table 13: Results of linear regression analyses (r^2 values, p values, degrees of freedom (df)) to detect correlations between number of pituitary basophils and either body weight or GSI in rainbow trout.

Experiment	Pituitary basophil/body weight correlation			Pituitary basophil/GSI correlation		
	r^2	df	p value	r^2	df	p value
February/March	0.15	12	0.19	0.153	12	0.19
April	0.573	8	0.12	0.54	8	0.25
September	0.0008	10	0.16	0.509	10	0.04

Table 14: Blood lead levels (μg Pb/L whole blood ± standard error) of rainbow trout exposed to 10 or 25 μg/L Pb

Month	Day 12	Day 20
February/March		
25 μg/L Pb	332 ± 22.9 (n = 9)	
April		
10 μg/L Pb	181 ± 15.9 (n = 9)	
September		
10 μg/L Pb	256 ± 22.5 (n = 5)	406 ± 42.9 (n = 4)
25 μg/L Pb	492 ± 47.4 (n = 6)	581 ± 53.5 (n = 4)

Control fish blood contained no detectable levels of Pb.

There was no evidence of lead accumulation in the ovary or liver of fish exposed to either 10 or 25 μg/L Pb, during early or late vitellogenesis.

DISCUSSION

The present study is the first report of Pb having an effect on the ovary of rainbow trout at the low concentrations of 10 and 25 μg/L.

Lead had no impact on reproductive function, as measured by GSI, maximum oocyte diameter, and oocyte stage frequency, in female rainbow trout exposed during endogenous vitellogenesis (February/March). Pb also did not affect the number of pituitary basophils. However, during endogenous vitellogenesis, the number of GtH cells is low and only GtH I cells are present.

At the transition period from endogenous to exogenous vitellogenesis (April), there were trends indicating an adverse impact from Pb on oocyte development. Although not statistically significant, GSI and maximum oocyte diameters were greater in controls relative to Pb-exposed fish after 12 days of exposure. Also, control oocytes were continuing the development process, progressing from early stage (stage 2,3) to later stage (stage 4,5,6) oocytes. Although no effects were observed on pituitary basophils, the trends in the GSI, oocyte diameter, and oocyte stage data indicate that the beginning of exogenous vitellogenesis may be a sensitive time for Pb exposure of fish.

The late exogenous vitellogenesis experiment (September) data suggested that although late stage oocytes were able to complete maturation, this process was delayed by exposure to Pb. At the end of the experiment, both the GSI and maximum oocyte diameter were the same in Pb-treated and control fish

groups. However, the presence of more early stage and atretic oocytes in Pb-exposed fish suggests that Pb may affect fecundity. The large number of basophils in the pituitary of Pb-exposed fish suggests that Pb may be acting by inhibiting or decreasing the amount of GtH released from the pituitary.

Results of the present study will be compared to studies which exposed rainbow trout to cyanide, as well as studies of other fish species exposed to Pb and other toxicants. A comparison of reproductive effects caused by Pb and cyanide are of particular interest. It has been suggested that these toxicants may both act on the hypothalamus-pituitary-gonadal axis (Szabo et al., 1991; Thomas, 1988; 1990) and produce certain identical toxic responses (Ruby et al., 1993), although exact mechanisms and some responses may differ.

Using GSI as a parameter, the response of the ovary of sexually maturing females varied relative to the physiological stage of reproduction following exposure to low levels of inorganic lead. During endogenous vitellogenesis there was no significant change. During exogenous vitellogenesis the GSI of 10 µg/L Pb-treated fish was significantly different relative to controls at Day 12, but there was no significant difference between controls and lead-treated fish at the end of the experiment.

In the February/March and April experiments, Pb at concentrations of 25 or 10 µg/L had no observable effect on GSI after 12 days. Unfortunately, it is not known if an effect would have been observed after 20 days. The February/March experiment was conducted during endogenous

vitellogenesis. During the RNA synthesizing endogenous phase, oocytes do not increase in size as rapidly when compared to exogenous vitellogenesis. Ovarian size increases significantly during exogenous vitellogenesis when the oocytes rapidly sequester Vg from the blood. In the April experiment, oocytes were depositing secondary yolk. There was a trend indicating growth in the control group, although this trend was not statistically significant. The power of the ANOVA was low (0.30) indicating that there was a 70% chance that the null hypothesis was wrongly accepted and that there was, in fact, a difference. In both the Day 12 control and Pb-treated groups, a similar number of fish were entering the phase of secondary yolk deposition (exogenous vitellogenesis). This experiment was conducted during the transition period between endogenous and early exogenous vitellogenesis. The lack of a significant increase in GSI may be because the fish were in this transitional phase, between endogenous vitellogenesis, characterized by relatively slower ovarian growth, and exogenous vitellogenesis in which the ovary grows more rapidly.

Impaired ovarian growth was evident by the lack of significant difference between the GSI of Day 0 control fish and Day 12 or 20 Pb-treated fish exposed during late exogenous vitellogenesis (September). GSI in control fish increased over the first 12 days of the experiment, but not between Day 12 and 20. These differences suggest delayed development in Pb-treated fish. At Day 20, the large variance in the

controls and 10 μg/L Pb-treated fish, along with small sample sizes may have contributed to a lack of significant difference. The power of the ANOVA was only 0.35 indicating that there was a 65% chance that the null hypothesis was incorrectly accepted and there is, in fact, a difference. The minimum detectable difference required was 1.62. Large variance may explain the lack of difference between the Pb treatments. The large variance particularly in the Pb-treated Day 20 10 μg/L fish data may occur because this is a low Pb level which lies below a toxic threshold level. At this low concentration, physiological mechanisms may be modified in an attempt to cope with the stress. Reduced GSI in fish exposed to toxicants during exogenous vitellogenesis has been reported by Ruby et al. (1986), Thomas (1988) and Tulasi et al. (1989). Ruby and co-workers (1986) studied effects of sublethal cyanide on rainbow trout, Thomas (1988) exposed Atlantic croaker Micropogonias undulatus to dietary lead, and Tulasi and co-workers dosed the freshwater fish Anabas testudineus with higher concentrations (1-20 mg/L) of waterborne lead.

The physiological stage of the reproductive cycle is also important when evaluating Pb effects on oocyte development based on the maximum oocyte diameter. There was no significant change during endogenous vitellogenesis. During exogenous vitellogenesis, Pb-exposed oocyte growth was delayed.

Lead had no effect on maximum oocyte diameter during endogenous vitellogenesis (February/March). An effect may not

have been observed because during the RNA synthesizing phase, oocytes do not increase in size as rapidly as during exogenous vitellogenesis. Lead also had no statistically significant effect in the April experiment, although there was a trend indicating growth over the 12 days in the controls. The variance in the size of the fish oocytes in the April experiment may have contributed to the lack of a significant change over the 12 days. The fish were in a period of transition from endogenous to exogenous vitellogenesis, which may have contributed to the difficulty in observing a statistical difference. Lead may be inhibiting the uptake of Vg by the oocytes.

In September, the largest control oocytes did not continue to increase in size from Day 12 to Day 20. These oocytes were at the end of oogenesis, and would soon be released into the body cavity for storage until spawning. The 10 and 25 µg/L Pb-treated fish oocytes required up to eight more days to reach the same diameter as control oocytes. However, the power of the ANOVA for Day 20 comparisons was low (0.20), indicating that there was an 80% chance that the null hypothesis was incorrectly accepted and that there is a difference. A decrease in the oocyte diameter relative to controls also has been observed in rainbow trout exposed to sublethal levels of cyanide (Lesniak and Ruby, 1982), and acid and aluminium (EPRI, 1991), and in another teleost fish exposed to endosulfan (Pandey, 1988). The delay in oogenesis may result in an asynchronism between when the males and

females are prepared to spawn. Egg maturation and spawning were delayed by 12 days in rainbow trout exposed to acid (pH 4.83 and 5.25) for 157 days (EPRI, 1991). In the same study, rainbow trout exposed to acid and aluminium (50 and 100 µg/L) spawned 19 days later than the control group. Despite the delay in ovulation in these studies, minor differences in egg diameter, and the accompanying physiological disturbances, no differences in fecundity were observed among exposure groups (EPRI, 1991).

The effects of Pb on GSI and maximum oocyte diameter were similar for all three experiments. There were no effects in the February/March experiment for either parameter. In the April experiment, no changes were statistically significant, but for both GSI and oocyte diameter, there was a trend indicating an increase in controls over the 12 days, but no increase in Pb-treated fish. In the September experiment significant differences occurred at Day 12 but by Day 20 there were no differences. This suggested delayed development. It is not unexpected that oocyte growth and gonad growth would be related. Only the largest oocytes were measured, and these would occupy the largest volume of the ovary. The impact of Pb on early stage oocytes may not be observed in GSI since the early stage oocytes would contribute to a lesser extent to the entire mass of the ovary.

Every year at the beginning of the reproductive cycle, the developing oocytes show a considerable disparity in size and stage of development. It is not until later in

vitellogenesis that a uniformity occurs such that all oocytes are of a similar size and developmental stage at ovulation (Tyler et al., 1990).

In the February/March early vitellogenesis experiment, there was no change ($p>0.05$) in the frequency of individual oocyte stages over the course of the experiment. Differences were not large enough to detect a significant change and the power of the ANOVA was always < 0.30, indicating there was a >70% chance that the null hypothesis was incorrectly rejected and that there was a difference. The 12 day length of the experiment may have been insufficient to observe a significant change. When oocyte stages were evaluated separately for the April experiment, there was no statistically significant difference, but there was a trend indicating fewer stage 1 and 3 oocytes in the control group after 12 days relative to the Day 0 controls. There also was a trend indicating an increase in stage 5 and 6 oocytes in controls from Day 0 to Day 12. This may suggest that control oocytes are continuing in their development, advancing to later stages, whereas this development is delayed in the Pb-treated fish. Although this trend was not statistically significant, power analyses showed that for stages 1 and 3, there was a 70% and 52% probability, respectively, that the null hypothesis (population means are equal) was incorrectly accepted. This trend was supported by the results of combining the endogenous vitellogenic stages (stages 2,3) and exogenous vitellogenic stages (stages 4,5,6). Although not statistically significant, there was a decrease

in the number of stage 2,3 oocytes in controls, with a corresponding increase in stage 4,5,6 oocytes. This was not observed in Pb-treated fish. Again, there is a 69% probability that the null hypothesis (population means are equal) was incorrectly accepted.

During late vitellogenesis, most oocytes of control fish were at the final stage of oogenesis, ready for ovulation. Pb-treated fish ovaries contained oocytes in all stages of oogenesis. The presence of early-stage oocytes in Pb-treated fish has also been observed by Thomas (1988). With fewer oocytes reaching maturity, a decrease in fecundity may result for these fish. A decrease in fecundity has been reported for fish exposed to higher levels of lead (1-20 mg/L) (Tulasi et al., 1989).

During late vitellogenesis, the similar size of largest oocytes in control and Pb-treated fish but the presence of more early stage oocytes in Pb-treated fish suggests that oocytes that have reached a certain size are able to complete development, but the early stage oocytes are delayed in their development. Several mechanisms may exist whereby lead negatively impacts oocyte development. These include the perturbation of GtH synthesis or secretion, or an inhibition by Pb of Vg synthesis in the liver, resulting in an inadequate supply of Vg for the developing oocytes. A decrease in plasma Vg levels has been reported in rainbow trout exposed to sublethal cyanide (Ruby et al., 1986). Lead could also inhibit the uptake of vitellogenin by the oocytes.

It is difficult to elucidate the exact mechanism of Pb toxicity in the present study. Pb may be having an effect on the calcium dynamics in the fish. It has been illustrated that Ca and Pb are taken up by the same system in the mammalian gastrointestinal tract (Six and Goyer, 1970). It is unknown whether this would occur in fish exposed to low level lead which is taken up primarily at the gill, as is the case in the present study. Small amounts of dietary calcium decrease uptake of water-borne Pb by fish. However, Ca has little effect in reducing the retention of Pb in most fish tissues, with the exception of skin and skeleton (Varanasi and Gmur, 1978). The trout in the present experiment were fed daily with a feed which contained 0.6 % calcium; Ca should not have been limiting.

Vitellogenin is a calcium-bound glycolipophosphoprotein complex (Ng and Idler, 1983). The calcium content of rainbow trout Vg is 0.5% (Sumpter, 1985 cited in Nagler et al., 1987). Wallace (1970) has shown that one atom of Ca is associated with every protein phosphate group in the Vg complex. Thus, as maturation proceeds, gross changes occur in the total serum Ca levels (Whitehead et al., 1978). Prior to vitellogenesis, the total plasma Ca concentration of rainbow trout is approximately 10mg%. Levels of plasma Ca reach up to 58mg% in rainbow trout prior to spawning (Whitehead et al., 1978). Estrogen has been shown to cause hypercalcaemia (elevated plasma Ca concentrations) in a number of species, including rainbow trout (Elliot et al., 1979 cited in Ng and Idler,

1983). Bone Ca deposition is also inhibited by estrogen treatment. Estrogen elevates the serum content of nonultrafiltrable Ca and phosphorus and proteins without affecting the content of ultrafiltrable Ca and phosphorus. A part of the increase in the nonultrafiltrable Ca is attributable to the increase in the serum concentration of Vg. The measurement of Ca in the blood alone may not reveal any information on the mechanism of action of Pb; the Ca measured represents total Ca, including Ca bound to Vg. However, a measure of both Ca and Vg levels in rainbow trout blood during exogenous vitellogenesis would yield additional information regarding the mechanism of Pb toxic action.

Lead was shown to lower plasma estradiol concentrations in Atlantic croaker exposed to Pb in the diet for 30 days (Thomas, 1988). Lower estradiol may reduce Vg levels. The decreased estrogen levels may not be adequate to stimulate an increase in Ca concentrations in the blood (i.e., hypercalcaemia may not occur). Pb exposure has been shown to reduce serum Ca concentrations in rats (Six and Goyer, 1970). Therefore, there may not be adequate Ca for the Vg. Da Costa and Ruby (1984) suggest that for cyanide-exposed fish, insufficient calcium during the critical phase of early vitellogenesis would block exogenous yolk production and release from the liver subsequently making secondary yolk unavailable for uptake by the ovary. This would result in a decrease in or blockage of oocyte exogenous vitellogenesis. Oocytes would not be able to complete development, or would

have their development slowed. This hypothesis is supported by the results of the September experiment in the present study. Pb-treated oocytes required up to 8 more days to complete development (reach the same diameter as controls).

There is extensive oocyte atresia in Pb-treated fish in the September experiment. Oocyte atresia is a common phenomenon during the vitellogenic phases of oocyte development, and amongst the unovulated follicles of post-ovulatory fish (Braekevelt and McMillan, 1967). Atresia also is an important determinant of fecundity (Bromage and Cumaranatunga, 1987). Increased atresia was also seen in fish exposed during exogenous vitellogenesis to dietary lead (Thomas, 1988), and a variety of other toxicants including endosulfan (Pandey, 1988), pentachlorophenol (Nagler et al., 1986), cyanide (Lesniak and Ruby, 1982), and acid stress (McCormick et al., 1989). Guraya (1986) suggested that atresia in the fish ovary was either due to the lack of proper gonadotropic stimulation or due to imperfect balance of various hormones including steroids. Gonadotropins stimulate the release of estrogens which have a direct protective effect on oocyte follicles (Guraya, 1986). Therefore, if Pb is inhibiting the release of GtH, this would result in a decrease in estrogen production and therefore decrease the protection against oocyte atresia. The result is an increase in atresia, as was observed in the September experiment. Since atretic oocytes are not spawned, they constitute an energy consumptive process without reproductive output (McCormick et al., 1989).

It would be interesting to study the fecundity of rainbow trout exposed to low level Pb to see if rainbow trout populations may be impacted.

Lead-exposed oocytes in the late vitellogenesis experiment had an irregular membrane contour, and the zona pellucida was detached from the follicular epithelium. The zona pellucida is formed by the invagination and interdigitation of the plasma membrane of the oocyte and the surrounding follicular cells. The integrity of the zona pellucida is necessary for transport of materials, such as the yolk proteins lipovitellin and phosvitin, between the outside and inside of the oocyte. Alterations in the zona pellucida would affect the permeability of the oocyte. Similar to the present study, oocytes of rainbow trout exposed to sublethal cyanide showed an irregular membrane contour and almost complete separation of membranes from the egg surface (Lesniak and Ruby, 1982). The extensive thickening (hypertrophy) of egg membranes, and hypertrophied and wavy zona pellucida observed in cyanide treated oocytes (Lesniak and Ruby, 1982) was not observed in the present study. McCormick et al. (1989) observed that the zona pellucida of fathead minnow oocytes exposed to acid water was eroded. Oocytes in the present study with alterations in the follicular wall may not be able to sequester vitellogenin and hence not reach final maturation or be viable if spawned. Again, this would result in decreased fecundity.

The evaluation of ovarian characteristics (e.g., GSI,

oocyte diameter, stage frequency) enabled the determination that the February/March experiment occurred during endogenous vitellogenesis, the April experiment during the transition period from endogenous to exogenous vitellogenesis, and the September experiment in late exogenous vitellogenesis. This information is important to the understanding of potential effects Pb may have on the pituitary during these three physiological stages.

Pituitary granular basophils have been implicated in gonadotropin (GtH) production. It is well established that there are two types of pituitary gonadotroph cells which vary in their activity during the reproductive cycle of salmonids, and two distinct gonadotropins, referred to as GtH I (or Con AI or vitellogenic GtH) and GtH II (or Con AII or maturational GtH) (reviews by Ng and Idler, 1983 and van Oordt and Peute, 1983; Nozaki et al., 1990a). GtH I cells are present and appear to be active prior to the onset of exogenous vitellogenesis. Therefore, basophils present in the February/March experiment were GtH I cells. The appearance of GtH II cells coincides with the appearance of eosinophilic material in the oocytes at the onset of exogenous vitellogenesis (Nozaki et al., 1990b). There were GtH II cells as well as GtH I cells present in the April experiment pituitaries. GtH I may be primarily but not exclusively involved in stimulating steroidogenesis during vitellogenesis, while GtH II is associated primarily with final reproductive maturation (Suzuki et al., 1988). Levels of GtH are low

during the first half of the year in fall spawning rainbow trout, although a pulse of GtH is thought to result in the beginning of exogenous vitellogenesis. The levels of GtH increase in September and October, and fall through November (Scott and Sumpter, 1983b). In general, the gonadotropins induce liver Vg synthesis (Ng and Idler, 1983), and enhance vitellogenin uptake from the blood by eliciting micropinocytotic activity at the oocyte surface (de Vlaming, 1983).

No effects were evident in the number of pituitary granular basophils during the endogenous vitellogenesis experiment (February/March). These are likely GtH I cells since GtH II cells were never found in previtellogenesis rainbow trout in the study by Nozaki et al. (1990b). Sixty per cent of pituitaries contained less than 100 granular basophils. The small number of basophils is consistent with the results of Nozaki et al. (1990b) who observed a low number of GtH I cells in pituitaries of early vitellogenic rainbow trout. In the April experiment, control or lead-treated fish which had larger GSIs and ovaries containing oocytes in the secondary yolk deposition phase also had pituitaries with a greater number of granular basophils. The presence of granular basophils suggests that the pituitaries were becoming active producing, storing and secreting GtH (Nozaki et al., 1990b). This experiment was conducted at the onset of exogenous vitellogenesis, and may have lasted for too short a duration to observe any effect of Pb on the number of

pituitary basophils. Also, since the experiment occurred during the transition from endogenous to exogenous vitellogenesis, the pituitary may have been in the early phase of GtH II formation. The power for both of these experiments was low ($\leq$ 0.20), indicating a >80% chance that the null hypothesis was incorrectly accepted and that there may be a difference.

At the end of vitellogenesis, the release of GtH would result in fewer pituitary basophils containing GtH granules. There is a low number of basophils in the September experiment Day 0 control fish. The large number of basophils at Day 12 and 20 may represent the formation of GtH cells for the next reproductive cycle. At a lead concentration of 25 µg/L during late vitellogenesis, there was a consistently large number of pituitary granular basophils. At Day 20, although there also was a large numbers of pituitary basophils in some control fish pituitaries, the maximum number of basophils in a pituitary was greatest in the Pb-treated fish. It is possible that GtH was not being released from the basophils of the Pb-treated fish, or was being released in smaller quantities. This cannot be shown statistically in this study, but this hypothesis is supported by the work of Thomas (1990) who reported decreased in vitro GtH secretion from pituitaries of Atlantic croaker exposed to Pb administered in food. Also, the exposure time of the present study may not have been long enough or the concentration not high enough to see an impact. Thomas' studies (1988; 1990) revealed that female Atlantic

croaker exposed to dietary lead exhibited decreased plasma steroid (estrogen and estradiol) concentrations. Estrogen has a negative feedback effect on the pituitary, and hence lower estrogen levels would cause GtH to continue to be released by the pituitary. With these stimulatory and inhibitory effects, it would be difficult to see a correlation between number of pituitary basophils and Pb exposure. This also may explain the large variance in the number of basophils, especially in the 10 μg/L Pb-treated fish.

There was a correlation between the number of pituitary basophils and GSI in the April and September experiments. This result is contrary to that of Nozaki et al. (1990b) who found no correlation between number of pituitary GtH I or GtH II cells and GSI. They did find a relationship between the number of GtH I cells and body size. In the current study, there was a correlation between the number of basophils and body weight in the April experiment. Unfortunately, the staining methods employed in the present study were unable to distinguish between GtH I and GtH II cells.

Another complication for the correlation analysis between the number of basophils and other parameters is the presence of thyrotropic cells in the pituitary. There is a difficulty in differentiating between gonadotropic and thyrotropic basophils on the basis of staining because they are both glycoprotein hormones. However, Ruby and Bryant (1990) found a high coefficient of correlation between basophils and cysts of spermatocytes in rainbow trout which suggested that most of

the granular basophils represented gonadotrophs, while the number of thyrotrophs were negligible. There was no correlation between number of granular basophils and cysts of spermatogonia (Ruby and Bryant, 1990), and so the above conclusion may be specific to particular periods in the reproductive cycle. If this relationship holds for female rainbow trout, the presence of thyrotropic cells should not affect the results of the late vitellogenesis experiment.

The presence of large numbers of basophils in Pb-exposed late vitellogenesis fish pituitaries, combined with the delayed oocyte development observed in treated fish from that experiment suggests Pb is altering GtH release from the pituitary. Specifically, Pb may be acting on GtH II, since there were no effects on the pituitary or ovary of fish exposed to lead during endogenous vitellogenesis, which is the physiological stage under GtH I control. However, the reproductive changes may not result from a direct action of Pb on the pituitary, but by an effect on the hypothalamus causing changes in pituitary GtH release. Thomas (1991) proposed that Pb acts at the level of the hypothalamus in Atlantic croaker exposed to dietary lead.

The hypothesis of lead acting at the level of the hypothalamus may be supported by evidence from Katti and Sathyanesan (1986). An exposure of 5 ppm Pb for 150 days caused degenerative changes in the neurons of the nucleus preopticus (NPO) and nucleus lateralis tuberis (NLT). Neurons of the NPO give rise to the left and right preoptico-

neurohypophyseal neurosecretory tracts which join together prior to entering the pituitary stalk. The NLT is at the base of the hypothalamus, adjacent to the pituitary stalk. The NLT and probably part of the NPO are involved in the regulation of GtH secretion from the pituitary (Peter, 1982).

Based on the experimental results presented in the current study, lead may have an effect on rainbow trout oocyte development in exogenous vitellogenesis. The endogenous vitellogenesis experiment resulted in no adverse impact to oocyte development. However, the early and late exogenous vitellogenesis experiments suggest that the early stage oocytes are more susceptible than later stage oocytes and may not be able to complete development. If lead does have a major impact on the hypothalamus-pituitary-gonad axis resulting in altered GtH production and release, this effect would be more pronounced during exogenous vitellogenesis when oocyte development requires the hormonal control of the hypothalamus and pituitary. This also would be true if Pb specifically targeted GtH II cells and not GtH I cells. During endogenous vitellogenesis, only GtH I cells are present.

Lead did not accumulate in the liver or ovary of fish in the present study. This may be due to the short exposure duration, the low exposure concentrations, or analytical detection limits or errors. Pb was detected in the blood of fish.

Obtaining meaningful information from the measurement of

the accumulation of Pb in tissues can be difficult, especially during short exposure periods. For example, accumulation in the opercular bone of fish was greater in smaller fish than larger, and opercular bone uptake was typical of other tissues (Hodson, 1982). Therefore smaller fish should show signs of toxicity before larger fish. At a constant rate of uptake, individual tissues of large fish will take longer to equilibrate with blood than those of smaller fish. Thus, short-term (e.g., days) uptake rates by specific tissues may vary inversely with fish size, but long-term (e.g., weeks) equilibrium concentrations should not (Hodson et al., 1982). Thus, concentrations of lead in tissues of fish exposed for only 12 or 20 days, as in this study, probably will provide little toxicologically or mechanistically useful information.

Pb is a non-essential element. The use of concentrations of lead in tissues also may not be useful because dose-response relationships do not always occur for non-essential metals such as cadmium (Jenkins, 1991). The compartmentalization of the metal within the cell may be important to the evaluation of the toxicity of the metal. Metals in the cell bind high molecular weight proteins, very low molecular weight proteins, lysozymes, mitochondria, and possibly DNA. Thus, it has been suggested that the total amount of metal is not important, but the amount bound to specific cell components is important (Jenkins, 1991). It is important here to mention that Pb does not induce metallothionein (Heath, 1987) as do other metals.

Metallothionein, a low molecular weight protein, binds a variety of metals and makes them less toxic to other cellular constituents. However, a recent study (Conner and Fowler, 1994) has isolated a fish hepatic protein, called the lead-binding protein (PbBP), which is immunologically distinct from metallothionein. Such a PbBP may be present in other tissues of the fish as well.

At the present time, it is impossible to determine if Pb is directly affecting the pituitary. The ability of lead to accumulate in the brain, and hence act directly on this organ, remains controversial. Lead was considered unlikely to cross the blood-brain barrier, and did not accumulate in brain tissue of brook trout at any exposure concentration in one experiment (Holcombe et al., 1976). Hodson and co-workers (1978) measured low levels of lead (1.2 μg/g wet weight) in brain tissues of rainbow trout exposed to approximately 10 and 25 μg/L Pb, but found that lead levels did not correlate with water lead concentrations, and hence postulated that the accumulation may have been anomalous. However, Tulasi and co-workers (1989) measured significant levels of lead (between 29 and 143 μg/g dry weight) in brain tissues of fish exposed to between 1.25 and 20 mg/L lead.

It is also unknown whether or not Pb is acting directly on the ovary of rainbow trout. Gonads of fish in this study showed no detectable levels of lead. The gonads of fish are not considered major organs of Pb accumulation. Holcombe et al., (1976) measured very small amounts of Pb (8 μg/g dry

weight) in the gonads of brook trout, but only at a higher water Pb concentration (235 μg/L). Dallinger and Kautzky (1985) also measured very small concentrations of gonadal Pb during their field study of two contaminated rivers. Water lead levels were between 5 and 50 μg/L, and lead levels in gonads were 8.0 ± 3.8 and 12.4 ± 17.9 μg/g dry weight. Fish exposed to between 1.25 and 20 mg/L lead contained between 3.4 and 8.1 μg/g dry weight lead in the ovary (Tulasi et al., 1989). Thus, although waterborne lead levels in these studies varied from 5 μg/L to 20 mg/L, gonad lead levels were consistently between 8 and 12 μg/g dry weight. More importantly, Thomas (1988) found no evidence of a direct suppressive action of Pb on ovarian steroidogenesis from in vitro experiments on Atlantic croaker exposed to dietary Pb.

Lead also did not concentrate in the livers of fish in the present study. Lead may not accumulate to a great extent in the liver because it is rapidly cleared to other sites of accumulation (Hodson et al., 1978). Over a 32-week exposure period, lead concentrations in the liver of rainbow trout were approximately 1.2 and 1.5 μg/g wet weight for water-borne exposure concentrations of 10 and 25 μg/L Pb, respectively (Hodson et al., 1978). A two-week exposure of brook trout to 34 μg/L Pb resulted in liver Pb accumulation of 7 μg/g dry weight (Holcombe et al., 1976). This variability may be due to analytical error, species or sex differences, or differences in physiology. A Pb level of 1.5 μg/g would have been below the limit of detection for the method employed in

this study.

Considerable variability exists in the blood Pb concentration data of the present study, as well as in the studies by Hodson et al., (1977; 1978), where the standard deviation of all blood measurements over all concentrations was ± 200 µg/L. Lead enters the fish circulatory system at the gill. Blood does accumulate Pb, and blood Pb levels do reflect Pb exposure at equilibrium. However, occasional measurements of blood Pb cannot be relied upon to give more than a crude index of exposure except when the levels are very high (Schubert, 1973). There can be considerable variability in blood Pb measurements. This may be due to analytical error and variation between fish. Hodson and Blunt (unpublished data cited in Hodson et al., 1984) tested one fish on 10 occasions and found the relative standard deviation between obtained values was 11.1 %. Variation could result in part because 90 % of blood Pb is bound to red blood cells (rbc), and hence reporting the blood Pb levels as µg Pb / volume rbc may be more accurate than µg/L whole blood. Most researchers have used the latter notation. There are also problems with sample contamination. Hodson et al. (1984) recommend the use of ALA-D (δ-aminolevulinic acid dehydratase) measurements alone or in conjunction with blood lead measurements for determining fish exposure to Pb (i.e., ALA-D inhibition can be related to Pb exposure levels). ALA-D inhibition is a highly specific response to environmental Pb contamination. The assay for ALA-D activity is simple and

sensitive to water levels as low as 5 μg/L Pb or blood levels as low as 300 μg/L. There are no significant time effects, but there are definite species differences, with rainbow trout being more sensitive than brook trout (Hodson et al., 1984).

An equation relating blood Pb concentrations to waterborne Pb concentrations was formulated by Hodson et al. (1977) for constant Pb exposures of up to 100 μg/L for two weeks, to rainbow trout which were six to eighteen months old. This equation is as follows:

$$\log_{10} [\text{blood Pb}] = 1.9730 + 0.6099 \log_{10} [\text{water Pb}]$$

Substituting in 10 and 25 μg/L as the water Pb concentrations of the present study, this equation yields blood concentrations of 383 and 669 μg/L Pb. These levels are comparable to those obtained in the late vitellogenesis experiment of the present study. In another study, approximate values of lead in blood of fish exposed to 10 and 25 μg/L Pb were 200 and 350 μg/L, respectively (Hodson et al., 1978), which is similar to Pb levels measured in blood of the current study early vitellogenesis experiments.

Hodson et al. (1977) do not state whether their experimental conditions would result in a dynamic equilibrium, resulting from net uptake of Pb from water at the gills and transfer from blood to other tissues. However, because the exposure durations of the present study were similar to those of Hodson et al. (1977; 1978), and the present study used Pb concentrations that were within the range of those used by Hodson and co-workers to derive their equation, this author

feels the comparison between the present study results and those of Hodson et al. (1977; 1978) a: valid.

The greater Pb concentrations in blood in the late vitellogenesis experiment relative to the early vitellogenesis experiment may be due to analytical variability and error or physiological differences due to the different season. It probably is not due to the fact that the fish in the September experiment were larger. Hodson et al. (1982) found that there was no relationship between Pb uptake by blood and rainbow trout body weight.

Fluctuating water Pb levels have been shown to cause greater Pb accumulation than constant Pb exposure (Hodson et al., 1983). This could have serious consequences for fish exposed to episodic high Pb levels, such as at spring run-off. This would occur as fish were entering exogenous vitellogenesis. The present study suggests this would be a sensitive time since Pb may cause the impairment of early stage oocyte development. Maximum acceptable toxicant concentrations (MATCs) have been calculated for rainbow trout exposed to Pb. A MATC is calculated based on the idea that certain life stages of an organism will be more sensitive to a toxicant than others. To determine a MATC, breeding members of a population of fish are exposed in the laboratory to a graded series of toxicant concentrations and this is maintained through two generations. Therefore, the MATC should be protective of fish during all stages of reproductive development. The MATC for Pb has been estimated at between 3

and 13 μg/L for rainbow trout in water with a hardness of 135 mg/L (Hodson, 1976), which is similar to the hardness of the water used in the present study. Davies and co-workers (1976) noted that fish were more sensitive when exposed as eggs. Thus, if reproductive effects are being observed on adult fish at the low levels of this study (10 and 25 μg/L in hard water (125 mg/L)), present MATC or water quality guidelines may be inadequate in the event of fluctuating exposures.

Biochemical changes in nutrients besides calcium may interfere with reproductive function in Pb-exposed fish. Some of the interactions of Pb with Ca may be influenced by Vitamin D or its metabolites. Vitamin D mediates intestinal Ca absorption and bone Ca metabolism. It usually acts as a hormone precursor, as it requires two stages of metabolism, first to 25-hydroxycholcalciferol and then to 1,25-dihydroxy cholecalciferol before reaching actual hormonal form. Pb may have an adverse effect on the formation of 1,25-dihydroxy cholecalciferol by renal tubular cells and therefore cause decreased intestinal absorption of calcium (Sandstead, 1977). Vitamin C chelates Pb and increases its depuration rate in humans, but this is not seen in rainbow trout (Hodson et al., 1980). It has been suggested that Pb may interfere with iodine metabolism in mammals, by adversel effecting the uptake of iodine by the thyroid and the conversion of iodine to protein-bound iodine (Sandstead, 1977).

Due to the complexity of the hypothalamus-pituitary-gonad-liver axis, with all of its feedback mechanisms and

chain-responses, it is very difficult to elucidate a mechanism for the toxic action of lead. The present study is the first to histologically observe negative impacts on the female fish reproductive system at low levels of inorganic lead. Exposure to lead in the environment may inhibit the release of GtH from the pituitary, lower estrogen and estradiol production, and alter liver Vg production, and hence affect ovarian growth. The results of the endogenous and early and late exogenous vitellogenesis experiments suggest that lead may affect early stage oocyte development, blocking oocytes in the endogenous phase, while allowing later stage oocytes to complete oogenesis. Lead may retard oocyte development in late vitellogenesis, and thus delay spawning readiness. It also may increase the number of atretic oocytes and decrease the total number of oocytes that complete oogenesis causing a significant reduction in fecundity. Further studies involving measurements of plasma estradiol, vitellogenin, Ca and GtH concentrations would provide more information regarding the mechanism of the toxic effects of lead. This could be accompanied by histological and biochemical studies of the liver. Because the Pb exposed fish oocytes appeared to reach maturation, the impact on fecundity could be investigated.

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Appendix 1: GSI and mean size of the five largest oocytes for the February/March 1991 early vitellogenesis experiment in which two-year old rainbow trout were exposed to 25 µg/L lead nitrate for 12 days.

Fish Number *	GSI	Average Size of Five Largest Oocytes (µm)
DAY 0 CONTROL:		
F 0 5	0.17	227.0
F 0 7	0.29	248.5
F 0 1	0.65	391.9
F 0 4	1.12	359.6
DAY 12 CONTROL:		
F 12 11	0.20	226.3
F 12 21	0.30	298.2
F 12 30	0.30	296.4
F 12 18	0.31	285.4
F 12 26	0.35	340.3
F 12 33	0.44	319.7
F 12 22	0.44	333.6
F 12 10	0.59	288.3
F 12 6	0.71	376.2
DAY 12 25 µg/L Pb:		
F 12 8	0.22	283.5
F 12 19	0.29	325.4
F 12 7	0.31	297.1
F 12 34	0.32	325.7
F 12 9	0.39	273.2
F 12 3	0.40	434.7
F 12 24	0.41	342.0
F 12 25	0.43	301.6
F 21 16	0.70	390.0

* Explanation of Sample Identification Code- e.g., F05: F=February/March experiment; 0=Day zero sampling; 5=fish number.

Appendix 2: GSI and mean size of the five largest oocytes for the April 1991 early vitellogenesis experiment in which two-year old rainbow trout were exposed to 10 μg/L lead nitrate for 12 days.

Fish Number	GSI	Oocyte Size (μm)	Oocyte Condition
DAY 0 CONTROL:			
*			
A 0 6	0.19	387.9	basophilic
A 0 9	0.31	329.1	basophilic
A 0 5	0.33	303.9	basophilic
A 0 2	0.45	259.7	basophilic
A 0 10	0.76	396.6	acidophilic
A 0 4	0.80	413.7	acidophilic
DAY 12 CONTROL:			
A 12 13	0.46	411.1	acidophilic
A 12 18	0.50	378.4	basophilic
A 12 29	0.54	396.7	basophilic
A 12 16	0.76	454.5	acidophilic
A 12 8	0.76	460.7	acidophilic
A 12 28	0.86	428.7	acidophilic
A 12 3	0.96	589.9	acidophilic
DAY 12 10 μg/L Pb:			
A 12 24	0.35	268.6	basophilic
A 12 12	0.35	337.7	basophilic
A 12 21	0.55	406.8	acidophilic
A 12 6	0.62	487.7	acidophilic
A 12 4	0.63	551.8	acidophilic
A 12 25	0.65	489.1	acidophilic
A 12 5	0.70	445.1	acidophilic
A 12 27	0.71	403.7	acidophilic
A 12 17	0.73	246.2	basophilic, atretic

* Explanation of Sample Identification Code- e.g., A06: A=April experiment; 0=Day zero sampling; 6=fish number.

Appendix 3: GSI and mean size of the five largest oocytes for the September 1990 late vitellogenesis experiment in which two-year old rainbow trout were exposed to 10 and 25 µg/L lead nitrate for 12 or 20 days.

Fish Number	GSI	Average Size of Five Largest Oocytes (µm)
DAY 0 CONTROL:		
*S 0 2	1.00	591.9
S 0 6	1.00	533.3
S 0 1	1.17	595.6
S 0 3	1.31	608.6
DAY 12 CONTROL:		
S 12 10	1.55	615.6
S 12 4	2.04	758.4
S 12 9	2.92	867.3
S 12 3	2.99	859.9
S 12 14	3.09	768.6
DAY 12 10 µg/L Pb:		
S 12 11	0.40	441.5
S 12 6	0.75	544.9
S 12 15	0.98	590.9
S 12 17	0.98	565.2
S 12 21	1.47	588.6
DAY 12 25 µg/L Pb:		
S 12 2	0.90	465.3
S 12 7	0.97	548.1
S 12 8	1.16	553.1
S 12 13	1.50	668.7
S 12 1	2.28	749.6
S 12 18	2.69	752.5
DAY 20 CONTROL:		
S 20 16	1.80	700.6
S 20 17	3.73	829.9
S 20 5	4.08	775.6
DAY 20 10 µg/L Pb:		
S 20 9	0.65	573.5
S 20 21	0.96	630.3
S 20 10	1.54	780.3
S 20 7	3.61	756.5
DAY 20 25 µg/L Pb:		
S 20 14	1.25	829.9
S 20 3	1.43	709.7
S 20 11	1.52	715.5
S 20 12	1.78	740.2

* Explanation of Sample Identification Code- e.g., S02: S=September experiment; 0=Day zero sampling; 2=fish number.

Appendix 4: Mean number of pituitary basophils in the February/March early vitellogenesis experiment in which two-year old female rainbow trout were exposed to 25 µg/L lead nitrate for 12 days.

FISH *	GSI	NO. BASOPHILS
Day 0 Control		
F 0 1	0.65	619
F 0 4	1.12	1131
F 0 5	0.17	12
F 0 7	0.29	20
Day 12 Control		
F 12 6	0.77	976
F 12 11	0.20	694
F 12 18	0.31	0
F 12 21	0.30	40
F 12 22	0.44	35
F 12 26	0.35	16
F 12 30	0.23	0
F 12 32	0.83	27
F 12 33	0.48	2363
Day 12 25 µg/L Lead		
F 12 3	0.40	25
F 12 7	0.31	21
F 12 8	0.22	213
F 12 19	0.29	0
F 12 24	0.41	0
F 12 25	0.49	703
F 12 34	0.32	992

* Explanation of Sample Identification Code- e.g., F01: F=February/March experiment; 0=Day zero sampling; 1=fish number.

Appendix 5: Mean number of pituitary basophils in the April early vitellogenesis experiment in which two-year old female rainbow trout were exposed to 10 µg/L lead nitrate for 12 days.

FISH	GSI	NO. BASOPHILS
Day 0 Control		
*		
A 0 2	0.45	908
A 0 4	0.80	2248
A 0 5	0.33	232
A 0 9	0.31	1031
A 0 10	0.76	1047
Day 12 Control		
A 12 8	0.75	500
A 12 13	0.46	528
A 12 16	0.76	467
Day 12 10 µg/L Lead		
A 12 5	0.68	767
A 12 12	0.35	388
A 12 21	0.55	747
A 12 25	0.65	226

* Explanation of Sample Identification Code- e.g., A02: A=April experiment; 0=Day zero sampling; 2=fish number.

Appendix 6: Number of granular pituitary basophils (September 1990) in two-year old female rainbow trout exposed to 10 or 25 µg/L lead nitrate for 12 or 20 days.

Fish	# Basophils	GSI	Oocyte Characteristics
Day 0 Control			
*S 0 1	49	1.17	- mostly large, fully
S 0 2	38	1.00	developed oocytes,
S 0 3	55	1.31	deeply eosinophilic,
S 0 6	247	1.00	shattered yolk
			- few early stages
Day 12 Control			
S 12 3	3641	2.99	- similar to Day 0,
S 12 4	1968	2.04	but oocytes are
S 12 10	735	1.55	larger
S 12 14	1300	3.09	
Day 12 10 µg/L Pb			
S 12 6	3028	0.75	- yolk shattered
S 12 11	665	0.40	- basophilic oocytes
S 12 15	599	0.98	- like Day 0
S 12 17	146	0.98	- like Day 0, early stages
S 12 21	1132	1.47	- like Day 0
Day 12 25 µg/L Pb			
S 12 1	3558	2.28	- like Day 12 control
S 12 2	2109	0.90	- yolk not shattered
S 12 7	1439	0.97	- like Day 0
S 12 8	2046	1.16	- like Day 0
S 12 13	1244	1.50	- much atresia
S 12 18	317	2.69	- like Day 12 control
S 12 20	2072	0.42	- basophilic oocytes, atresia
Day 20 Control			
S 20 5	2674	4.08	- all fully mature
S 20 16	1041	1.80	oocytes
S 20 17	1150	3.93	
Day 20 10 µg/L Pb			
S 20 7	7343	3.61	- like Day 20 control
S 20 8	343	0.14	- small, late stage
S 20 9	1355	0.65	- like Day 0
S 20 10	2006	1.54	- like Day 20 control
S 20 20	2665	6.56	- like Day 20 control
S 20 21	593	0.96	- more atresia
Day 20 25 µg/L Pb			
S 20 3	1716	1.43	- atresia
S 20 12	3894	1.78	- like Day 20 control

* Explanation of Sample Identification Code- e.g., S01: S=September experiment; 0=Day zero sampling; 1=fish number.