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The Effects of Sublethal Pentachlorophenol
Exposure on Reproduction in
Rainbow Trout (Salmo gairdneri).

James Joseph Nagler

A Thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada.

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ABSTRACT

The Effects of Sublethal Pentachlorophenol
Exposure on Reproduction in
Rainbow Trout (Salmo gairdneri).

James Joseph Nagler

Rainbow trout (Salmo gairdneri) were exposed to sublethal levels of pentachlorophenol (PCP) and the effects upon oocyte development in the ovary were assessed. A significant increase in atresia among the Stage 2 oocytes (Balbiani stage) was recorded after an 18-day exposure of maturing female fish to 22 and 49 ug PCP/L during July. The number of viable Stage 2 oocytes were reduced to 52% and 19% at 22 and 49 ug PCP/L respectively relative to the controls, thus significantly reducing the number of oocytes available to complete oogenesis. This study indicates that Stage 2 oocytes are highly sensitive to sublethal PCP during early summer.

The effect of sublethal PCP on exogenous yolk precursors (vitellogenin) formed in the liver was investigated in both mature naturally reproducing female rainbow trout and in a model system in which exogenous yolk was artificially induced in immature rainbow trout by estradiol (E2) injection. Changes in exogenous yolk were measured indirectly by total serum phosphoprotein phosphorus (SPP) and total serum calcium (SCa) or directly utilizing a

radioimmunoassay for vitellogenin (Vg).

A significant decrease in exogenous yolk was recorded in the serum of immature rainbow trout treated with 10 ug E2 after 19 days exposure to 25 ug PCP/L. This reduction occurred when levels of SPP and SCa were above 240 ug P/ml and 32 mg Ca/100 ml respectively. Mature naturally reproducing female fish exposed for 18 days to 25 ug PCP/L showed a similar trend to that observed in the model but levels of SPP and SCa in PCP exposed fish were not significantly different from the controls. In the model system, the weight of the fish and the E2 dose used significantly altered the response of the fish to the toxicant. Collectively results from the exogenous yolk experiments suggest that SPP and SCa are more critically affected by sublethal PCP exposure when levels of these exogenous yolk precursors are high in the serum than when they are low.

This study demonstrates for the first time a high correlation between the indirect indicators SPP and SCa with vitellogenin. Predictive regression equations were developed from the data which permitted estimation of vitellogenin levels from SPP and SCa in the range of 4 - 22 mg Vg/ml for E2 treated immature rainbow trout.

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INTRODUCTION

Pentachlorophenol belongs to the chlorophenol group of chemicals which are widely known and utilized extensively for a variety of biocidal purposes in North America. Canada's current annual consumption of pentachlorophenol (PCP) is estimated at 20,000 tonnes (NRCC, 1982), while in excess of 36,000 tonnes are used each year in the United States (Cirelli, 1978). The forest-products industry consumes more than 80% of all PCP as a wood preservative in wood treatment processes and as a slimeicide in pulp and paper mills (Hoos, 1978). It is now being tested as a component for drilling fluids in ocean petroleum exploration (Williams, 1982). Due to its general biocidal characteristics PCP is also incorporated into a wide range of domestic products where fungicide, insecticide or herbicidal qualities are required (Bevenue and Beckman, 1967).

Pentachlorophenol enters the aquatic ecosystem primarily through effluent discharges of the user industries. It has a relatively short residence time in the water, 24 hours, where it is photolysed, but may remain in bottom sediments bound to organic matter for periods up to one year (Pierce et al., 1977). In biological tissue PCP is a persistent chemical since it contains five chlorine atoms on a phenol ring. A log octanol/water partition coefficient of 5.01 (Williams, 1982) makes PCP very lipophilic and it has

been found to bioaccumulate in aquatic organisms and may biomagnify through the food chain (NRCC, 1982). The chlorophenols, especially the commercial formulations, are frequently contaminated with up to 5% polychlorinated dibenzodioxins and dibenzofurans (Nilsson et al., 1978 and Williams, 1982). These compounds, particularly the tetrachlorodibenzodioxins are among the most toxic chemicals known. Based on the wide use of PCP it may represent a major source of dioxin input into the environment (NRCC, 1982).

As an aquatic pollutant PCP is very toxic to fish at low levels and fish kills due to accidental PCP spills have been reported (Pierce et al., 1977). The reported acute lethal 96 - hour LC50 varies between the salmonids at 60 ug PCP/L and the guppy (Lebistes reticulatus) at 2 mg PCP/L (NRCC, 1982). At lethal concentrations the pesticide inhibits oxidative phosphorylation in the mitochondria. Pentachlorophenol tends to bind very strongly to mitochondrial protein and mitochondria will accumulate this chemical and concentrate it by a factor of 150 (Weinbach and Garbus, 1965). Studies have determined that one mole of PCP uncouples one functional ATP production site containing four moles of ATP (NRCC, 1982). In effect it stops energy supply to the cell. Death rapidly results when toxic levels are reached in life supporting tissues after sufficient poison has accumulated.

The sublethal toxicity of PCP has been examined in

teleost fish and detrimental effects on bioenergetics (Krueger et al., 1968), metabolism (Holmberg et al., 1972, Bostrom and Johansson, 1972, Chapman and Shumway, 1978, and Dalela et al., 1980), histological changes in the liver (Owen and Rosso, 1981), food conversion efficiency (Webb and Brett, 1973) and growth (Crandall and Goodnight, 1962, Chapman, 1969, Hodson and Blunt, 1981, Holcombe et al., 1982 and Dominguez and Chapman, 1984) have been recorded. The toxicology of sublethal PCP on reproduction is restricted to effects on early life history stages. Hodson and Blunt (1981) found eggs of rainbow trout (Salmo gairdneri) chronically exposed to PCP from fertilization to hatch to have elevated mortality and reduced weight at hatch. Continued exposure after hatch significantly increased mortality, reduced growth rates and yolk sac reabsorption efficiency. The estimated threshold concentration of 16 ug PCP/L produced the overall effect of a large reduction in biomass of the total population up to the fourth week of exogenous feeding. In a similar study using steelhead trout Dominguez and Chapman (1984) determined thresholds of 34 ug PCP/L for embryo mortality and 19 ug PCP/L for yolk sac larvae mortality and growth. Exposure of early life history stages of rainbow trout to sublethal PCP concentrations as low as 16 ug PCP/L could reduce the number of viable fish maturing. There is no information on the sublethal effects of PCP involving reproduction in maturing or adult rainbow trout.

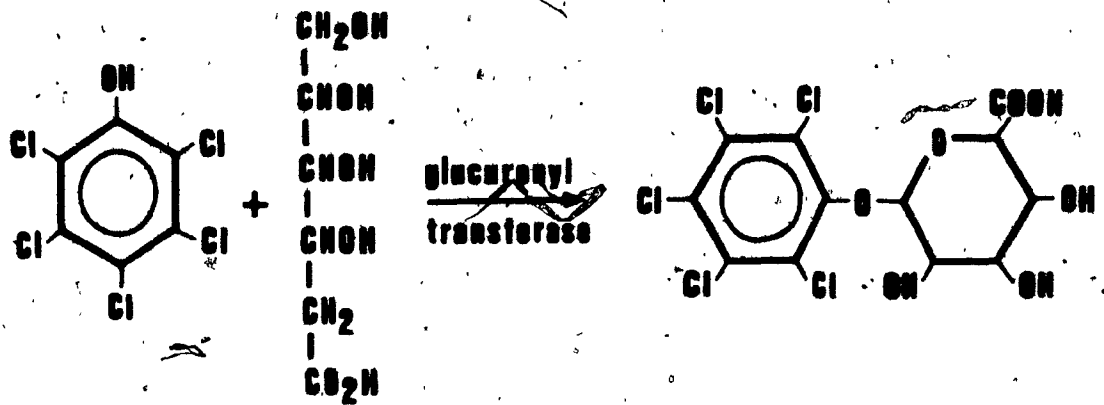
Rainbow trout will concentrate waterborne PCP at very low levels (ng/L) (Niimi and McFadden, 1982). Fish exposed to sublethal PCP rapidly absorb it and most body tissues reach steady state levels within 24 hours (Kobayashi, 1978). The mode of action at sublethal levels is uncoupling of oxidative phosphorylation (Weinbach, 1956) producing low concentrations of ATP and elevated levels of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Stockdale and Selwyn, 1971). This uncoupling prevents the capture of the free energy released in electron transport and cellular requirements for oxygen increase. A state of hypermetabolism occurs and lipid reserves are oxidized to maintain energy levels.

At sublethal levels PCP can be metabolized by rainbow trout primarily in the liver (Glickman et al., 1977 and Lech et al., 1978). The main metabolite is pentachlorophenyl-beta-glucuronide (Figure 1) which is accumulated in the gall bladder. This glucuronic acid conjugate, a result of Phase 2 biotransformation, is an organic anion and is actively concentrated by the biliary anionic transport system. The biliary route is used for elimination of this conjugated PCP form. A secondary metabolite is free-PCP which is present in the blood at low levels, and is effectively removed across the gill membrane (Kobayashi, 1978).

Rainbow trout generally mature at two years of age although some females do not breed until their third year

Figure 1 : The formation of the primary metabolite pentachlorophenyl - beta - glucuronide by Phase 2 biotransformation during pentachlorophenol metabolism in rainbow trout (Kobayashi, 1978).

Glucuronic acid



Pentachlorophenol

Pentachlorophenyl -
beta - glucuronide

FIGURE 1

(Scott and Sumpter, 1983a). Once mature they have an annual reproductive rhythm synchronized by environmental factors chiefly photoperiod, temperature and food availability (de Vlaming, 1974). Actively reproducing mature females can be classed throughout the year into four physiological stages 1) previtellogenesis 2) endogenous vitellogenesis 3) exogenous vitellogenesis and 4) ovulation and spawning (van Bohemen et al., 1981). They are seasonal breeders that may spawn three or more consecutive years.

The period of exogenous vitellogenesis entails the ovarian accumulation of yolk protein derived from the liver. Estrogens, of which estradiol is the most potent (Sundararaj and Nath, 1981 and van Bohemen et al., 1982), are released from the ovarian follicles and induce the hepatic synthesis of a calcium binding glycolipophosphoprotein (Emmersen and Petersen, 1976 and Hori et al., 1979). This female specific plasma protein called vitellogenin is rapidly produced and released into the circulation in large amounts (Sundararaj and Nath, 1981) where it is actively sequestered by micropinocytotic activity at the oocyte surface (Droller and Roth, 1966). Vitellogenin is incorporated into the developing oocyte as the yolk proteins lipovitellin and phosvitin (Ng and Idler, 1983). The process of vitellogenesis is a universal phenomenon in nonmammalian vertebrates and is well established in birds and amphibians (Follet and Redshaw, 1974). Although the contribution of

vitellogenin to the overall quantity of ovarian yolk has not been measured in teleost fish, studies on the toad (Xenopus laevis) have indicated greater than 99% of yolk protein is derived from vitellogenin (Wallace and Bergink, 1974).

Exogenous yolk production can be induced in immature (Elliott et al., 1979), male (Emmersen et al., 1979) and non-vitellogenic female teleost fish (Bailey, 1957) by estradiol treatment. Characteristically the liver weight (van Bohemen et al., 1982), plasma calcium and phosphoprotein phosphorus all increase (Elliott et al., 1979) and vitellogenin appears in the blood within days (Idler and Campbell, 1980). Plasma phosphoprotein phosphorus and calcium are often used as indirect indicators of vitellogenin in experimentally or naturally induced fish (Emmersen and Petersen, 1976, Whitehead et al., 1978, Elliott et al., 1979 and Tinsley, 1985). The vitellogenin induced by E2 in non-vitellogenic females is not removed from the blood by the ovary presumably due to the lack of proper endocrine priming and elevated plasma levels will persist in both sexes for weeks (Sundararaj and Nath, 1981 and Hori et al., 1979).

The development of the female gametes or oogenesis is a process of differentiation and growth of oogonia to mature oocytes (Nagahama, 1983). The various oocyte stages in the rainbow trout have been classified on the basis of morphological and/or functional criteria seen at the light

microscope level (Yamamoto et al., 1965, Beams and Kessel, 1973, Lesniak, 1977 and van den Hurk and Peute, 1979). Oogenesis initially starts with a primary growth phase involving oogonial proliferation into early stage oocytes up to the point of yolk deposition. A cytoplasmic feature of these early stage oocytes is the Balbiani body (Beams and Kessel, 1973). The secondary oocyte growth phase where large amounts of yolk is accumulated in the ooplasm finishes with maturation of the eggs which are then ready for spawning.

Atresia or ovarian follicular degeneration can occur at any oocyte stage during oogenesis. It is found as pre-ovulatory or post-ovulatory forms with or without phagocytic involvement (Lesniak, 1977). During pre-ovulatory atresia the follicular cells usually hypertrophy, absorb the oocyte contents and collapse into an irregular mass (Khoo, 1975). Although this is a very common feature of the teleost ovary (Ball, 1960) it may be enhanced by environmental stress (de Vlaming, 1983) or pollutants (Lesniak and Ruby, 1982).

The primary objectives of this research were 1) to examine the effect of sublethal PCP exposure on exogenous vitellogenesis in rainbow trout and 2) to determine any effects of sublethal PCP on oogenesis in maturing female rainbow trout.

To study the consequences of sublethal PCP exposure on exogenous vitellogenesis two approaches were taken. 1) An experimental model was used whereby immature rainbow trout

are treated with E2 to artificially induce vitellogenin production. The effect of sublethal PCP on exogenous yolk production was measured using the levels of total serum phosphoprotein phosphorus and total serum calcium. The factors of E2 dose and fish weight were considered as modifiers of SPP, SCa and hepatosomatic index levels in E2 treated immature rainbow trout. The validity of using SPP and SCa as indirect indicators of vitellogenin was also examined. 2) Then, mature naturally vitellogenic female rainbow trout were exposed to sublethal PCP. This was used to determine effects on the naturally induced levels of SPP and SCa and serve as a comparison to the results obtained from the experimental yolk induction model.

The second objective of this study was to examine the histological effects of sublethal PCP on oogenesis in female rainbow trout maturing for the first time. Fish were chronically exposed at two different times of the year to sublethal PCP. The quantitative effects involving oocyte atresia and oocyte growth were recorded.

MATERIALS AND METHODS

1. MATERIALS

i) Test Organisms

Immature (15 - 120g) and mature (400 - 600g) rainbow trout (Salmo gairdneri Richardson) were obtained from La Pisciculture Mont Sutton, RR#1 Sutton, Québec. They were received in aerated pond water at ambient temperature and transferred to the holding facilities of the Water Pollution Research Laboratory, Concordia University. The immature fish were placed in 175 litre (L) and the mature fish in 620 L rectangular fibreglass holding tanks. The holding tanks were supplied with a continuous flow of dechlorinated City of Montreal water (activated charcoal filter) at 12 +/- 1°C providing > 99% replacement in four hours (Sprague, 1973). Each tank was supplied with compressed air aeration and covered with a fibreglass lid. A photoperiod of 12-hour light : 12-hour dark was controlled by an automatic timer clock. The fish, depending on their length, were fed an appropriate size dry pelleted trout food (Table 1) ad libitum every morning. The fish remained in the holding facilities for at least one month before experimental use. During the holding period mortality was always < 1%.

Table 1 : The analysis of dry pelleted trout food.

Grower Pellets For Salmonids

Martin's 83G

Martin's Feed Mills Ltd.

Elmira, Ontario.

Crude protein (min.)	-	40.0 %
Crude fat (min.)	-	10.0 %
Crude fibre (max.)	-	3.0 %
Vitamin A (min.)	-	7500 i.u./kg
Vitamin D3 (min.)	-	3000 i.u./kg
Vitamin E (min.)	-	100 i.u./kg
Ascorbic Acid (min.)	-	800 mg/kg

ii) Experimental Apparatus

The test apparatus consisted of a series of four translucent 350 L rectangular polyethylene tanks painted black on the outside, with a cover of similar material. The laboratory water entered the test tanks from a common aerated head tank via PVC pipe through graduated flowmeters (Manostat Corp., New York) delivering 2 L/minute (90% replacement every six hours). The water left the test tanks through a standpipe at the end opposite water entry.

The physico-chemical characteristics of the laboratory water during the experimental periods is reported in Table 2. Water temperature was recorded daily in the head tank with a temperature probe (Yellow Springs Instrument Co., Ohio) and/or mercury thermometer. Dissolved oxygen was measured with a D.O. probe (Yellow Springs Instrument Co., Ohio) and pH with a combination glass calomel electrode (Canlab) in the head tank every four days throughout the experimental periods. To prevent photodegradation, pentachlorophenol (PCP) solutions were siphoned out of light shrouded Mariotte bottles (Leduc, 1966) using \emptyset .58 mm Intramedic polyethylene tubing (Clay Adams), mixing directly with the laboratory water entering the test tanks. Lighting was provided by overhead 40-watt fluorescent tubes (warm white, Westinghouse) controlled by an automatic timer clock to create a 12-hour light : 12-hour dark photoperiod. The

Experimental Periods	Total Hardness (mg/L as CaCO ₃) ^a	Alkalinity (mg/L as CaCO ₃) ^a	pH (\bar{X} +/- SD)	Temperature (° C) (\bar{X} +/- SD)	Dissolved Oxygen (mg/L) (\bar{X} +/- SD)
1. June, 1983	126	84	7.77 +/- 0.05	12.0 +/- 1.0	10.8 +/- 0.2
2. December, 1983	125	83	7.50 +/- 0.06	12.5 +/- 0.3	10.7 +/- 0.2
3. March, 1984	129	88	7.65 +/- 0.10	12.5 +/- 0.3	10.7 +/- 0.2
4. June, 1984	126	84	7.27 +/- 0.02	12.0 +/- 0.7	10.4 +/- 0.3
5. July, 1984	125	84	7.46 +/- 0.09	12.8 +/- 0.8	10.2 +/- 0.3
6. December, 1984	128	85	7.49 +/- 0.04	11.8 +/- 0.2	10.8 +/- 0.2

a - monthly means provided by the City of Montreal, Public Works Service, Waterworks Division.

Table 2 : Physico - chemical characteristics of laboratory water during the experimental periods.

whole apparatus was shielded from external disturbances by a dark black plastic curtain.

2. METHODS

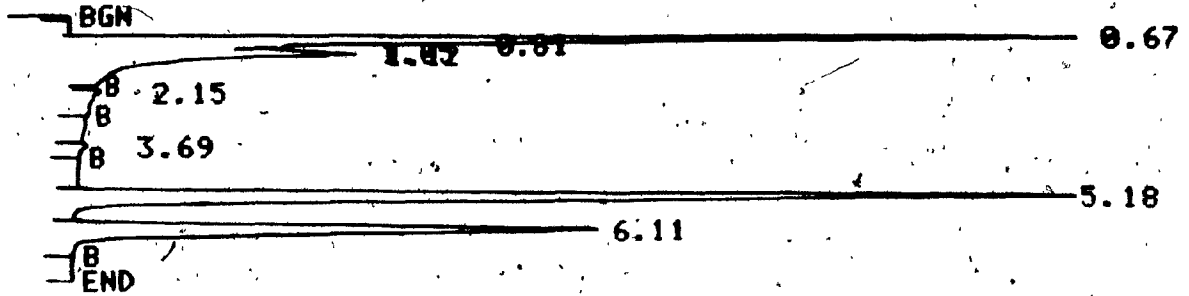
In all experiments the fish were randomized into the test tanks to acclimate at least two weeks before experimentation. A twenty fish subsample was weighed at the beginning of the acclimation period to determine the feeding ration per tank. Throughout the experiments the fish were fed a size specific dry pelleted trout food (Table 1) at 2.0% wet body weight every morning. The fish were not fed the day prior to any sampling and the ration adjusted per tank after fish were removed. The test tanks were cleaned with a siphon once each week.

i) Toxicant Preparation and Measurement

The toxicant employed was pentachlorophenol (99+% PCP, Sigma) which was recrystallized once in pure methanol (Fisher, pesticide residue grade) to remove impurities. A chromatogram of the purified PCP is shown in Figure 2. The concentrations of chlorinated dibenzofurans and dibenzodioxins found as contaminants in the PCP after purification is given in Table 3 (Nova Labs, Montreal).

The PCP test solutions were prepared by dissolving

Figure 2 : Chromatogram of purified pentachlorophenol used in toxicology experiments. The lindane (hexachlorobenzene) added to the solvent hexane was used in the extraction procedure as an internal standard.



TIME	AREA	NAME
0.67	8.2809	HEXANE
1.05	0.2453	
2.15	0.1172	
3.69	0.1133	
5.18	24.9177	LINDANE
6.11	14.5235	PCP ACETATE

FIGURE 2

weighed amounts of PCP in sodium hydroxide (NaOH) (1.0 ml 0.36 M NaOH/5 mg PCP) which was made up to 18 L with distilled water in glass Mariotte bottles. The concentrations thus produced were diluted with laboratory water (2 L/minute) before entering the test tanks to provide nominal levels of 12.5, 25.0 or 50.0 ug PCP/L. The nominal and measured PCP concentrations in the test tanks during the experimental periods are given in Table 4.

The concentration of PCP in the test tank water was monitored every two days during the exposure periods. The water concentration of PCP was determined following the procedure of Chau and Coburg (1974) with some modifications. Basically PCP in a sample of test tank water is converted to PCP-acetate by the addition of acetic anhydride in the presence of a basic medium and extracted into hexane. The hexane extract is separated from the water and analyzed by electron capture gas chromatography. Test tank water samples (200 ml) were collected in glass stoppered Erlenmeyer flasks (previously washed with hot water and IsocleanTM (Mandel Scientific), rinsed with acetone and dried). To each flask the following reagents were added in succession : 1) 1.38 g potassium carbonate (Fisher, reagent grade). 2) 1 ml acetic anhydride (Fisher, reagent grade) which was redistilled three times beforehand in an all glass system until the reagent blank showed no impurities on the gas chromatograph (GC). 3) 10 ml hexane (Fisher, pesticide residue grade) that contained

Table 3 : The concentrations of chlorinated dibenzofurans and dibenzodioxins in purified pentachlorophenol used in toxicology experiments.

<u>Compound</u>	<u>Concentration</u> (ug/gm PCP)
Pentachlorodibenzofurans	0.014
Pentachlorodibenzodioxins	N.D.
Hexachlorodibenzofurans	0.20
Hexachlorodibenzodioxins	N.D.
Heptachlorodibenzofurans	0.72
Heptachlorodibenzodioxins	2.02
Octachlorodibenzofuran	0.68
Octachlorodibenzodioxin	4.72
Total	8.36 ug

Experimental Periods	Duration of PCP Exposure (days)	Tank #	Nominal PCP Concentration (ug PCP/L)	Measured PCP Concentration (ug PCP/L) (X +/- SD)
1. December, 1983	18	1	0.0 (control)	0.03 +/- 0.06
		2	12.5	18.2 +/- 9.3
		3	25.0	23.8 +/- 5.5
		4	50.0	51.0 +/- 11.6
2. March, 1984	19	1	0.0 (control)	0.35 +/- 0.94
		2	0.0 (control)	0.22 +/- 0.60
		3	25.0	25.1 +/- 1.8
		4	25.0	25.8 +/- 4.3
3. July, 1984	18	1	0.0 (control)	0.04 +/- 0.05
		2	12.5	11.5 +/- 0.3
		3	25.0	21.8 +/- 1.9
		4	50.0	48.7 +/- 8.7
4. December, 1984	18	1	0.0 (control)	0.10 +/- 0.10
		2	0.0 (control)	0.15 +/- 0.21
		3	25.0	28.0 +/- 6.4
		4	25.0	22.1 +/- 5.7

Table 4 : Nominal and measured pentachlorophenol concentrations in test tanks during the experimental periods.

0.5 mg/L hexachlorobenzene used as an internal standard with which to measure extraction losses. A teflon stirring bar was added to the mixture and it was stirred at moderate speed for at least one hour. The hexane layer was separated and 2.0 ul quantities injected into a Perkin-Elmer Model Sigma 4B gas chromatograph equipped with a 63 Ni electron capture detector. The GC used a 6' x 1/4" o.d. glass column packed with 3.6% OV-101, 5% OV-210 on 80-100 mesh Chromosorb Q, acid washed and DMCS treated. The operating conditions were injector 300°C, column 210°C, detector 300°C with a carrier gas of nitrogen or P5 at 60 ml/minute. Data was collected on a Perkin-Elmer Sigma 10 integrator interfaced with the GC.

The unknowns were compared with equal volumes of PCP-acetate standard dissolved in hexane. This standard was prepared by dissolving 10 g (0.04 mole) of purified PCP in 25 ml pyridine (Fisher, reagent), adding 100 g (0.28 mole) acetic anhydride and the mixture maintained at 100°C in an oil bath for 45 minutes. The product was cooled, diluted with 200 ml distilled water and extracted three times with 100 ml portions of benzene (Fisher, pesticide residue grade). The combined benzene extracts were washed with 200 ml 5% (w/v) NaOH and dried over anhydrous sodium sulfate (Fisher, reagent). The benzene extract was evaporated to get solid PCP-acetate which was recrystallized three times from hexane to obtain pure white crystals (m.p. 150-152°C) that gave a

single peak in the GC.

ii) Estradiol Treatment

The experiments involving estradiol (E2) treatment of immature rainbow trout utilized crystalline beta-estradiol-3-benzoate (Sigma). The E2 was dissolved and suspended in peanut oil (Sigma) by mechanical stirring for at least 24 hours prior to use. Previous studies indicate that the peanut oil carrier has no effect on the parameters under study (Cyr, 1984). The fish chosen for E2 treatment were removed and placed in 300 L tanks provided with a continuous flow of laboratory water in a room equipped with red fluorescent lighting (40-watt, Sylvania) to minimize stress.

The fish were individually anaesthetized in a separate container with 0.4 mg/L 2-phenoxyethanol (Sigma), blotted dry and weighed to determine the appropriate E2 dosage. They were given a 50 ul intraperitoneal (i.p.) injection of 2.5 or 5.0 ug E2/g wet body weight on the midline of the belly behind the pectoral fins. A 50 ul glass syringe (Hamilton) with a disposable 26G 13mm Yale needle (Becton-Dickinson) was slowly inserted on an angle toward the posterior of the fish to deliver the E2 solution. The E2 treated fish were immediately placed in a recovery tank for 2 hours before being returned to the test tanks.

iii) Tissue Sampling

On the morning of specific sampling days five immature fish were removed from each tank, killed by a blow to the head, blotted dry and weighed to the nearest 0.01 g. The mature fish were handled identically except that they were anaesthetized (0.4 mg/L 2-phenoxyethanol) upon removal from the test tanks. Blood was collected in sodium citrate (25% w/v, Fisher) rinsed 1 cc tuberculine syringes (Becton-Dickinson) following caudal severance. The whole blood was placed in 1.5 ml plastic micro-centrifuge tubes (Sarstedt) on crushed ice for no longer than three hours. The clotted blood was subsequently centrifuged at 12,000 g for 3 minutes, the serum decanted into clean 1.5 ml plastic micro-centrifuge tubes, sealed and quickly frozen in liquid nitrogen. The tubes were stored at -80 °C until required for serum analysis.

The fish were opened mid-ventrally and the liver (gall bladder excised) and the gonads removed and weighed to the nearest 0.01 g. The liver weight was used with the body weight in determining the hepatosomatic index (HSI). The ovaries of the female fish were preserved with Bouin's fluid in glass vials for histological study.

Calculation of hepatosomatic index (HSI)

$$\text{HSI} = \frac{\text{Wet weight of the liver}}{\text{Wet body weight}} \times 100$$

iv) Serum Analysis

Serum samples were assayed for total serum phosphoprotein phosphorus, total serum calcium and in one experiment vitellogenin.

The total serum phosphoprotein phosphorus concentration was determined using 50 ul or 100 ul of freshly thawed serum in a 15 ml glass centrifuge tube to which 5 ml of cold 10% (w/v) trichloroacetic acid (Sigma) was added. The protein fraction was precipitated and the solution spun for 15 minutes in a desk top clinical centrifuge (Damon, IEC) at top speed. The supernatant was discarded and the resulting protein pellet delipidated with successive organic solvents according to the method of Wallace and Jared (1968). The pellet remaining was analysed for total phosphorus content (ug P/ml serum) using the wet-ashing spectrophotometric procedure outlined in Boehringer-Mannheim Corporation (1969).

The analysis of total serum calcium consisted of 50 ul of freshly thawed serum diluted to 5.0 ml with 2000 mg/L potassium chloride (Fisher). Total calcium concentration (mg

Ca/100 ml serum) was determined by flame atomic absorption using a Perkin-Elmer Flame Atomic Absorption Model 503 following Bhattacharya (1977).

The vitellogenin analysis (mg Vg/ml serum) was performed at the Marine Sciences Research Laboratory, Memorial University of Newfoundland, St. John's, Newfoundland using a homologous radioimmunoassay (RIA) system for rainbow trout (So et al., 1985).

v) Histological Preparation and Analysis

Mid-ovarian portions of the Bouin's fixed ovaries from female fish were chosen as they have been shown to provide a homogeneous frequency of the oocyte stages present (Lesniak, 1977). The tissues were washed three times in 70% ethanol to remove the fixative. They were subsequently dehydrated (Tissuematon, Dual Unit, Fisher) for two hours in each of a gradient ethanol series (70, 80, 90, 95, 100%), cleared for two hours in 1:1 100% ethanol:toluene and 100% toluene and infiltrated for two hours in paraffin (Tissue Prep., m.p. 56 +/- 0.5°C, Fisher). The individual tissues were oriented and embedded in paraffin cassettes on a Tissue Tek II Embedding Center (Lab Tek, Model 4603, Fisher). The tissue blocks were cut in cross-section at 24 serial 10 micron (um) sections using a rotary microtome (Spencer AO '820' Model). The sections were stained Gill's #2 formulation hematoxylin

(Fisher) and counterstained with eosin Y (Baker) before mounting with Permount resin (Fisher).

A binocular Leitz orthoplan microscope (Leitz-Wetzlar), fitted with an ocular micrometer (10 mm) and grid (7 mm sq.), was used for histological examination. A camera (Leica) using Panatomic X print film (32 ASA, Kodak) was attached to the microscope for photography.

The histological analysis consisted of quantitative measurement of oocyte atresia and oocyte diameter in mid-ovarian sections of control and PCP-exposed female rainbow trout.

The different oocyte stages present in the maturing female rainbow trout have been classified from Stage 0 - 4 (Appendix A), and this classification scheme was followed in identifying oocyte stages found. Stage 2 oocytes were selected and recorded as being viable or atretic. Counts were conducted on every third serial section from 1 to 21 using an ocular grid. The grid was moved randomly over the section for counting by alternately throwing the microscope stage out of focus, moving the stage and bringing the section back into view (Chalkley, 1943). All Stage 2 oocytes with a visible nucleus falling by more than 1/2 within the grid were recorded and the count terminated when 20 viable oocytes had been scored. In each treatment group the number of atretic Stage 2 oocytes were compared to viable Stage 2 oocytes and presented as a percent of the total.

The ellipsoid nature of trout oocytes necessitates the measurement of both the major and minor axes (Lesniak, 1977). The maximum and minimum diameters of the ten largest viable oocytes in which the nucleus was visible on mid-ovarian cross-sections were recorded with an ocular micrometer from each fish in control and PCP exposed groups. The diameter values were standardized by using the square root of the product of the maximum diameter multiplied by the minimum diameter (Braekevelt and McMillan, 1967). The diameter values calculated were used in comparison of oocyte size between treatment groups over the 18-day exposure periods.

vi) Statistical Analysis

All the data involving total serum phosphoprotein phosphorus, total serum calcium, serum vitellogenin and hepatosomatic index values from individuals of each test group for specific sampling days were calculated together as a mean (\bar{X}) +/- standard error of the mean (SEM). When differences between groups were compared all values were log-transformed ($X = \log(X+1)$). The histological data pertaining to oocyte diameter values and Stage 2 oocyte atresia levels were calculated as a mean +/- 95% confidence limits. To test for differences between groups, Student's t-test or Analysis of Variance (ANOVA) (1-Factor or 2-Factor) at the 95% level of significance was performed.

When statistical differences occurred with ANOVA the Student Newman-Keuls (SNK) test for multiple comparisons was used ($p < 0.05$). Simple linear correlations were determined between log-transformed values of total serum phosphoprotein phosphorus, total serum calcium and serum vitellogenin. The linear functions of serum vitellogenin with total serum phosphoprotein phosphorus and total serum calcium were calculated with log-transformed values by simple linear regression. All computations were performed with the Statistical Program packages available for the Apple II e computer (Apple Personal Computers).

RESULTS

The first experiment was performed to examine the effect of sublethal PCP exposure (25 ug PCP/L) on the levels of total serum phosphoprotein phosphorus (SPP) and total serum calcium (SCa) in immature rainbow trout treated with 10 ug E2, (5 ug E2/g body wt. on Days 0 and 3). It was observed, following exposure of fish to 25 ug PCP/L for 19 days, that there was a significant decline in both mean SPP and mean SCa levels in the PCP exposed E2 treated group (Figure 3). The mean SPP and SCa levels attained maximums of 245.4 ug P/ml and 32.9 mg Ca/100 ml respectively on Day 17 and then decreased significantly ($p < 0.05$). By Day 24 in the PCP exposed E2 treated group the mean levels of SPP were 192.3 ug P/ml and SCa 31.4 mg Ca/100 ml, relative to the E2 treated control group where the mean SPP and SCa levels were still increasing at 372.1 ug P/ml and 49.6 mg Ca/100 ml respectively.

The effect of injecting three different doses of E2, 2.5, 5 and 10 ug (2.5 and 5 ug E2/g body wt.) on the levels of SPP, SCa and the HSI in immature rainbow trout was tested. As the dose of E2 increased, so did the magnitude of the mean SPP and SCa levels (Figure 4 and 5). The lowest dose of 2.5 ug E2 gave maximum levels of SPP, 49.8 ug P/ml, on Day 7 (Figure 4A) and SCa, 14.1 mg Ca/100 ml, on Day 17 (Figure 5A). In the E2 treated group receiving the

Figure 3 : The effect of sublethal pentachlorophenol exposure (25 ug PCP/L) on the levels of total serum phosphoprotein phosphorus and total serum calcium in 10 ug E2 treated immature rainbow trout (mean wt. = 88.0 g) during March, 1984. Each point represents a mean +/- SEM for 5 fish. (* - significantly different from E2 treated control group with Student's t-test at $p < 0.05$).

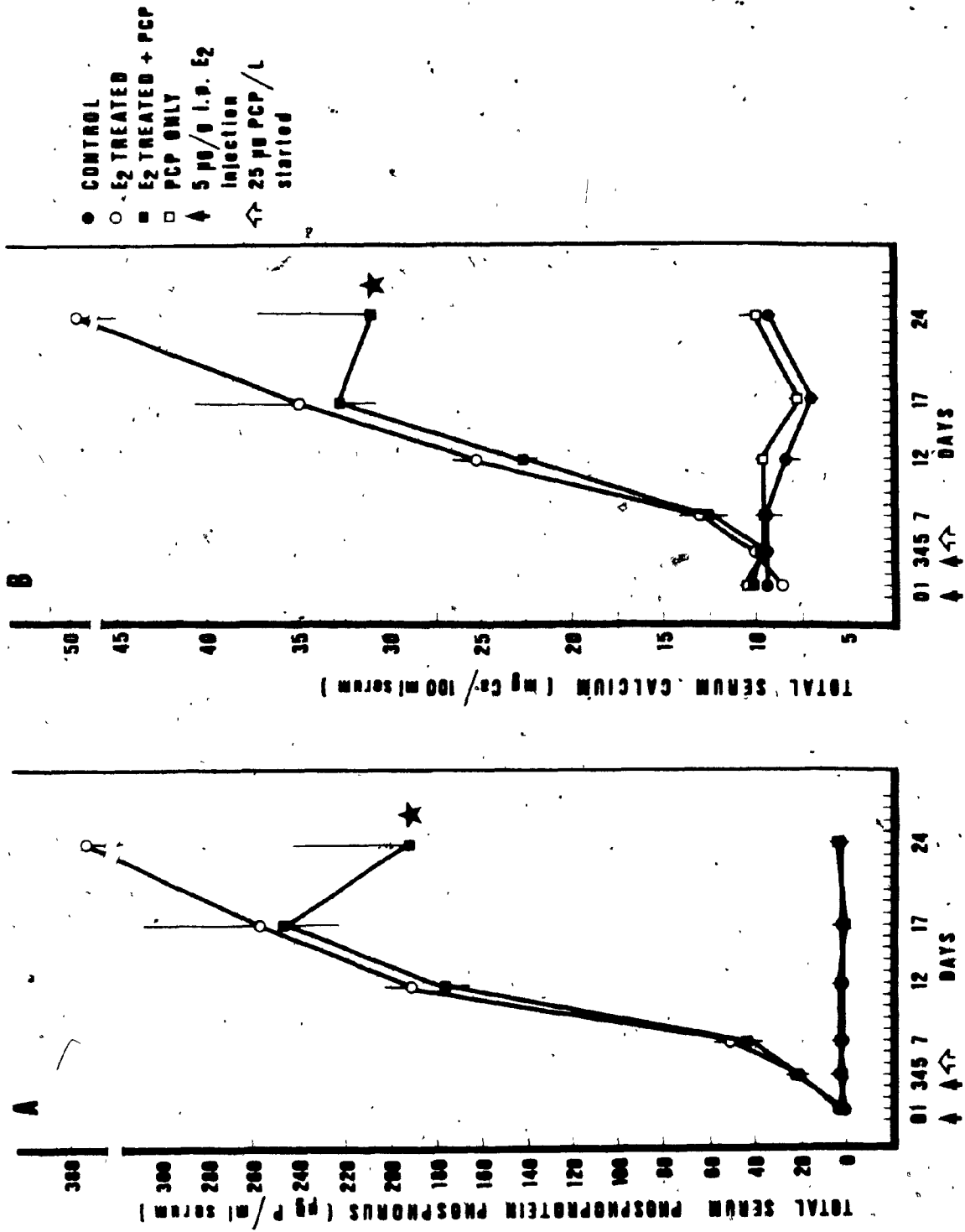


FIGURE 3

Figure 4 : The effect of 2.5, 5 and 10 ug E2 on the levels of total serum phosphoprotein phosphorus in immature rainbow trout (mean wt. = 66.7 g) during June, 1984. Each point represents a mean +/- SEM for 5 fish.

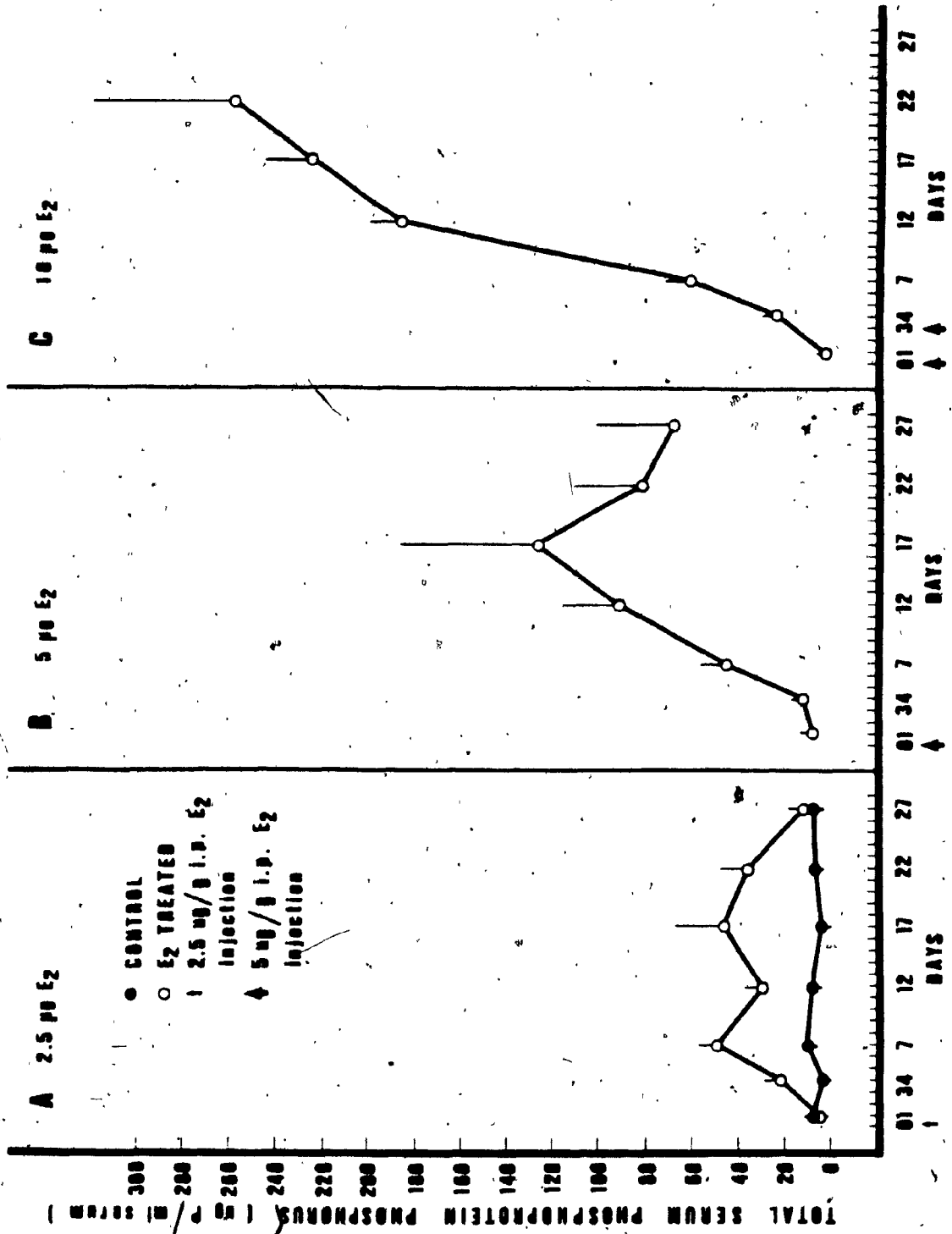


FIGURE 4

Figure 5 : The effect of 2.5, 5 and 10 E2 on the levels of total serum calcium in immature rainbow trout (mean wt. = 66.7 g) during June, 1984. Each point represents a mean \pm SEM for 5 fish.

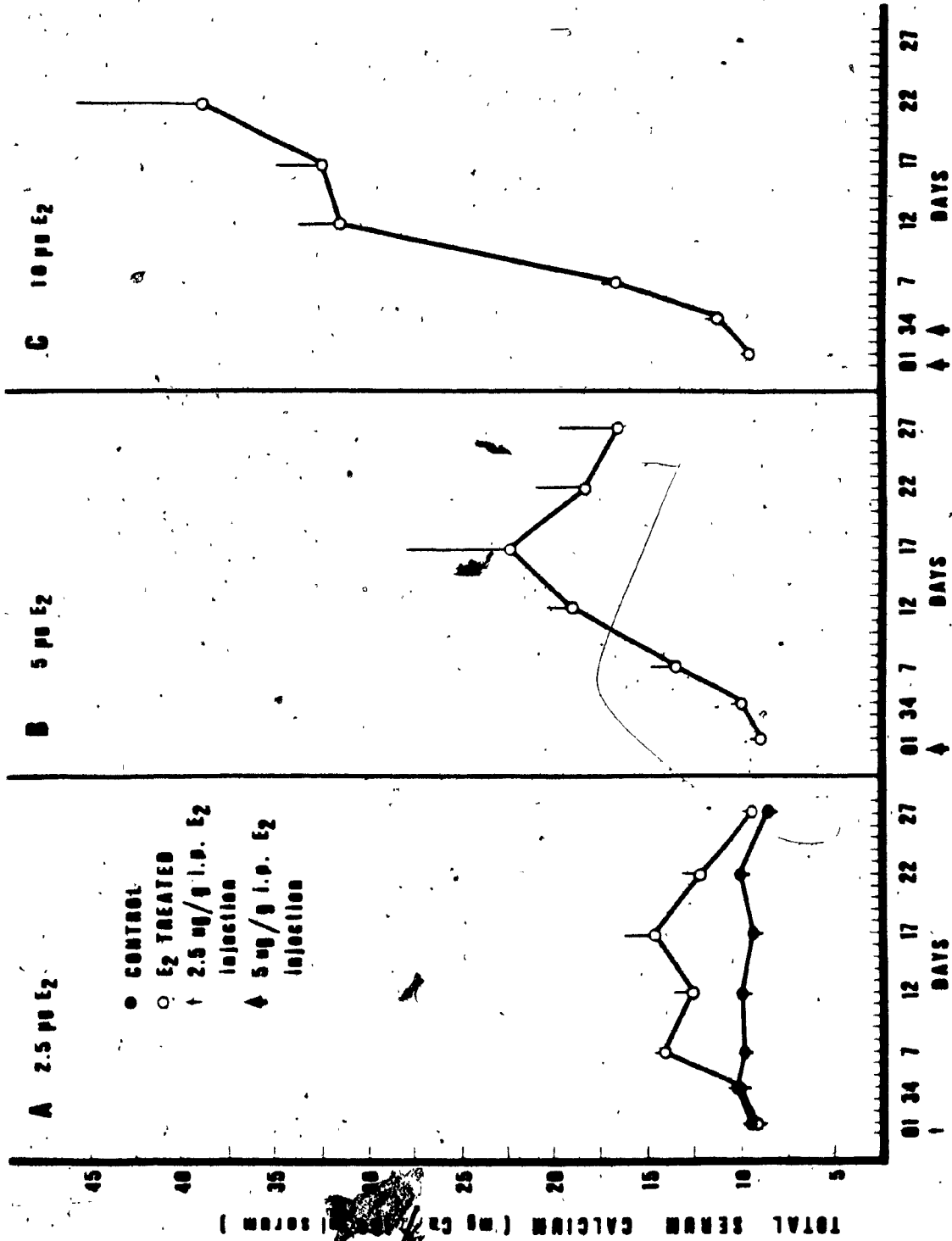


FIGURE 5

intermediate dose of 5 ug E2 mean SPP, 129.6 ug P/ml and SCA, 22.5 mg Ca/100 ml, peaked on Day 17 (Figure 4B and 5B). The highest mean levels of SPP and SCA, 258.7 ug P/ml, and 38.6 mg Ca/100 ml respectively were recorded on Day 22 in the 10 ug E2 treated group (Figure 4C and 5C). The magnitude of the mean HSI levels increased as the E2 dose increased (Figure 6), similar to that observed for SPP and SCA. At the lowest E2 dose, 2.5 ug E2, the mean HSI level, 1.7, peaked on Day 4 (Figure 6A). The mean HSI peaked later at 1.8 on Day 7 at the E2 dose of 5 ug E2 (Figure 6B). The highest mean HSI value 2.1 was found on Day 12 in the 10 ug E2 treated group (Figure 6C).

A further study separated immature rainbow trout into two weight classes 18.9 g (wt. range 15-25 g) and 66.7 g (wt. range 55-80 g) and treated them with an identical dose of 10 ug E2, (5 ug E2/g body wt. on Days 0 and 3) to determine the effect weight has on the levels of SPP, SCA and the HSI. The SPP and SCA levels peaked earlier and at lower mean values in the lighter E2 treated fish compared to the heavier E2 treated individuals (Figure 7 and 8). The mean SPP and SCA levels in fish from the smaller E2 treated fish peaked on Day 12 at 179.8 ug P/ml and 29.4 mg Ca/100 ml respectively (Figure 7A and 8A). In the heavier E2 treated group the mean levels of SPP, 258.7 ug P/ml and SCA, 38.6 mg Ca/100 ml, were still increasing on Day 22 (Figure 7B and 8B). The pattern of mean HSI values for both E2 treated

Figure 6 : The effect of 2.5, 5 and 10 E2 on the hepatosomatic index in immature rainbow trout (mean wt. = 66.7 g) during June, 1984. Each point represents a mean \pm SEM for 5 fish.

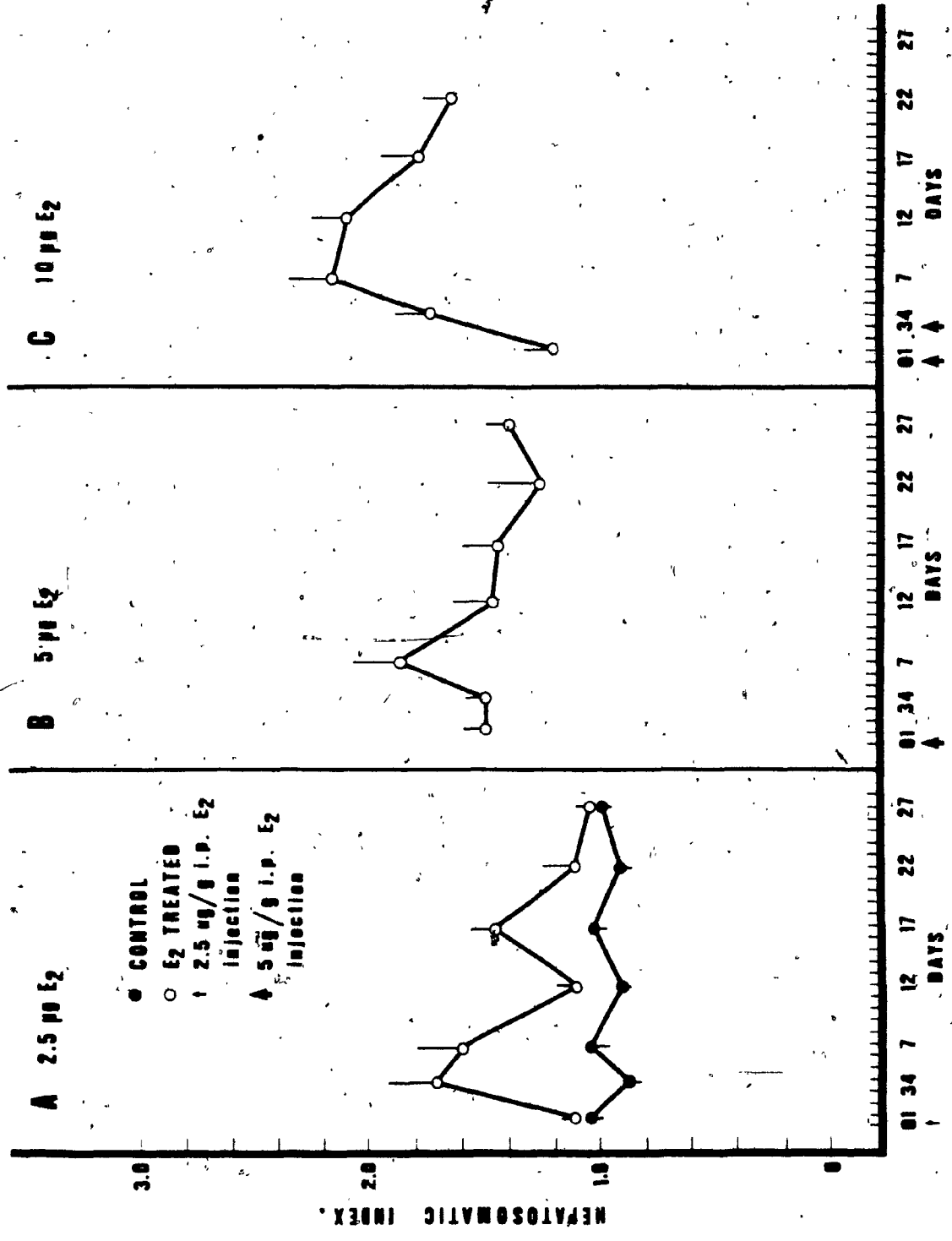


FIGURE 6

Figure 7 : The effect of fish weight (mean wts. = 18.9 and 66.7 g) on the levels of total serum phosphoprotein phosphorus in immature rainbow trout treated with 10 ug E2. The experiment using 18.9 g fish was performed during June, 1983, the other using 66.7 g fish in June, 1984. Each point represents a mean +/- SEM for 5 fish.

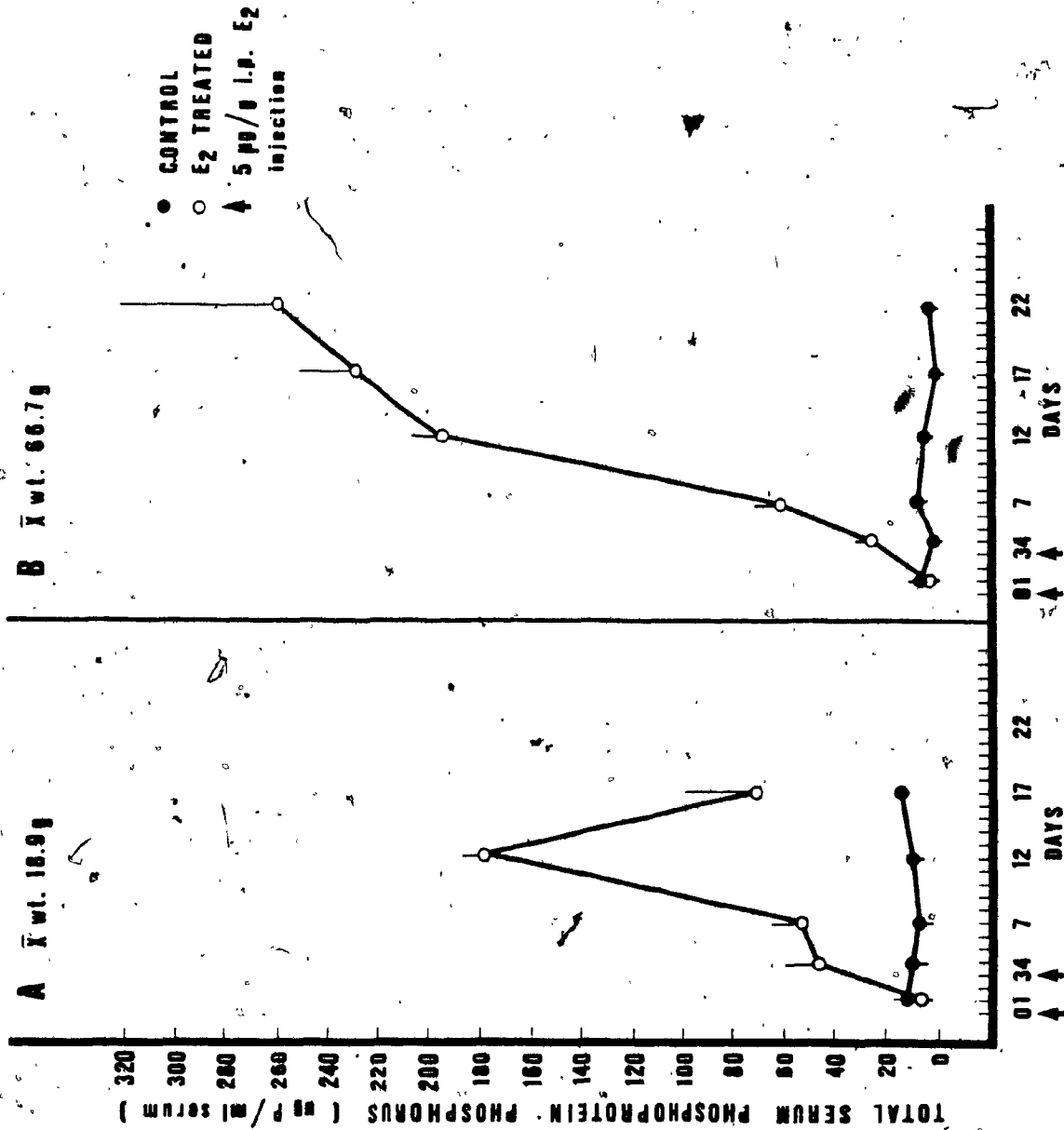


FIGURE 7

Figure 8 : The effect of fish weight (mean wts. = 18.9 and 66.7 g) on the levels of total serum calcium in immature rainbow trout treated with 10 ug E₂. The experiment using 18.9 g fish was performed during June, 1983, the other using 66.7 g fish in June, 1984. Each point represents a mean +/- SEM for 5 fish.

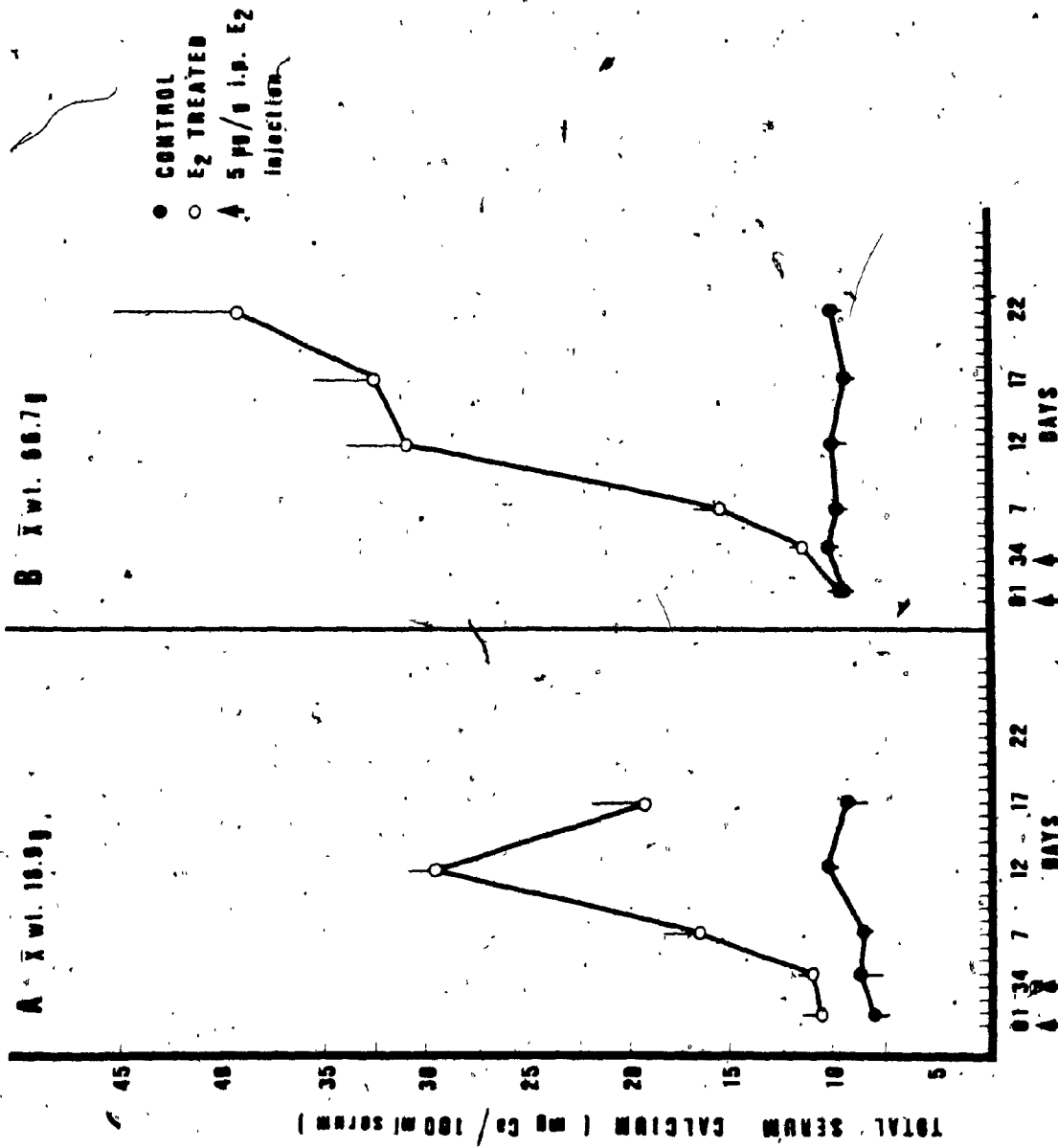


FIGURE 8

groups were similar (Figure 9), each reaching a maximum of 2.1 on Day 7.

The validity of using SPP and SCa as indirect measures of vitellogenin (Vg) in E2 treated immature rainbow trout was examined. To achieve this, SPP and SCa levels were obtained and compared with the serum vitellogenin levels in the same fish injected with 5 ug E2 (5 ug E2/g body wt.). The overall response was the same for all three parameters SPP, SCa and vitellogenin in the E2 treated group (Figure 10). There was a steady increase after injection to peak levels on Day 12, followed by a decline to control levels on Day 30. The mean maximum levels of SPP, SCa and vitellogenin were 105.0 ug P/ml, 20.1 mg Ca/100 ml and 17 mg Vg/ml respectively on Day 12. A strong correlation exists between SPP, SCa and vitellogenin values in individual fish. The correlation coefficient for SPP and SCa is 0.89 (n=28, p<0.0001). When SPP and SCa were correlated with vitellogenin, the correlation coefficient was 0.91 (n=17, p<0.0001) for both parameters. The predictive linear regression equations to estimate individual vitellogenin values from SPP and SCa determinations are respectively :

$$1) \log y = -0.72 \pm 0.36 + 1.02 \pm 0.24 \log x$$

where x = SPP and y = vitellogenin, (Figure 11).

and

Figure 9 : The effect of fish weight (mean wts. = 18.9 and 66.7 g) on the hepatosomatic index in immature rainbow trout treated with 10 ug E2. The experiment using 18.9 g fish was performed during June, 1983, the other using 66.7 g fish in June, 1984. Each point represents a mean +/- SEM for 5 fish.

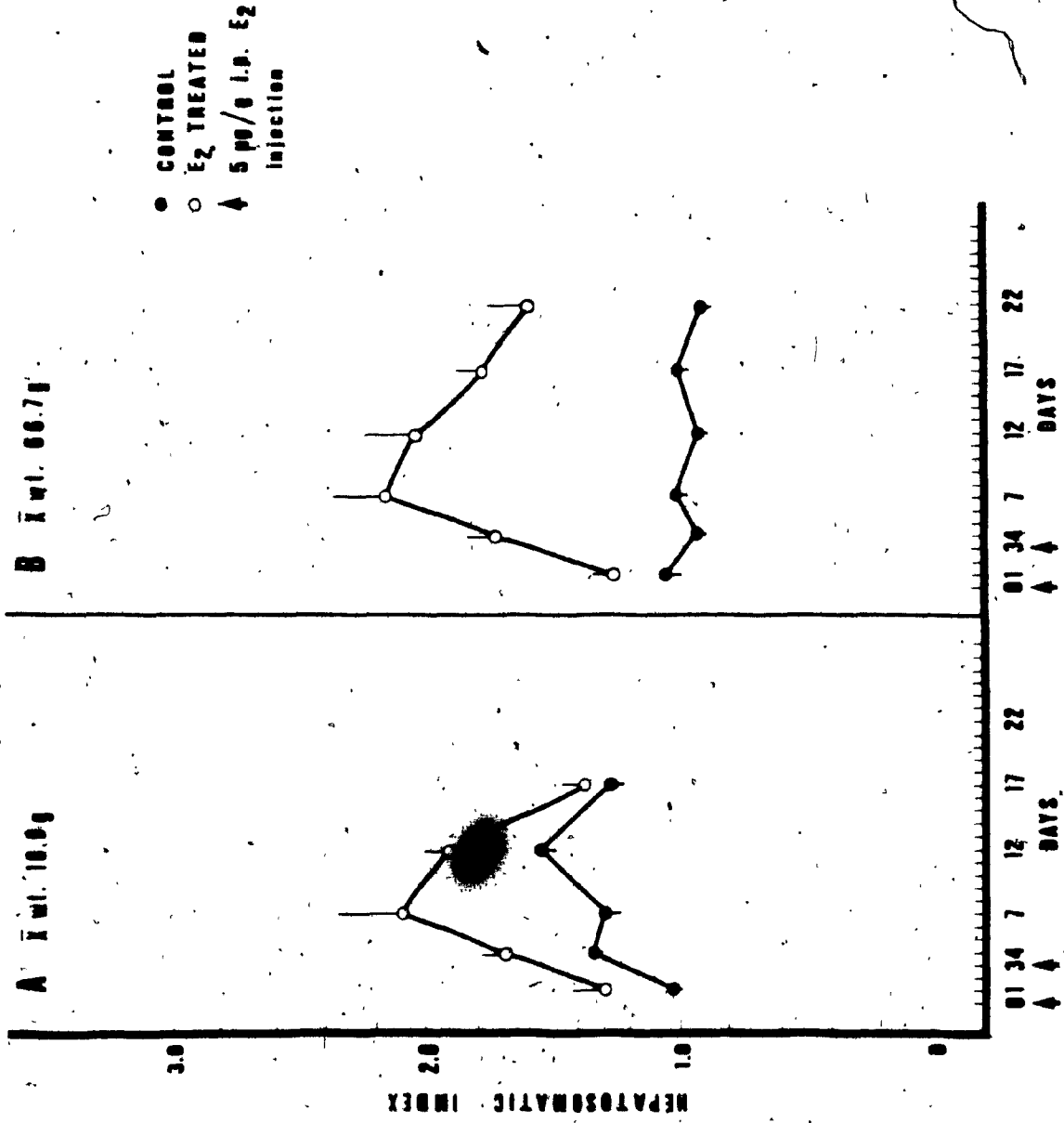


FIGURE 9

Figure 10 : The effect of 5 μ g E2 on the levels of total serum phosphoprotein phosphorus, total serum calcium and serum vitellogenin in immature rainbow trout (mean wt. = 35.4 g) during July, 1984. Each point represents a mean \pm SEM for 5 fish.

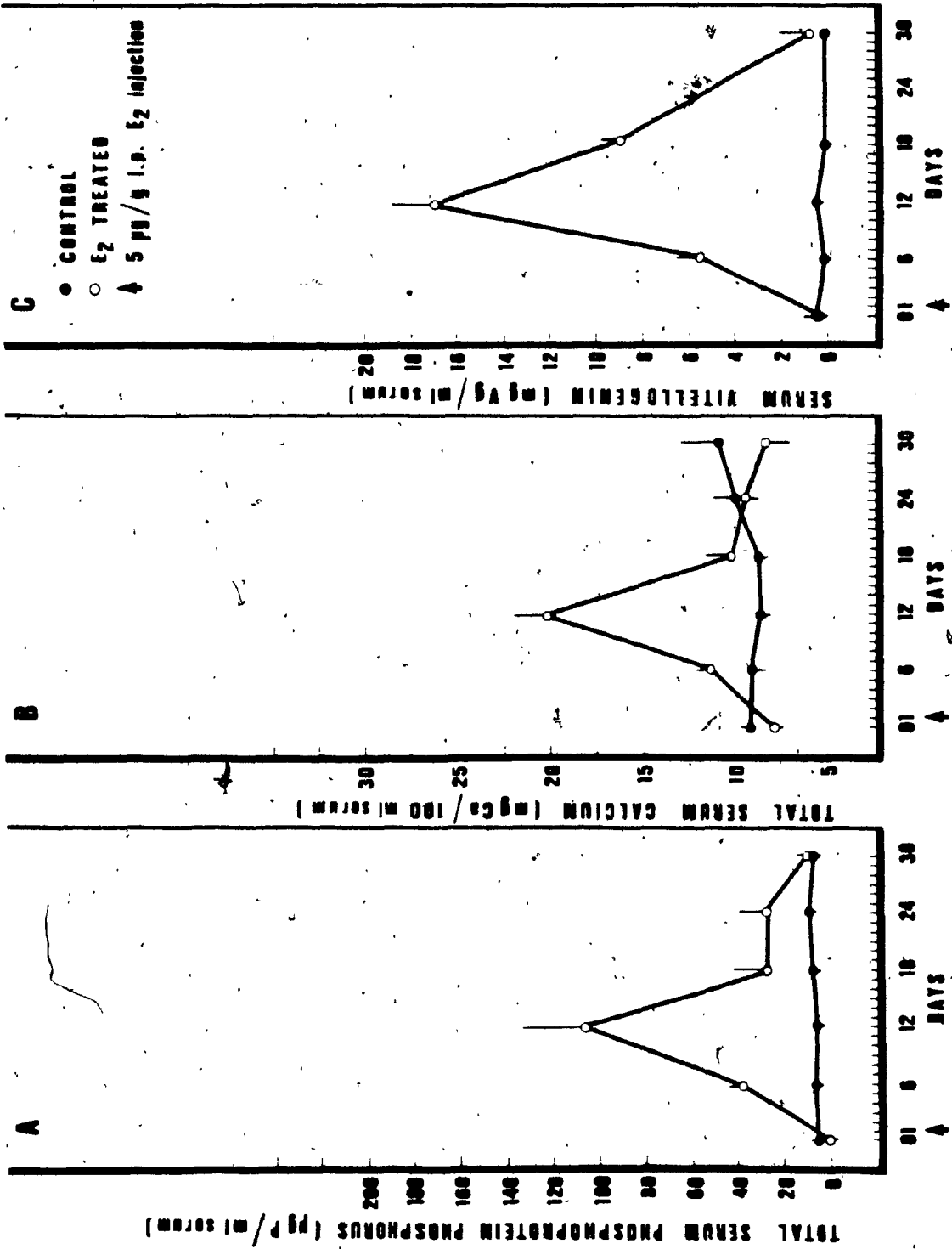


FIGURE 10

Figure 11 : The simple linear regression relationship between total serum phosphoprotein phosphorus (x) and serum vitellogenin (y) in 5 ug E2 treated immature rainbow trout (mean wt. = 35.4 g) during July, 1984. (n=17).

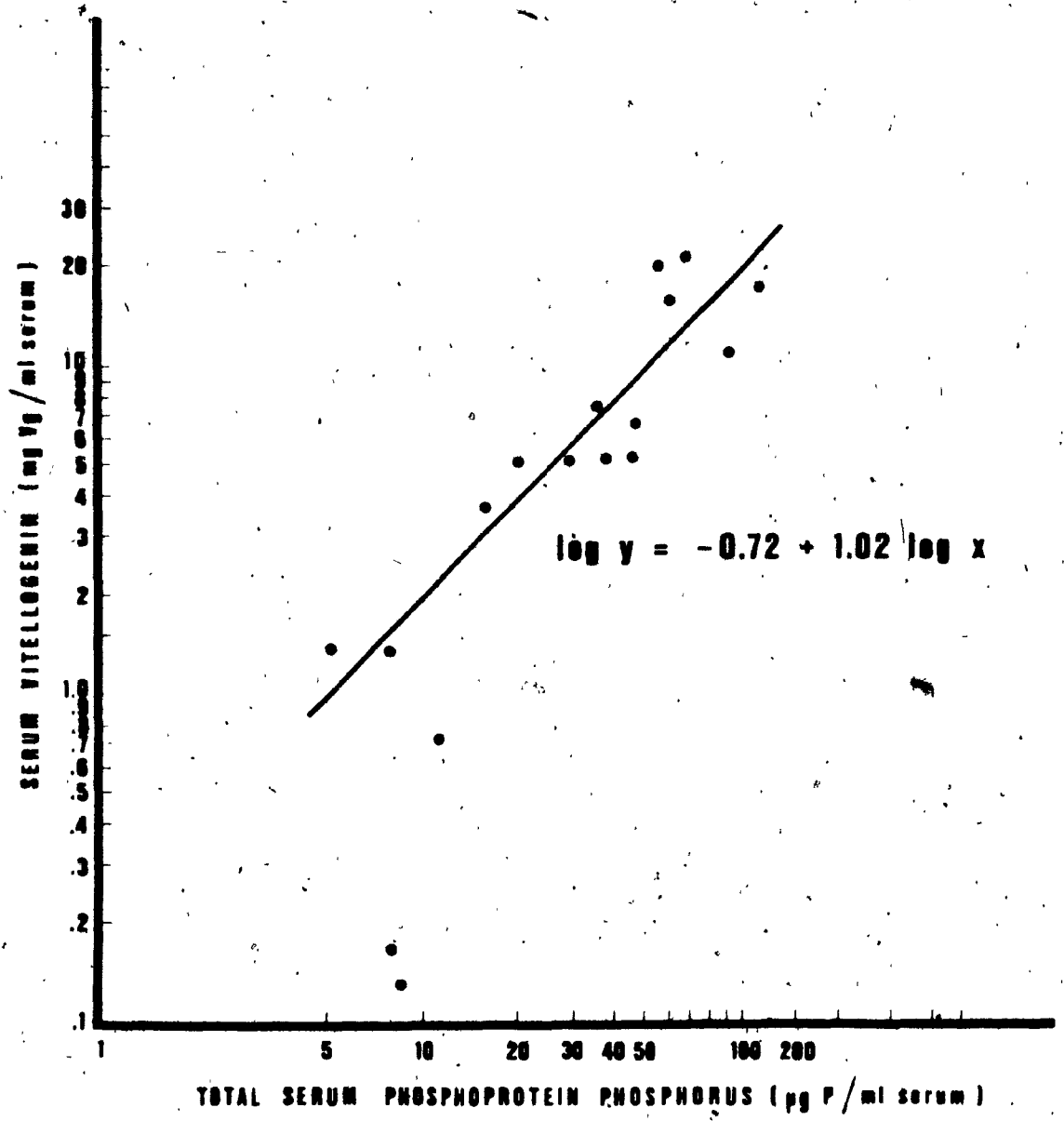


FIGURE 11

$$2) \log y = -2.16 \pm 0.73 + 2.73 \pm 0.68 \log x$$

where $x = \text{SCa}$ and $y = \text{vitellogenin}$, (Figure 12).

Following testing of the model, the effect of sublethal PCP (25 ug PCP/L) on SPP and SCa in mature female rainbow trout undergoing natural exogenous vitellogenesis was examined. At 9 and 18 days after sublethal PCP exposure began there were lower mean levels of SPP and SCa in PCP exposed female rainbow trout compared to control fish but no statistically significant differences occurred ($p > 0.05$) (Figure 13). The PCP exposed group had mean levels of SPP, 59.9 ug P/ml and SCa, 13.0 mg Ca/ml, relative to 89.0 ug P/ml and 14.1 mg Ca/100 ml respectively for the control group on Day 19.

The histological effects of an 18-day exposure to sublethal levels of PCP (12.5, 25 and 50 ug PCP/L) on oocyte growth and development in maturing female rainbow trout was studied in two experiments during December, 1983 and July, 1984. The Stage 2 oocyte characterized by the presence of the Balbiani body (Figure 14) was the most numerous stage in the ovaries of these female rainbow trout maturing for the first time. An abnormally high incidence of pre-ovulatory, non-hypertrophic atresia was observed at this oocyte stage in females exposed to sublethal PCP. Atretic oocytes were identified by disintegration of the cytoplasm with a large space developing between the nucleus and the oocyte

Figure 12 : The simple linear regression relationship between total serum calcium (x) and serum vitellogenin (y) in 5 ug E2 treated immature rainbow trout (mean wt. = 35.4 g) during July, 1984. (n=17)..

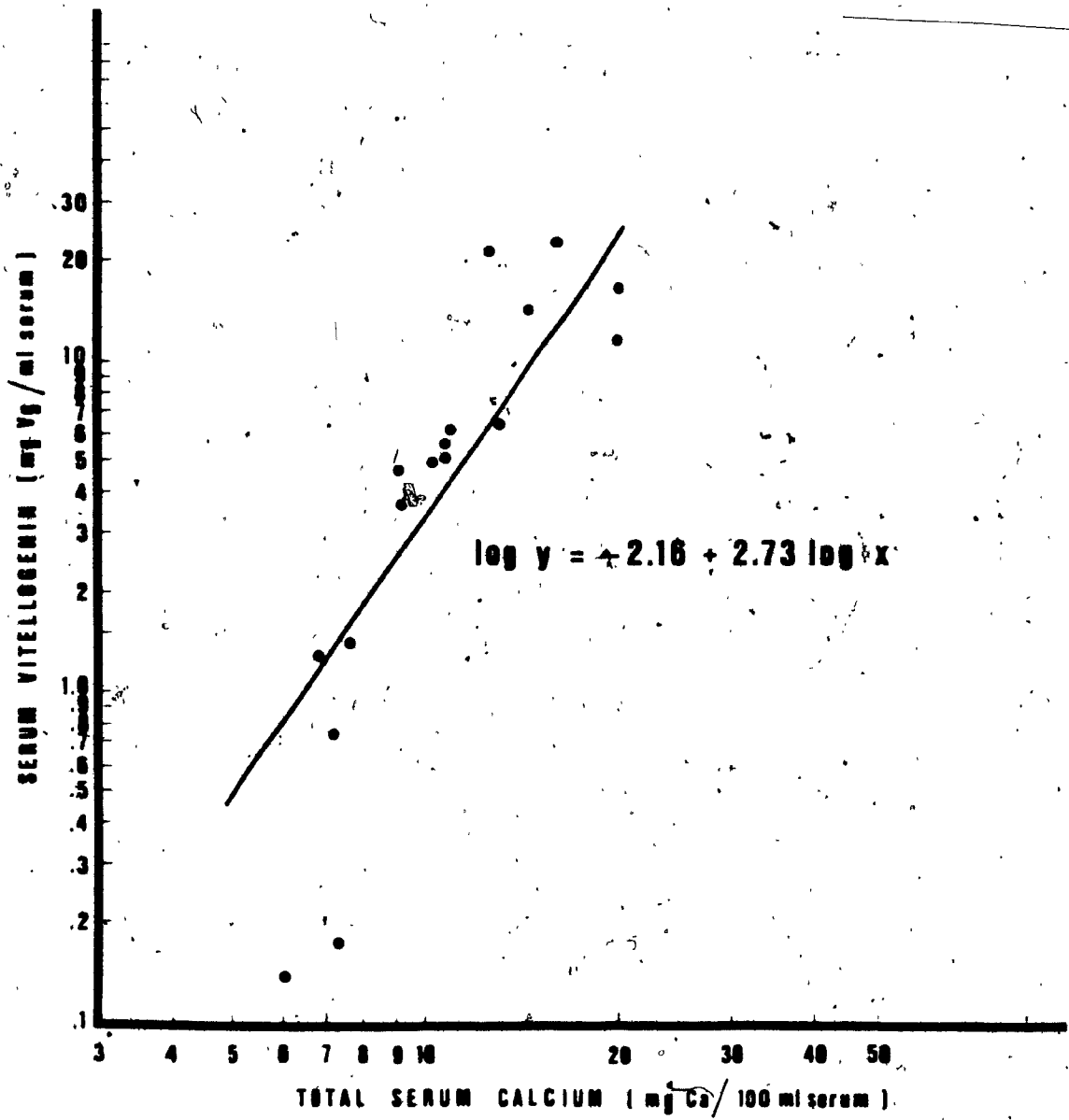


FIGURE 12

Figure 13 : The effect of sublethal pentachlorophenol exposure (25 ug PCP/L) on the levels of total serum phosphoprotein phosphorus and total serum calcium in mature naturally vitellogenic female rainbow trout (mean wt. = 550 g) during December, 1984. Each point represents a mean +/- SEM.

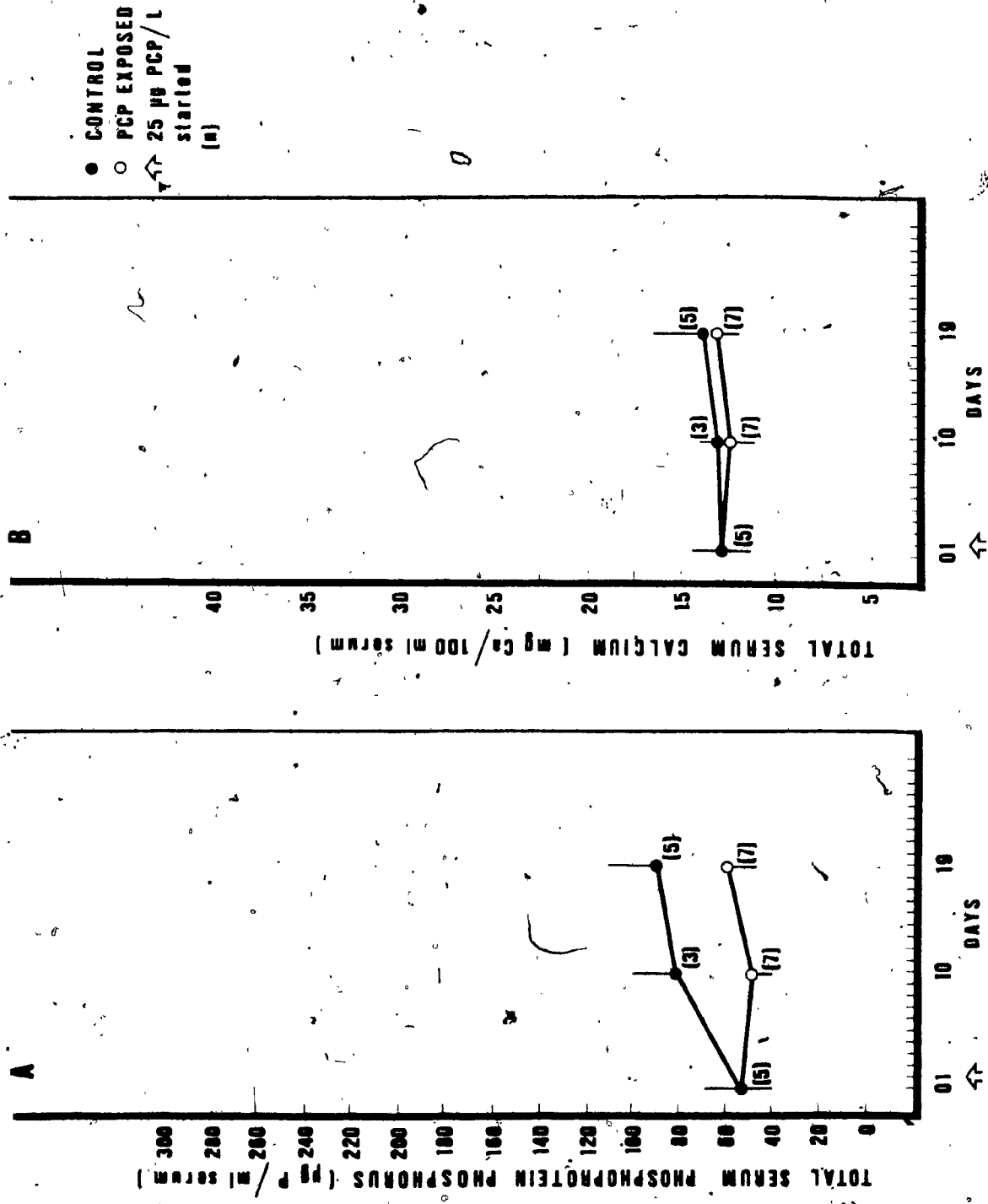


FIGURE 13

Figure 14 : Mid-ovarian section from maturing female rainbow trout (control) showing viable Stage 2 oocytes containing Balbiani bodies (↔) in various stages of development. Early Stage 2 oocyte (a), later Stage 2 oocyte with Balbiani body dispersing to the periphery (b) and Stage 3 oocyte (c) where Balbiani body has completely dispersed. Haematoxylin and eosin, 100 x.

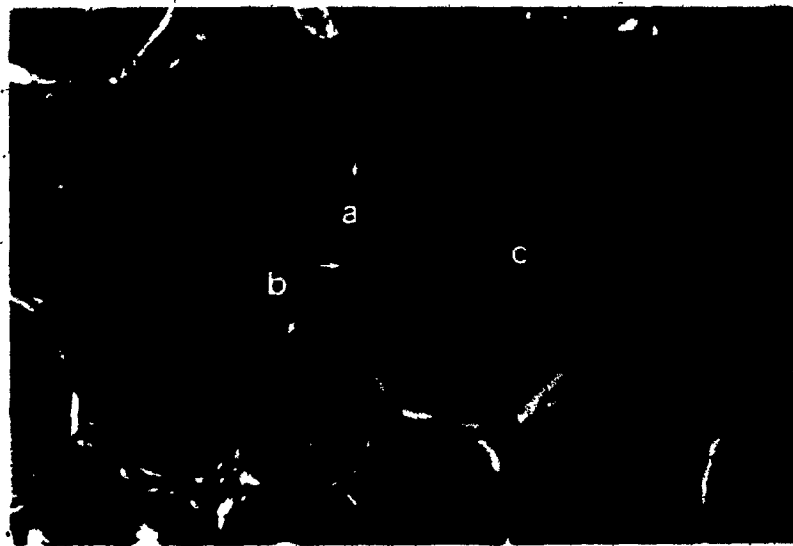


FIGURE 14

periphery. In most cases the nucleus was displaced to one side of the oocyte and the Balbiani body had shrunk against it (Figure 15). The Stage 2 oocytes were assessed as a group for the proportion of viable or atretic (Figure 16) oocytes present in control and exposed fish. The level of atresia among Stage 2 oocytes in female fish exposed to sublethal concentrations of PCP during December, 1983 was similar to the controls ($p > 0.05$) (Figure 17). During the second experiment (July, 1984) significantly ($p < 0.05$) higher levels of atresia among Stage 2 oocytes were found at the two highest PCP concentrations employed (Figure 18). At the levels of 22 and 49 $\mu\text{g PCP/L}$ there were 71% and 89% atretic Stage 2 oocytes respectively, relative to 48% atresia in the control fish.

The mean diameters for the ten largest viable oocytes apparent in the ovaries of these maturing female rainbow trout were measured in control and exposed individuals. In both experiments there were decreases in mean oocyte diameter for female fish exposed to sublethal PCP, but these differences were not significant ($p > 0.05$) from the control fish (Table 5 and 6).

Figure 15 : Mid-ovarian section from a female rainbow trout chronically exposed to sublethal pentachlorophenol (22 ug PCP/L) for 18 days showing atretic Stage 2 oocytes (A) with a large space developing in the cytoplasm and the Balbiani body (→) clustered around the nucleus (n).
Haematoxylin and eosin, 250 x.



FIGURE 15

Figure 16 : Mid-ovarian section from a female rainbow trout chronically exposed to sublethal pentachlorophenol (22 ug PCP/L) for 18 days showing viable (V) and atretic (A) Stage 2 oocytes. Haematoxylin and eosin, 100 x.



FIGURE 10

Figure 17 : The relative proportion of viable Stage 2/
atretic Stage 2 oocytes in maturing female
rainbow trout (mean wt. = 70.8 g) exposed
to sublethal levels of pentachlorophenol
(17, 25 and 51 ug PCP/L) for 18 days during
December, 1983. Each treatment group
represents a mean +/- 95% confidence
interval for 7 fish.

V - VIABLE STAGE 2
A - ATRETIC STAGE 2
DECEMBER 1993

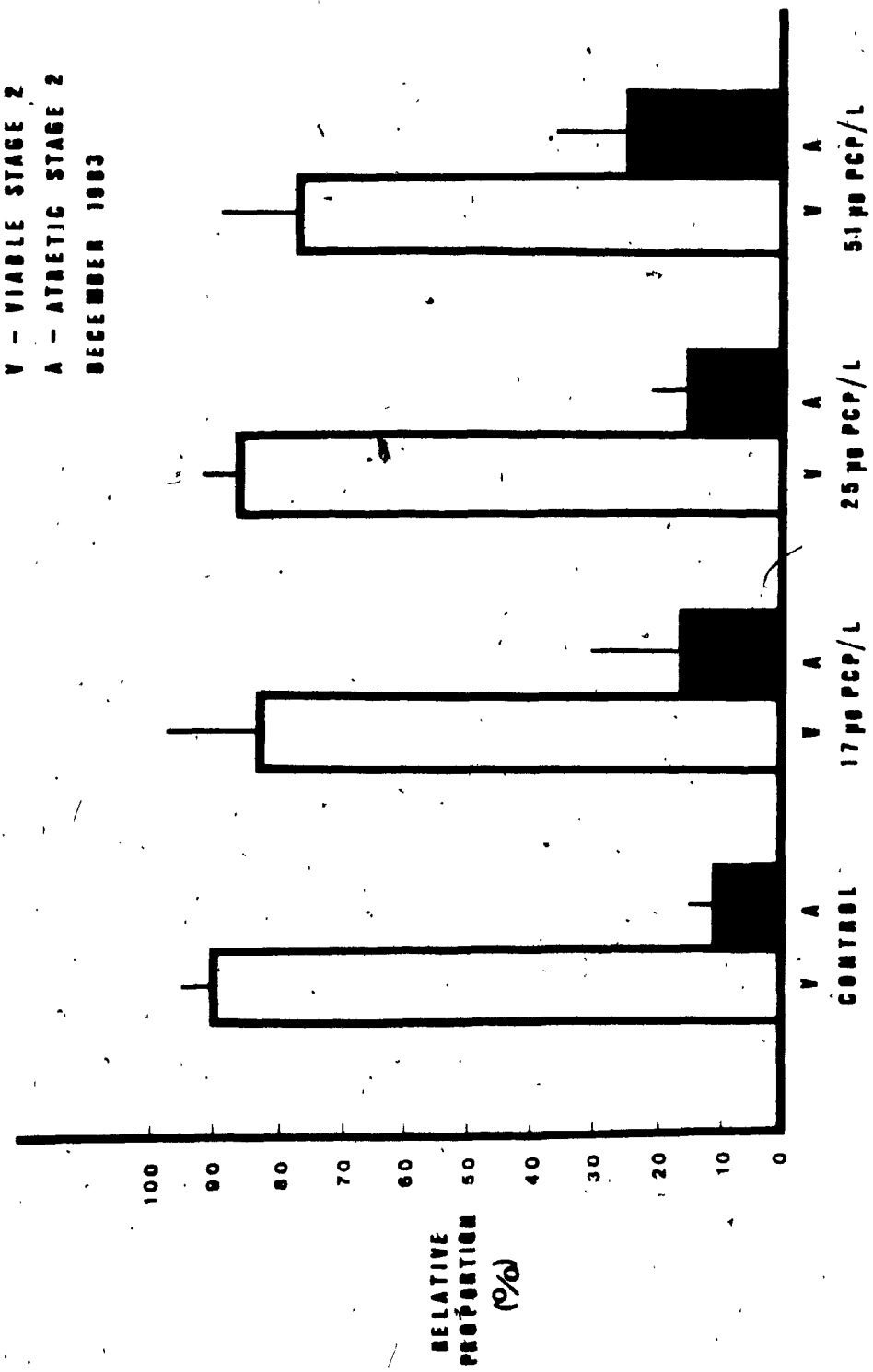


FIGURE 17

Figure 18 : The relative proportion of viable Stage 2/ atretic Stage 2 oocytes in maturing female rainbow trout (mean wt. = 113.4 g) exposed to sublethal levels of pentachlorophenol (12, 22 and 49 ug PCP/L) for 18 days during July, 1984. Each treatment group represents a mean +/- 95% confidence interval for 7 fish. (* - significantly different from the control group with ANOVA and SNK multiple comparison test at $p < 0.05$).

V - VIABLE STAGE 2
A - ATRETIC STAGE 2
JULY 1984

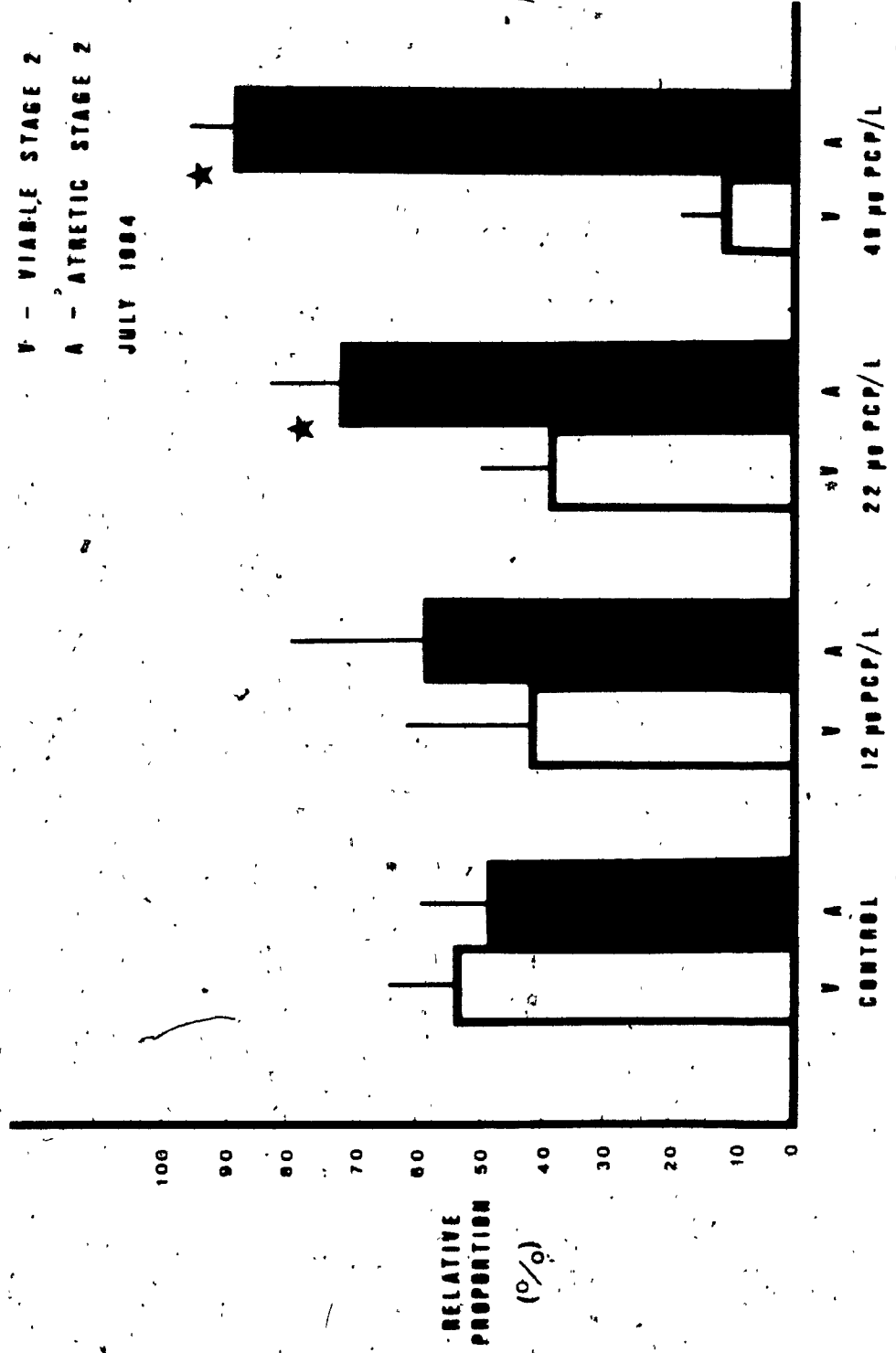


FIGURE 18

Table 5.: The effect of 18 day sublethal exposure to different levels of pentachlorophenol (17, 25 and 51 ug PCP/L) on the mean diameters (μm) of the ten largest viable oocytes in the ovaries of female rainbow trout.

December 1983

Control Day 0	Control Day 18	17 ug PCP/L Day 18	25 ug PCP/L Day 18	51 ug PCP/L Day 18
$\bar{X}=194.7$	$\bar{X}=216.5$	$\bar{X}=214.9$	$\bar{X}=212.0$	$\bar{X}=209.5$
± 9.1	± 11.9	± 11.5	± 12.0	± 14.7
n = 12	n = 10	n = 7	n = 7	n = 7
N.S.	N.S.	N.S.	N.S.	N.S.

\bar{X} (mean) \pm 95% confidence limits.

N.S. - not significant with ANOVA, $p < 0.05$.

Table 6: The effect of 18 day sublethal exposure to different levels of pentachlorophenol (12, 22 and 49 ug PCP/L) on the mean diameters (um) of the ten largest viable oocytes in the ovaries of female rainbow trout.

July 1984

Control Day 0	Control Day 18	12 ug PCP/L Day 18	22 ug PCP/L Day 18	49 ug PCP/L Day 18
$\bar{X}=264.6$	$\bar{X}=296.2$	$\bar{X}=282.7$	$\bar{X}=263.8$	$\bar{X}=268.1$
$\pm/-21.1$	$\pm/-31.0$	$\pm/-13.6$	$\pm/-22.5$	$\pm/-34.0$
n = 10	n = 9	n = 7	n = 8	n = 7
N.S.	N.S.	N.S.	N.S.	N.S.

\bar{X} (mean), $\pm/-$ 95% confidence limits.

N.S. - not significant with ANOVA, $p < 0.05$.

DISCUSSION

The effect of sublethal PCP exposure on exogenous yolk production was examined utilizing a model in which exogenous yolk formation was induced by E2 injection in immature rainbow trout. It was found that sublethal PCP, 25 ug PCP/L, could significantly reduce SPP and SCa when levels were greater than 240 ug P/ml and 32 mg Ca/100 ml respectively (Figure 3). This occurred after 19 days of toxicant exposure following an E2 dose of 10 ug E2. It appears that the sublethal concentration of 25 ug PCP/L can, in E2 treated immature rainbow trout, significantly reduce SPP and SCa when levels have reached a critical point.

A similar model was used previously to examine the effect of sublethal hydrogen cyanide (HCN) exposure on exogenous yolk (Cyr, 1984) but this marks the first application of this model to study sublethal PCP toxicity. To date there is no information on the consequences of sublethal PCP exposure on exogenous vitellogenesis in experimentally or naturally induced fish and little involving other water pollutants. Singh and Singh (1980, 1982) found the organochlorine compounds, aldrin and endrin reduced ovarian 32P uptake in the Indian catfish (Heteropneustes fossilis Bloch) at lethal and sublethal concentrations. Cyr (1984) showed sublethal HCN to significantly increase SPP and SCa levels in October, but

have no effect during June in immature rainbow trout injected with 10 ug E2 after 17 days. Alternatively, SCA levels were reduced and SPP not affected after 7 days of sublethal HCN in mature female rainbow trout during October (da Costa and Ruby, 1984). Finally, in a lake (George Lake, Northern Ontario) undergoing acidification it was demonstrated (Beamish et al., 1975 and Lockhart and Lutz, 1977) that the seasonal rise in SCA associated with exogenous vitellogenesis did not occur in female white suckers (Catostomus commersoni) living there.

Sublethal PCP interferes with SPP and SCA once the serum levels attain 240 ug P/ml and 32 ug Ca/100 ml respectively. Based on the SPP and SCA values the liver is equally induced in both E2 treated groups but only sustains exogenous yolk production to a certain point following 25 ug PCP/L exposure. To produce the reduction in SPP and SCA levels PCP could be acting in three different manners. It may be at the level of the hepatocyte by limiting energy production or secondly by preventing the biological expression of the hormone E2. The third possibility is generally accelerating the catabolism of high exogenous yolk levels in the blood.

The capacity for the hepatocyte to produce vitellogenin could be limited by the inhibitory action of sublethal PCP on energy production. The liver comes in rapid contact with PCP as exposed rainbow trout concentrate it there for

52.

detoxification (Lech et al., 1978). PCP would be readily absorbed across the hepatocyte cell membrane where it would bind to mitochondria. A number of liver enzymes that are directly involved with key energy producing pathways have been shown to be affected by PCP. Boström and Johansson (1972) demonstrated that acute PCP poisoning decreased the hepatic activities of pyruvate kinase and lactate dehydrogenase and increased the activities of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, fumarase and cytochrome oxidase in vivo in the yellow eel (Anguilla anguilla). The cumulative biologic response to sublethal PCP intoxication is an uncoupling of oxidative phosphorylation. The net effect is less chemical energy for the cell and this is crucial for the hepatocyte in this instance which is stimulated by E2 for vitellogenin production. The hepatocyte has enough energy to sustain vitellogenin production, as indicated by SPP and SCA levels, up to a certain limit under sublethal PCP exposure, but above this point the levels are not maintained. It is of interest to note that following sublethal PCP exposure SPP and SCA levels decrease suggesting that energy reserves are depleted and the hepatocyte can no longer sustain exogenous yolk production.

The second means by which sublethal PCP could limit exogenous yolk production at the hepatocyte is by preventing the biological expression of E2. Pentachlorophenol may

affect the formation of hormone-receptor complexation or gene regulation although there is no toxicological work to support this. Estradiol treatment quickly stimulates exogenous yolk production in the liver and release into the blood. Black et al. (1971) found elevated serum egg proteins in E2 treated immature cod (Gadus mornua) within 2 days and Sundararaj and Nath (1981) induced serum vitellogenin in E2 treated mature female Indian catfish after 24 hours. The tissue uptake of PCP is rapid in exposed fish. Decker et al. (1978) reports a level of 16 ug PCP/g in liver of rainbow trout subjected to 25 ug PCP/L for 24 hours. If sublethal PCP does limit E2 directly in the present study, the effects should be evident in at most 3 days and this was not the case at either E2 dose used as evidenced by similar SPE and SCA levels in both PCP exposed and control groups for up to two weeks post-injection.

Lastly sublethal PCP poisoning may cause catabolism of exogenous yolk present in the blood due to the lipid component in vitellogenin. By it's mode of action of uncoupling oxidative phosphorylation sublethal PCP increases basal metabolic rate (Holmberg and Saunders, 1979). Sufficient ATP energy from the electron transport chain is not provided by oxidative phosphorylation. Catabolism of lipid, providing more ATP/g than carbohydrate, occurs to supplement energy deficiency (NRCC, 1982). Total body lipids were dramatically reduced in young coho salmon (Oncorhynchus

Kisutch exposed to lethal threshold levels of PIP (Hanes et al., 1968). The reduced levels of exogenous yolk in E2 treated immature rainbow trout exposed to sublethal PIP in this study, may be the result of vitellogenin sequestered by various tissues to meet energy demands.

The advantages of this exogenous yolk induction model for studying the effects of aquatic toxicants on exogenous vitellogenesis have been previously described (Yr, 1984). The use of this experimental model involving sublethal PIP raised no consideration. This work has determined that the levels of SPP and SCA must be above 140 μ g/ml and 12 mg/100 ml, respectively, before significant changes due to sublethal PIP exposure can be recorded.

The E2 dose injected into the fish and fish weight were two variables that changed the SPP and SCA levels and subsequently altered the response observed when utilizing the induction model. It was demonstrated that altering the E2 dose from 2.5 to 10 μ g E2 had different effects on the levels of SPP and SCA (Figures 5, and 6). There is a clear dose-dependent response with increasing E2 dose and higher maximum SPP and SCA levels in E2 treated immature rainbow trout. This is supported by a similar response involving the HSI which complements the changes observed in the serum (Figure 7). The E2 dose-response involving some of the previous parameters has been demonstrated in a number of immature teleosts. van Bohemen et al., (1982) have indicated

an E2 dose-response with plasma vitellogenin, total plasma proteins and albumin in immature rainbow trout and fundulus heteroclitus. Similar relationships with E2 in body wt. Similar E2 dose-dependent relationships have been established for other indirect indicators of vitellogenin in aquatic species: alkaline phosphatase in immature founder Platichthys flesus L. (Lamberson et al., 1974), serum egg proteins in immature cod Gadus morhua L. (1977) and to plasma proteins, specific plasma protein fractions and plasma albumin in immature Salmo gairdneri Richardson (Alla et al., 1977).

Fish weight was also shown to affect the level of E2 exogenous yolk induction in E2 treated immature rainbow trout. Heavier, older immature fish attained higher maximum NP and albumin levels compared to smaller (younger) fish given the same dose of E2 (Figures 8 and 9). This phenomenon has not been previously documented in fish, but a probable hypothesis for it relates to the hormone-receptor interaction at the target tissue as a function of age. In mammals the uterine response in post-natal rats measured by induction of protein synthesis increased with age following a single E2 injection (Somjen et al., 1973). There are three factors which could affect this hormone-receptor interaction relative to the fish 1) the number of hepatic receptors, 2) affinity of the receptors for E2 and 3) specificity of the receptors for E2. Since the HSI was higher in control fish from the smaller weight group relative to the larger weight

group (Figure 13), there is no indication in these results that the number of E2 receptors based on liver size should be a factor. Unfortunately there are no known previous studies concerning E2 receptor affinity or specificity with regard to age in fish. It is clear from this study that, E2 dose and fish weight, are important variables in the experimental model because they determine the levels of SPP and SCA reached in E2 treated immature rainbow trout. The appropriate E2 dose - fish weight combination is important, demonstrated in this case by sublethal PCB exposure where an effect was only observed when SPP and SCA levels were above 240 ug P/ml and 32 mg Ca/100 ml respectively.

This study has found that SPP and SCA are reliable, indirect indicators of vitellogenin in E2 treated immature rainbow trout. A strong correlation exists between the levels of SPP and SCA and they correlate equally well to the vitellogenin levels in individual fish. This study represents the first direct comparison that has been made between the indirect measures of SPP and SCA with vitellogenin in individual E2 injected immature rainbow trout.

In the past many techniques have been used to measure vitellogenin in E2 induced immature fish. Elliott et al., (1979) used the same parameters as the present study to indicate the presence of vitellogenin. They observed peak SPP and SCA levels of 832 ug P/ml and 75.2 mg Ca/100 ml

respectively in E2 treated immature rainbow trout after 21 days. Idler and Campbell (1980) measured vitellogenin with a radioimmunoassay in E2 treated immature rainbow trout and found levels of 5.5 mg Vg/ml in males and 8.2 mg Vg/ml in females while untreated fish had 24 ug Vg/ml after 12 days. Using polyacrylamide gel electrophoresis van Bohemen et al. (1982) determined a female-specific polypeptide as vitellogenin in E2 treated immature rainbow trout at levels from 4.4-7.9 mg/ml after 7 days. In immature cod E2 treatment resulted in the appearance of serum egg proteins within two days that corresponded to the gel filtration pattern of mature female cod serum and cod egg extract (Plack et al., 1971). Aida et al. (1973) was able to increase plasma calcium and two specific plasma protein fractions characteristic of mature vitellogenic females using starch-gel electrophoresis in E2 treated immature male ayu after 7 days. Finally, immature goldfish (Carassius auratus) fed E2 showed elevated serum protein phosphorus, 1197 ug/ml, total serum calcium, 555.3 ug/ml and similar electrophoretic serum protein patterns to vitellogenic fish in a study by Hori et al. (1979). The levels of SPP and SCA induced by E2 treatment of immature rainbow trout in this study compare favourably to values reported here. Although the SPP and SCA levels are lower than those of Elliott et al., (1979), the variables of fish weight and age probably account for the differences observed. The vitellogenin levels in this study

were higher than those found by Idier and Campbell (1980), for rainbow trout most likely due to the sensitivity of the homologous vitellogenin radioimmunoassay for rainbow trout employed in this work (So et al., 1985).

The measures of SPP and SCa are valid indirect indicators of vitellogenin in E2 treated immature rainbow trout based on their correlation with one another, their high correlation to vitellogenin and the physiological levels found in E2 treated immature rainbow trout as compared to mature naturally vitellogenic female fish. The correlation between SPP and SCa is $r=0.89$ ($n=28$), indicating a 1:1 ratio. This has also been noted by Elliott et al., (1979) who reports a correlation, $r=0.96$ ($n=86$), for SPP and SCa in E2 treated immature rainbow trout. Further evidence is the fact that SPP and SCa respectively, correlate equally well with vitellogenin levels obtained in this study, $r=0.91$ ($n=17$), supporting the hypothesis that they represent strong indirect indicators of vitellogenin. The maximum SPP, SCa and vitellogenin levels are 105.0 ug P/ml, 20.1 mg Ca/100 ml and 17 mg Vg/ml respectively and well within the normal physiological range for mature females of this species actively undergoing exogenous vitellogenesis. Whitehead et al. (1978) report maximum levels of SPP and SCa to be 400 ug P/ml and 58 mg Ca/100 ml respectively, in mature vitellogenic female rainbow trout. Scott and Sumpter (1983b) indicate a maximum level for vitellogenin of close to 50 mg/ml and So

et al., (1985) of 22 mg/ml for female rainbow trout during the period of exogenous vitellogenesis.

From this work there are specific limits within which SPP and SCa values can be used in generating vitellogenin levels from the predictive regression equations and utilized in separating vitellogenic from non-vitellogenic fish. The determination of SPP is a measurement of total serum phosphorus due to protein phosphorus and nucleic acid phosphorus. The nucleic acid phosphorus component of the SPP in non-vitellogenic female rainbow trout has been shown to make up 64% of the total serum phosphorus content (Crak and Harvey, 1984). Though there is no evidence that serum nucleic acid phosphorus is affected by E2, high levels of SPP should be attained before it can be assumed that exogenous vitellogenesis is taking place. Likewise the determination of SCa is a total measure of serum calcium content, both bound and unbound, as E2 is a mobilizer of calcium reserves in fish (Ng and Idler, 1983). The measurement of SCa then represents calcium unbound and bound to serum proteins, specifically vitellogenin, which is a calcium binding protein. The strong correlation however between SPP and SCa can not be discounted and above certain limits in conjunction with SPP, the measurement of SCa can be expected to reflect calcium bound to vitellogenin. It is therefore recommended that the presence of vitellogenin in the serum can be assumed when SPP and SCa have reached values of 20 ug

P/ml and 12 mg Ca/100 ml respectively. These levels correspond to a vitellogenin value of 4 mg/ml (Figure 12 and 13). Craik and Harvey (1984) also suggest 20 ug/ml protein phosphorus as a lower limit for vitellogenesis, a value derived from a variety of marine and freshwater teleost fish species. The upper limits of 110 ug P/ml for SPP and 20 mg Ca/100 ml for SCA representing 22 mg/ml vitellogenin should be employed. Extrapolation beyond the limits of the respective predictive regression equations developed in this study has not been validated. Any values of SPP or SCA from fish below this given range may be classed as non-vitellogenic. When the levels of SPP and SCA together are high or very low these indirect indicators can be utilized in separating vitellogenic from non-vitellogenic E2 treated immature rainbow trout respectively.

The relationship between the levels of SPP, SCA and vitellogenin in vitellogenic rainbow trout can only be discussed hypothetically since comparative values for naturally reproducing rainbow trout are not available. If the level of vitellogenin in highly vitellogenic females is between 22-50 mg/ml (So et al., 1985 and Scott and Sumpter, 1983b) the corresponding maximum SPP and SCA levels would be in the order of 220 ug P/ml and 50 mg Ca/100 ml respectively, predicted from extrapolation of the regression equations following E2 induction of immature rainbow trout. There are two previous reports of peak levels of SPP, 400 ug

P/ml and SCA, 58 mg/ml, for naturally reproducing mature female rainbow trout (Whitehead et al., 1978) and SPP, 832 u/g P/ml and SCA, 75 mg Ca/100 ml respectively (Elliott et al., 1979) in E2 treated immature rainbow trout. These values would suggest vitellogenin in excess of 100 mg Vg/ml for mature naturally vitellogenic female fish and 200 mg Vg/ml for E2-treated immature fish if a linear relationship exists between SPP, SCA and vitellogenin as proposed from the E2 induction model for rainbow trout in the present study. Physiologically these predicted vitellogenin levels would appear to be excessive. It is possible that a plateau is eventually reached in the linear relationship between SPP and SCA with vitellogenin. At levels approximating 50 mg/ml vitellogenin the degree of phosphorylation and calcium binding would have to increase and the linear curve would flatten parallel to the abscissa. This change could account for the high SPP and SCA levels reported by some investigators relative to the maximum vitellogenin level of 50 mg/ml indicated in highly vitellogenic rainbow trout (Scott and Sumpter, 1983b).

The effect of sublethal PCP exposure on exogenous yolk production in mature naturally reproducing rainbow trout was determined, permitting a comparison with immature rainbow trout in which exogenous yolk had been artificially induced. There is an indication in mature female fish that 25 ug PCP/L exposure does reduce the levels of SPP and SCA after

18 days, but no statistically significant differences were recorded. Conversely, a significant decrease in SPP and SCA was recorded in immature rainbow trout injected with 10 µg E2 and exposed to 25 µg PCP/L for 19 days. This effect was noted however after SPP and SCA had attained levels of 249 µg P/ml and 32 mg Ca/100 ml respectively. The maximum SPP and SCA levels in the mature female control fish were less than half these values. Results from the E2 treated immature fish indicate there is a critical point for SPP and SCA levels above which 25 µg PCP/L produces a significant toxic effect. In mature female fish the maximum SPP and SCA levels were well below this point and this may explain why a statistically significant effect was not observed even though levels were reduced in PCP exposed fish.

During July, 1984 at the sublethal PCP concentrations, 22 and 49 µg PCP/L, Stage 2 oocytes were highly susceptible and showed a significant increase in atresia not observed in other stages among female rainbow trout maturing for the first time (Figure 19). While high levels of atresia among Stage 2 oocytes were recorded in PCP-exposed females undergoing oogenesis in early summer, a similar response was not observed in December, 1983. High levels of Stage 2 oocyte degeneration during early summer would significantly reduce the number of viable gametes available for fertilization.

The sublethal effects of PCP on early life history stages of rainbow trout have been previously reported

(Hodson and Blunt, 1981 and Dominguez and Chapman, 1984) however, there has been no research regarding the effects on developing eggs within the ovary of sexually maturing females. Sublethal sodium pentachlorophenate (NaPCP) caused a significant reduction in the fecundity and viability of eggs layed by the aquatic snail (Australorbis glabratus), following exposure to 50 and 100 ug NaPCP/L (Olivier and Maskins, 1960). Other organochlorine compounds, specifically polychlorinated biphenyls have caused proliferation of the smooth endoplasmic reticulum in developing rainbow trout and carp (Cyprinus carpio) oocytes (Freeman et al., 1980). The present study is the first report of the histopathological consequences of sublethal PCP exposure on oogenesis in maturing female rainbow trout.

In these female rainbow trout there are three possible explanations for the excessive atresia observed among Stage 2 oocytes following sublethal PCP exposure during early summer. They are 1) the inhibitory effects of PCP within the developing oocytes, 2) effects on hormones that control oogenesis and 3) effects on general metabolism and growth. In this study Stage 2 oocytes were the most numerous in the ovaries of these females maturing for the first time. The Stage 2 oocyte is characterized by the presence of the Balbiani body, an aggregate composed of ribonucleoproteins located in the perinuclear cytoplasm (Wallace and Selman, 1981). Organelles such as mitochondria, Golgi bodies, smooth

endoplasmic reticulum, multivesicular bodies and lipid granules have been shown to make up these aggregates (Beams and Kessel, 1973). Although their exact function remains unknown, they appear to be involved in active RNA-protein synthesis. Guraya (1979) suggests they are the center for formation and duplication of organelles and materials required by the oocyte prior to yolk deposition.

The Stage 2 oocyte containing the Balbiani body was severely affected during July probably because it was metabolically most active at this time. Pentachlorophenol is a metabolic poison as previously mentioned with a high affinity for mitochondria and a potent uncoupler of oxidative phosphorylation. At sublethal concentrations, PCP has been shown in vivo to significantly reduce hepatic succinic dehydrogenase (SD) in Notopterus notopterus (Dalela et al., 1980) and hepatic glucose-6-phosphate dehydrogenase (G-6-PD) in the yellow eel (Bostrom and Johansson, 1972). Both these enzymes, SD and G-6-PD have high activity in the mitochondria and Balbiani bodies respectively of immature oocytes in the following teleosts carp, Tilapia aurea and Mugil capito (Livni, 1971).

These enzymes provide crucial functions in the tricarboxylic acid cycle and oxidative phosphorylation at the mitochondrial level. Sublethal PCP may therefore affect the oocyte directly, disrupting the normal formation of the Balbiani body by binding to mitochondrial proteins and

inhibiting energy production.

The probability that hormones are involved in the significant increase of atresia among Stage 2 oocytes seems unlikely. The primary growth phase of oocytes in the teleost ovary, that is the period up to vitellogenesis, is said to occur independent of pituitary gonadotropin (de Vlaming, 1983 and Khoo, 1979) or gonadal steroid influence (Fostier et al., 1983). Any effects then of sublethal PCP on gonadotropins or steroids elaborated by the female should not manifest itself on oogenesis at this stage of development.

Sublethal PCP poisoning does significantly alter general metabolism and growth in fish and subsequently could affect ovarian development in sexually maturing females. The effects on metabolism and growth can be explained by PCP's mode of action which causes blockage of ATP production (NRCC, 1982) and inhibition of enzymes involved with energy metabolism in the liver (Bostrom and Johansson, 1972 and Holmberg et al., 1972). The effect of PCP toxicity has been measured in various studies and reflects the increased metabolic demands placed on the fish. Sublethal PCP exposure in the eel (Anguilla rostrata) doubled oxygen consumption (Holmberg and Saunders, 1979). Krueger et al. (1968) showed sublethal potassium pentachlorophenate to affect the cichlid (Cichlasoma bimaculatum) by increasing the cost of specific dynamic action, energy losses and the cost of

exercise due to swimming. Different authors have reported decreased growth in juvenile salmonids subjected to sublethal PCP (Webb and Brett, 1973, Chapman, 1969, and Dominguez and Chapman, 1984). The overall metabolic effects of sublethal PCP poisoning could affect ovarian development at the primary growth stage. Energy channelled to gonadal development in the maturing female rainbow trout may be reduced or re-directed during sublethal PCP exposure and this could account for the high incidence of oocyte degeneration found.

There was no statistically significant effect observed on the growth of the largest viable oocytes in the ovaries of maturing female rainbow trout exposed for 18 days to sublethal levels of PCP at either time of the year (Table 3 and 4). The exposure period at these PCP concentrations may not have been sufficient for significant ovarian growth effects to occur. By comparison, previous studies with salmonids on the significant effect sublethal PCP has on overall somatic growth involved similar ranges of toxicant concentrations but longer exposure periods (Webb and Brett, 1973 and Hodson and Blunt, 1981). A chronic sublethal PCP exposure of longer duration in maturing female rainbow trout may indicate significant ovarian growth effects.

In summary, this work has demonstrated that rainbow trout are sensitive to sublethal levels of PCP at specific stages in their reproductive cycle. Chronic sublethal PCP

produced toxic effects on early oogenesis in female rainbow trout maturing for the first time. This would reduce the number of viable gametes available for fertilization. Sublethal PCP may also affect the level of exogenous yolk during the period of exogenous vitellogenesis. Using a model where exogenous yolk was artificially induced by E2 treatment of immature rainbow trout, it was shown that 25 ug PCP/L could significantly reduce the levels of SPP and SCA when these yolk precursors are present at high levels in the blood. Finally, this study determined for the first time a high correlation between the indirect indicators SPP and SCA with vitellogenin. Regression equations were developed from the data that allow vitellogenin levels to be estimated from SPP and SCA in E2 treated immature rainbow trout.

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Appendix A

Stage	Class Range (um)	Nucleus	Nucleoli (\bar{X} #/10 um section)	Cytoplasm	Follicular layer
0	< 25	round, strongly basophilic	none visible	pale basophilic	none apparent
1	25 - 50	round, large and pale	1 - 3	lightly basophilic	scattered cells in the stroma
2	50 - 230	slightly ellipsoidal, eccentric	3 - 9, scattered	strongly basophilic Balbiani body, dispersing and fragmentation of the Balbiani body	incomplete single layer around oocyte
3	230 - 350	slightly ellipsoidal, central	9 - 20, mixed sizes, peripheral	Balbani body completely dispersed, cytoplasm lightly basophilic with strongly basophilic yolk nucleus	complete single layer surrounding oocyte
4	> 350	ellipsoidal, central, indented	> 20, peripheral	exogenous yolk vesicles in nuclear band, exogenous yolk vacuoles appear around oocyte periphery.	complete layer differentiating into cuboidal inner granulosa and outer thecal cells.

Appendix A: The morphological criteria used in classifying oocyte stages 0 - 4 in ovaries of maturing female rainbow trout (Salmo gairdneri).