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Exposure of Female Rainbow Trout (*Salmo gairdneri*) to Sublethal Cyanide During the Vitellogenic Stage of the Annual Reproductive Cycle

Helena Maria da Costa

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

June 1986

ISBN 0-315-35535-2

ABSTRACT

Exposure of Female Rainbow Trout (Salmo gairdneri) to Sublethal Cyanide During the Vitellogenic Stage of the Annual Reproductive Cycle.

Helena Maria da Costa

The annual reproductive cycle of female rainbow trout (Salmo gairdneri) was monitored using total serum calcium (TSCa), total serum phosphoprotein phosphorus (TSPP), the hepatosomatic (HSI) and gonadosomatic (GSI) indices. These parameters reflected the reproductive development of oocytes in the developing ovary.

The effect of sublethal cyanide exposure on female rainbow trout at different times of the vitellogenic period was investigated. A seasonal response was observed. In May, at the onset of vitellogenesis, exposure to a range of sublethal cyanide concentrations (0.01, 0.02 and 0.03 mg/L HCN) for 7 days resulted in higher TSCa and TSPP values. In October, elevated TSPP levels combined with declining TSCa and lower GSI, suggest an inhibition of exogenous yolk uptake by the ovary caused by low levels of calcium. In December, during late vitellogenesis, exposure to 0.01 mg/L HCN for 12 days of both mature naturally reproducing female rainbow trout and immature trout in which exogenous vitellogenesis was artificially induced by estradiol injections, resulted in lower TSCa and TSPP levels relative to controls. These results suggest a reduction in exogenous yolk production by the liver, hence, possibly decreasing the number of viable eggs available for the oncoming spawning time.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to everyone who assisted me. My sincere thanks to Dr. Sylvia M. Ruby for her guidance, criticism and encouragement throughout these studies. I would also like to extend my thanks to the members of my research advisory committee, Dr. Daphne Fairbairn and Dr. Gérard Leduc for their advice.

I am very grateful to Mr. Prasad Aysola for his valuable assistance, and also Ms. Diane Hastings who typed this thesis.

I would like to acknowledge my fellow graduate students, especially James Nagler, Daniel Cyr, Pierre Raymond, and Gonum Reddy-Williams for their help and support.

I would like to express my sincere appreciation to my father Alfredo M. da Costa and my uncle Fernando T. Perro, as well as the other members of my family and friends for their encouragement.

Finally, I am most indebted to my husband, Manouchehr Shahidi for his continual interest and support, and to my son Andrew Amir for being so patient.

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INTRODUCTION

In rainbow trout, gamete production is seasonal and requires precise coordination with environmental conditions. For salmonids, photoperiod appears to be the major controlling influence in the initiation of reproductive events, by triggering a series of neuroendocrine changes involving the hypothalamus, the pituitary gland and the gonads (Whitehead et al., 1978a). Female rainbow trout reach maturity for the first time in either their second or third year (Scott and Sumpter, 1983). The annual reproductive cycle can be divided into three physiological periods: (1) previtellogenesis; (2) vitellogenesis, which can be subdivided into an endogenous and exogenous phase, and (3) ovulation and spawning (van Bohemen et al. 1981).

The accumulation of yolk is responsible for the pronounced enlargement of oocytes during vitellogenesis. A part of the yolk is synthesized in the oocyte cytoplasm itself (endogenous yolk) whereas the remainder of the yolk is extraovarian in origin (exogenous yolk) (Wallace and Selman, 1981) and is the major contributor to ovarian growth. Figure 1 illustrates the sequence of events occurring during vitellogenesis. The hypothalamus produces a gonadotropin releasing hormone which stimulates the pituitary to secrete one or two gonadotropins (Burzawa-Gérard, 1982; Ng and Idler, 1983). Investigations by Ng and Idler (1983) suggest the existence of two gonadotropins: maturational and vitellogenic. The maturational gonadotropin is responsible for initiating vitellogenesis, at least

Figure 1: A schematic representation of the mechanism of vitellogenesis, illustrating the physiological interrelationship between the hypothalamus, pituitary, ovary and liver, in sexually maturing female rainbow trout.

VITELLOGENESIS

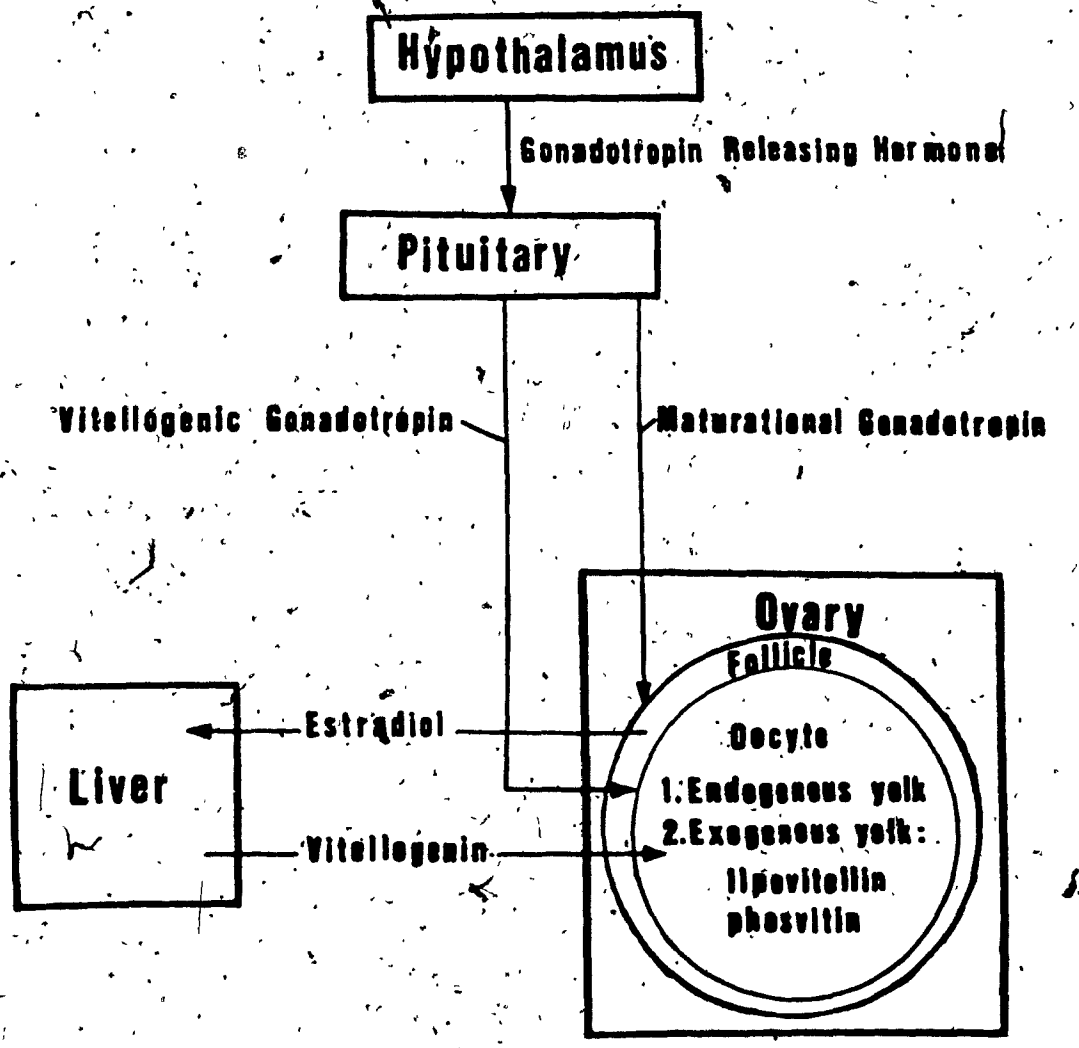

FIGURE 1

exogenous vitellogenesis, by inducing ovarian follicles to secrete estrogens, of which estradiol is the most active (Ng and Idler, 1983; van Bohemen and Lambert, 1981). Estradiol stimulates the liver to synthesize vitellogenin, which is then released into the circulation (Bailey, 1957). Vitellogenin is subsequently transported to the ovary, where it is incorporated by micropinocytosis into developing oocytes (Droller and Roth, 1966), and cleaved into the egg yolk proteins, phosvitin and lipovitellin, and deposited as yolk granules (Follet and Redshaw, 1974). This gonadal vitellogenin incorporation is under the control of the second pituitary hormone, the vitellogenic gonadotropin (Ng and Idler, 1983).

Vitellogenin is a female-specific calcium binding glycolipophosphoprotein (Wallace, 1978) and its presence in the blood of non-mammalian oviparous vertebrates is associated with increasing levels of serum components, especially calcium, phosphoprotein phosphorus and lipids (Black et al. 1971; Emmersen and Petersen, 1976), and with higher liver weights as expressed by the hepatosomatic index (van Bohemen et al., 1981). Different methods have been reported to determine serum vitellogenin concentrations in teleosts. Indirect methods, such as measurements of total serum calcium and total serum phosphoprotein phosphorus have been frequently reported (Bailey, 1957; Whitehead et al., 1978a,b; Elliott et al. 1979; Hori et al., 1979; Tinsley, 1985). Although, a direct radioimmunoassay has now been developed for vitellogenin (So et al. 1985), the technique was not available at the time of the present study. The indirect approach was utilized, measuring total serum calcium and total serum

phosphoprotein phosphorus as indicators of exogenous yolk. Nagler (1985) reported a strong correlation between these indirect indicators and vitellogenin levels measured by the radioimmunoassay in immature estradiol induced and naturally reproducing rainbow trout. So et al. (1985) also demonstrated a linear relationship between plasma vitellogenin levels and gonadal development expressed as the gonadosomatic index.

Exogenous yolk production can be induced in immature fish of both sexes by estradiol injections (Plack et al., 1971; Elliott et al., 1979; Cyr, 1984), thus, allowing the study of this process throughout the year.

The first objective of this study was to monitor the annual reproductive cycle of female rainbow trout by recording levels of total serum calcium and total serum phosphoprotein phosphorus and changes in the hepatosomatic and gonadosomatic indices during one year.

The second objective examines the effects of the toxicant cyanide on the annual reproductive cycle. Cyanide is a frequent, if not continuous, contaminant of terrestrial and aquatic ecosystems, and its presence can be attributed to both natural and man-made sources. Industrial sources of cyanide pollution in Canada include gold mining and milling industries, with common effluent discharges of cyanide in the mg/L range; photographic processing, ore extraction and iron and steel manufacturing (Leduc et al., 1982; Leduc, 1984).

Of the various cyanide compounds within the aquatic environment, free cyanide (HCN) is the most toxic, and is the form investigated in

most detail by researchers. In water, high concentrations of free cyanide rapidly kill aerobic organisms (Leduc, 1984). Several reports suggest that a concentration range of 0.003-0.005 mg/L HCN is an appropriate water quality objective for fresh water organisms in water at 10-13°C (Doudoroff et al., 1979; E.P.A., 1980). In the range of physiological pH normally encountered in surface water, HCN, a neutral hydrophilic molecule, will readily penetrate semipermeable membranes, such as the gills and egg membranes (Leduc, 1984). The majority of the HCN molecules will be carried to receptor sites via the bloodstream where toxic action and/or detoxification occurs. The mode of action of cyanide is generally attributed to the inhibition of the enzyme cytochrome oxidase, with consequent blockage of both electron transport and oxidative phosphorylation, thus depriving the cell of ATP energy and oxygen utilization (Solomonson, 1981). Raymond (1984) reported a 60-80% inhibition of cytochrome oxidase under sublethal cyanide exposure in rainbow trout. At sublethal levels, energy requirements may be partially maintained by anaerobic metabolism through glycolysis (Solomonson, 1981). At these levels, cyanide can also be detoxified to thiocyanate, a compound recently reported for rainbow trout by Raymond (1984), although thiocyanate itself is known to produce adverse effects in the thyroid gland (Way, 1981). Singh et al. (1977) described the pituitary-thyroid-gonad axis in the freshwater catfish (Heteropneustes fossilis) as particularly susceptible to thiocyanate toxicity.

Several reports in the literature deal with the sublethal effects of cyanide upon fish reproduction. Lind et al. (1977) exposed fathead

minnows (Pimephales promelas) for 256 days to various concentrations of cyanide and observed reduced egg production at 0.019 mg/L HCN and decreased hatchability at 0.044 mg/L HCN. A similar study by Koenst et al. (1977) also reported a reduction in the number of eggs spawned per female and in egg viability in brook trout (Salvelinus fontinalis) following exposure to 0.012 mg/L HCN for 144 days. Kimball et al. (1978) exposed bluegills (Lepomis macrochirus) to various concentrations of cyanide and determined that the highest concentration producing no adverse effects upon egg production was 0.0052 mg/L HCN. Leduc (1978) observed reduced hatching success and gross abnormalities of embryos at concentrations as low as 0.01 mg/L HCN in Atlantic salmon (Salmon salar) eggs.

More recently, Cheng and Ruby (1981) reported that flagfish (Jordanella floridae) exposed to intermittent sublethal cyanide concentrations (0.065-0.15 mg/L HCN) applied for 5-day periods during the embryonic, juvenile and adult stages of development, had egg production at sexual maturity reduced by 40%. In the same study, histological examination of the newly hatched fry revealed smaller pituitary glands caused by cyanide exposure which resulted in delayed sexual maturity. Lesniak and Ruby (1982) undertook a histological and quantitative study which demonstrated that oocyte maturation was seriously impaired in female rainbow trout exposed to 0.01-0.02 mg/L HCN for 15-20 days. In that study, the patterns of endogenous yolk formation were critically altered, but the stage that was the most seriously affected was exogenous yolk deposition.

Although these studies suggest that exposure to sublethal cyanide

interferes with egg production and development, little attention has been focused on the mechanisms that cause these effects. Based on the findings by Lesniak and Ruby (1982), the second objective of this study was to examine the effects of sublethal exposure to cyanide on the mechanisms of exogenous yolk production. Four parameters were selected for this purpose: total serum calcium, total serum phosphoprotein phosphorus, gonadosomatic and hepatosomatic indices. This objective was approached by: (i) exposing female rainbow trout to 0.01, 0.02 and 0.03 mg/L HCN for 7 days during early (May 1983) and mid-late (October 1982) vitellogenesis, to examine any seasonal effect of sublethal cyanide exposure on exogenous yolk production; (ii) exposing female rainbow trout to 0.01 mg/L HCN for 12 days at the peak of exogenous vitellogenesis (December 1982), to examine the effects of sublethal cyanide toxicity in highly vitellogenic females, prior to spawning; (iii) exposing rainbow trout, in which vitellogenesis was artifically induced with estradiol injections, to 0.01 mg/L HCN for 12 days in December 1983, to compare the response to sublethal cyanide exposure between naturally reproducing mature females (December 1982) and induced rainbow trout.

MATERIALS AND METHODS

A. Materials

Test Organisms

Rainbow trout, Salmo gairdneri, used in this study were obtained from La Pisciculture Mont Sutton, Sutton, Québec. Mean fish weights ($\bar{X}$ ± SEM) ranged from 226.37 ± 5.06 to 275.55 ± 10.06 grams, their length was 30-33 cm and age 2+.

Upon arriving at the Water Pollution Research Laboratory, fish were placed in fiberglass holding tanks and acclimated to laboratory conditions for a period of 2-4 weeks prior to the beginning of each experiment. Tanks were supplied with continuous flowing water, which was maintained at a constant temperature of 12 ± 1°C.

Diet

Fish were fed an artificial diet at all times. The diet, EWOS trout chow, consisted of dry pellets and was identical to that used by the hatchery where the fish were purchased. The components of the pelleted food as given by EWOS are listed in Table 1.

Fish were fed an ad libitum ration four times a week throughout the holding period, and 1% of the body weight per day, during the acclimation and experimental periods.

Table 1

Analysis of the dry trout chow pellets.

Component	Percentage, %
Crude protein (min.)	37.5
Crude fat (min.)	8.0
Crude fiber (max.)	5.0
Crude ash (max.)	13.0
Calcium (actual)	1.2
Phosphorus (actual)	1.0

Tanks

The test tanks consisted of four light blue fiberglass tanks. The tanks were oval with smooth inside surfaces. Tanks were drained by individual standpipes located in the middle of each tank and made of non-reactive PVC material. The capacity of these tanks was 243 liters. Adjustable flow meters (Manostat Corp., New York, N.Y.) maintained the desired flow rate of 4 L/min for each test tank. This flow of water provided a 99% replacement in 4.5 hours. This exceeded the minimum flow rate of 2.5 L of water/gram of fish/day as recommended by Sprague (1973). There were 8-10 fish per tank during the experimental period.

Each tank was covered with a hard translucent plastic top. Water entered the tank through a funnel placed in a hole at the top. The inflowing water from the flow meters and the toxicant from a Mariotte bottle entered the funnel simultaneously prior to entering the experimental tank. Their flow rates were monitored daily.

The toxicant was metered into the test tanks following the method outlined by Leduc (1966). Tanks were cleaned twice a week.

Illumination

Tanks were illuminated by fluorescent lights which were evenly distributed above the tanks. Lights were automatically controlled by a time-switch which provided a 12 hr light-12 hr dark photoperiod (7:30-19:30). The apparatus was enclosed by a black plastic curtain to minimize external disturbances to the fish.

Water Supply

The laboratory was supplied with water from the City of Montreal. Before reaching the test tanks it passed through activated charcoal filters which reduced the chlorine content to less than 0.01 ppm. The water was delivered to the tanks through PVC piping.

Measurements of dissolved oxygen indicated that, in general, O_2 levels were 90%, and never fell below 80% saturation throughout the experimental period.

Other chemical parameters of the water were provided by the City of Montreal Public Works Department, and are shown in the Table 2.

Table 2

Chemical characteristics of laboratory water used during the experimental period (March 1982 to December, 1983).

Alkalinity* $CaCO_3$ (ppm)	Total Hardness (ppm)	CO_2 (ppm)	pH
84	129	0.0	7.85

* mean values

B. Methods

Preparation of Cyanide Solutions

A stock solution of sodium cyanide was freshly prepared for each experiment and the concentration verified by titrations with silver nitrate following the method outlined by Epstein (1947).

Mariotte bottles containing the desired concentration of cyanide were prepared (Leduc, 1966) on the day prior to the start of the experiment. Sodium cyanide was introduced into the test tanks from the Mariotte bottles at a rate of 2 ml/min. The concentration of cyanide in the test water was determined every second day by the modified Konig reaction (Lambert et al., 1975) as follows: a sample of the test water was removed from the tank, placed in a 25 ml volumetric flask, 1 ml of a N-chlorosuccinimide-succinimide oxidizing reagent and 1 ml of a barbituric acid-pyridine reagent were added. The volumetric flasks were placed in a water bath at 25°C for 20 min. Following color development, absorbances were measured in a Bausch and Lomb spectrophotometer Model 70 at 595 nm. The concentrations were determined from a standard curve prepared in a similar manner and were within 10-12% of the predicted value.

Preparation and Injection of Estradiol 17B-3-Benzoate Solutions

Estradiol treatment in the December 1983 experiment involved the following procedures. Estradiol solutions were prepared by dissolving an appropriate amount of estradiol 17B-3-benzoate (Sigma Chemicals Co.) in peanut oil (Sigma Chemicals Co.) to produce a concentration of 5 mg of estradiol 17B-3-benzoate/Kg of body weight of fish. These

solutions were placed on a stirrer overnight. Prior to the injection each fish was lightly anesthetized with tricaine methane sulphonate (MS222) at a concentration of 30 mg/L, and based upon the weight given, an appropriate concentration of estradiol 17B-3-benzoate using a 100 ul Hamilton calibrated glass syringe. A disposable 26½ G needle (Yale stainless cannula) was placed on the syringe and the injection administered slightly below the right pectoral fin (intraperitoneal injection). Injections were delivered slowly to avoid backflow of the estradiol solution. Fish were placed in a tank containing continuously flowing water until they recovered from the anesthesia, and were then returned to the test tanks. Injections were administered on Days 0 and 3 of the experimental period.

Sampling Techniques

The following experimental sampling procedures were followed for all experiments. Fish were not fed on the day prior to sampling. Before sacrificing, each fish was anesthetized with tricaine methane sulphonate (MS222) at a concentration of 140 mg/L. The total body weight was recorded following removal of excess water with paper towel. The caudal peduncle was severed and blood was collected with a 5 cc disposable syringe. The plastic syringe was previously rinsed with an anticoagulant solution of 25% sodium citrate. The blood collected was gently forced out of the syringe into an Eppendorf microtest tube and placed on ice until centrifuging.

The sex of each fish was determined following dissection. Liver and gonad weights were recorded for later determinations of the

hepatosomatic index (HSI = wet liver weight x 100/wet body weight) and gonadosomatic index (GSI = wet ovary weight x 100/wet body weight). The midsection of one gonad was fixed in Bouin's solution for later histological observations.

The blood collected was centrifuged in a clinical centrifuge at full speed, for 15 minutes. The serum was separated and placed in a clean microtest tube and frozen in liquid nitrogen for 10 seconds. The tubes were stored at -70°C until further analysis.

Analysis of Serum for Total Calcium and Phosphoprotein Phosphorus

i) Total Calcium Determinations

Prior to determinations serum was thawed at room temperature. A 100 ul sample was placed in a 5 ml volumetric flask, using a 100 µl micropipette and diluted with a solution of 2000 ppm potassium chloride. The contents were mixed. Standards were freshly prepared. A Perkin-Elmer Atomic Absorption Spectrophotometer Model 503 equipped with a nitrous-oxide-acetylene flame was used for these determinations. The nitrous-oxide-acetylene flame was selected because of its greater reproducibility with higher signal output for calcium, and because it eliminates inter-elemental interferences (Bhattacharya, 1977). After a 20 minute warming period, the spectrophotometer was zeroed with the 2.0 g/L potassium chloride solution. The potassium chloride and the standard solutions were read after every four samples to maintain consistency of results.

Averages were calculated from standard and sample readings taken in triplicate. The Y-intercept, the slope and the correlation

coefficient were calculated for the absorbance-concentration curve by linear regression applied to the standards. The concentration of each sample was determined by the following equation:

$$\text{Concentration of sample} = \frac{\text{Absorbance of sample} - \text{Y intercept}}{\text{Slope}}$$

The results were converted to milligrams of calcium per 100 milliliters of serum (mg%).

ii) Determinations of Serum Phosphoprotein Phosphorus

100 ul of thawed serum of each sample were placed in a 15 ml Pyrex centrifuge tube. The proteins were precipitated with 5 ml of 10% trichloroacetic acid (TCA) solution. The precipitate formed was isolated by centrifugation and the supernatant discarded. The protein precipitate was washed successively with the following organic solvents to remove lipid (Wallace and Jared, 1968): (1) hot alcohol, (2) chloroform; petroleum ether:alcohol (1:2:2:), (3) acetone, and (4) ether. The resultant protein pellet was dried and assayed for phosphoprotein phosphorus using the Boehringer method (Boehringer Mannheim Test Handbook, 1970). In this method, proteins are digested with 0.5 ml of perchloric acid and 0.2 ml of hydrogen peroxide for 15 min in a paraffin bath at 180-200°C. The inorganic phosphate released forms a yellow complex in acid solution with ammonium vanadate and ammonium molybdate. The intensity of color is proportional to the concentration and was measured in a Bausch and Lomb spectrophotometer Model 70 at 390 nm.

Phosphorus standard solutions (10-120 µg/ml) were prepared and analyzed using the Boehringer method, but omitting the precipitation with TCA and the organic solvent washings.

Absorbance values measured were treated in a similar fashion as those for calcium. The phosphorus concentration of the samples was calculated by the same equation. The results were converted to micrograms of phosphorus per milliliter of serum (µg/ml).

Histological Preparations

The Bouin's fixative was removed from ovarian sections by several changes of 70% ethanol. Tissues were subsequently processed (dehydrated, cleared and infiltrated with paraffin) in a tissuematon (Dual Unit, Fisher Scientific Co.). Tissues were then embedded in paraffin using a tissue tek II Embedding Center (Lab Tek, Model 4603, Fisher Scientific Co.). Sectioning of the blocks was performed using a rotary microtome (Spencer AO 820 Model) set to yield 6 u sections. Sections were stained with the routine Hematoxylin and Eosin procedure for light microscopy and mounted with Permout resin.

Photographs were taken in a Leitz binocular and orthoplan microscope with a Leica camera, using a Panatomic-X film (ASA 32, Kodak).

Statistical Analysis

Data for each group is expressed as a mean ± standard error of the mean (SEM). Differences between groups were tested using a 1-factor or 2-factor analysis of variance (ANOVA) in an Apple II

personal computer. This was followed by the student-Newman-Keuls test (SNK) (Zar, 1984) for multiple comparisons among pairs of means, when significant differences occurred with ANOVA.

RESULTS

A. The Annual Reproductive Cycle of Female Rainbow Trout.

The annual reproductive cycle of female rainbow trout was monitored in the present study. Fish were sampled throughout the year. A new batch of fish was obtained each time and kept under a 12 hr light-12 hr dark photoperiod and temperature of 12 ± 1°C. The constant light-dark regime does not alter significantly ovarian development, since Whitehead et al. (1978b) reported that spawning occurred at approximately the same time in rainbow trout under the influence of a constant 12 hr light-12 hr dark photoperiod and those under a normal 12 month period. The male:female ratio was unpredictable accounting for the difference in sample sizes. The average weight of fish for this study was 265.91 ± 5.64 grams. Data were collected for the following parameters: hepatosomatic (HSI) and gonadosomatic (GSI) indices, total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) (Table 3, Fig. 2). Results indicate that there was a gradual increase in all parameters from April to October although the levels were not high enough to show a statistical difference. This was followed by a significant rise in GSI, TSCa and TSPP levels which peaked in December and returned to basal levels by February. Mean HSI values increased from 1.228 ± 0.022% in April to 1.610 ± 0.068% in December and returned to 1.384 ± 0.068% in February but differences were not significant. Changes were more visible for the remaining parameters. Mean GSI levels rose from 0.157 ± 0.008% in April to 0.777 ± 0.507% in October, reached a peak

of 13.558 ± 2.402% in December and declined to 0.247 ± 0.031% by February. Similarly, mean TSCa and TSPP levels increased from 16.572 ± 0.954 mg% and 9.212 ± 0.167 µg/ml, respectively, in April to 18.879 ± 0.769 mg% and 21.513 ± 2.794 µg/ml, respectively, in October. Between October and December there was a significant rise in mean levels of these two parameters, to maximum values of 27.713 ± 3.070 mg% for TSCa and 88.793 ± 16.647 µg/ml for TSPP. Levels declined thereafter to 18.169 mg% and 15.837 ± 3.191 µg/ml for TSCa and TSPP respectively, in February.

Histological observations of ovarian sections revealed gross morphological changes in oocytes throughout the year. The following series of pictures (Figs. 3 to 7) represents the various stages of oogenesis. The mean diameter of 10 randomly chosen oocytes/eggs was determined for each stage. In April, oocytes are predominantly small (mean diameter = 0.46 µ) (Fig. 3). In May, oocytes are larger (mean diameter = 1.32 µ), and have a well developed juxtanuclear Balbiani body (Fig. 4) and some oocytes have already started to exhibit yolk vesicles (Fig. 5). By October, the majority of the cells are bigger (mean diameter = 4.20 µ), and have various sizes of yolk vesicles within the ooplasm with the smallest spheres most peripheral (Fig. 6). In December, the ovaries occupy a large part of the coelom, and are full of developed eggs (mean diameter = 0.4 mm) (Fig. 7).

The present study also indicated that in December, the female population could be differentiated after sampling into two groups: the vitellogenic and non-vitellogenic based on the level of reproductive development. The vitellogenic group was discussed above. The

Table 3

The annual reproductive cycle of 2 year old female rainbow trout: means ± SEM for the hepatosomatic (HSI) and gonadosomatic (GSI) indices and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels (April 1982 to February 1983).

Month	n	HSI, %	GSI, %	TSCa, mg%	TSPP, ug/ml
April	3	1.228 ± 0.022	0.157 ± 0.008	16.572 ± 0.954	9.212 ± 0.167
May	5	1.416 ± 0.076	0.358 ± 0.189	17.265 ± 0.515	12.896 ± 2.792
June	12	1.338 ± 0.072	0.492 ± 0.077	19.325 ± 0.864	19.679 ± 2.789
October	6	1.407 ± 0.049	0.777 ± 0.507	18.879 ± 0.769	21.513 ± 2.794
December (vitellogenic)	7	1.610 ± 0.068	13.558 ± 2.402	27.713 ± 3.070	88.793 ± 16.647
December (non-vitellogenic)	7	1.421 ± 0.080	0.235 ± 0.371	16.833 ± 1.611	14.848 ± 0.383
February	10	1.384 ± 0.068	0.247 ± 0.031	18.169 ± 0.539	15.837 ± 3.191

Figure 2: The annual reproductive cycle of 2 year old female rainbow trout: profiles for the hepatosomatic and gonadosomatic indices, and total serum calcium and total serum phosphoprotein phosphorus levels from April, 1982 to February, 1983. Each point represents a mean.

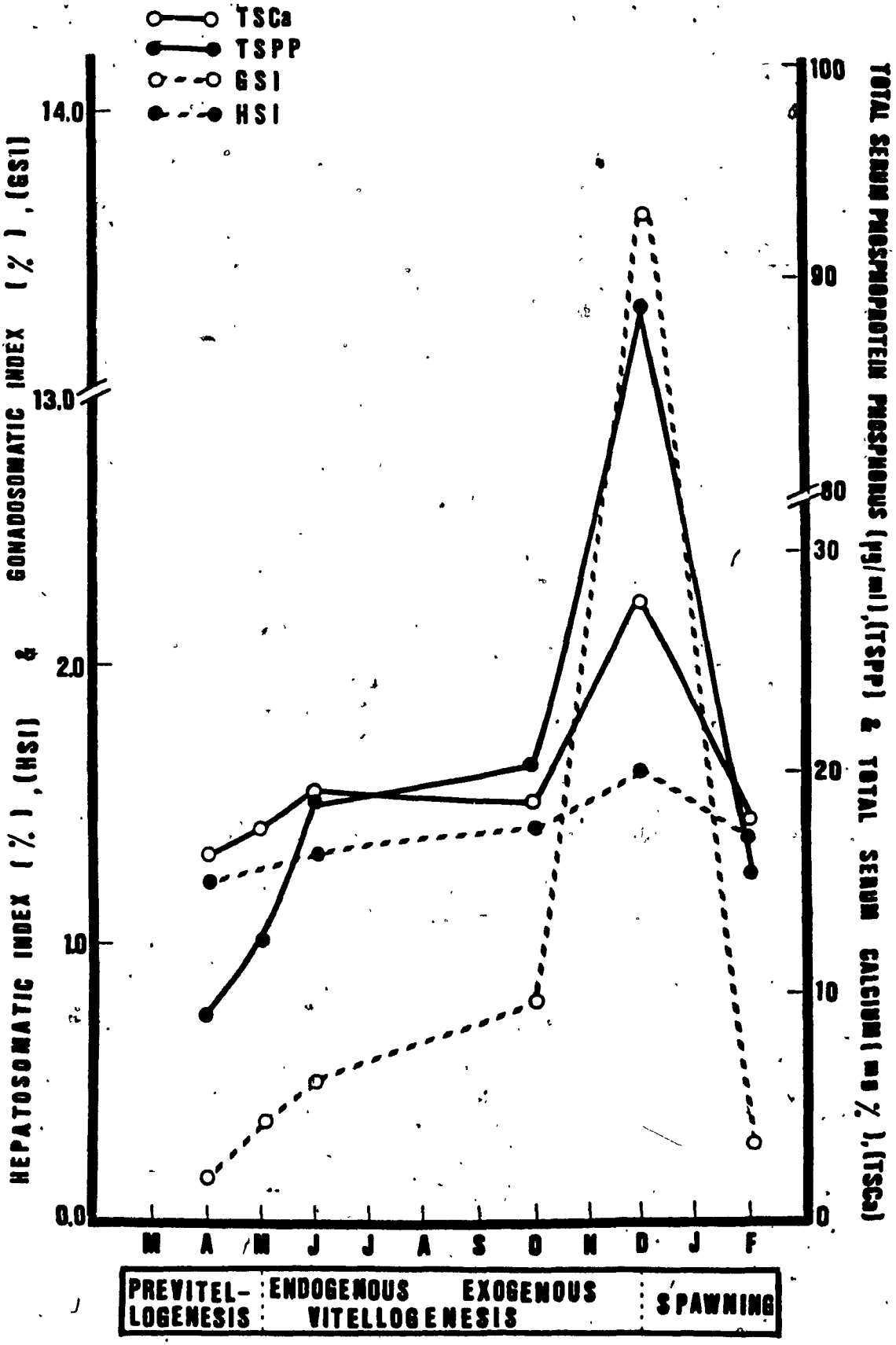
TSCa
TSPP
GSI
HSI
14.0
13.0
2.0
1.0
0.0
HEPATOSOMATIC INDEX (%), (HSI) & GONADOSOMATIC INDEX (%), (GSI)
100
90
80
30
20
10
0
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (mg/ml), (TSPP) & TOTAL SERUM CALCIUM (mg %), (TSCa)
M A M J J A S O N D J F
PREVITEL-LOGENESIS
ENDOGENOUS VITELLOGENESIS
EXOGENOUS
SPAWNING

FIGURE 2

Figure 3: Ovarian section from female rainbow trout taken in April, showing oocytes in early stages of development: (a) primary growth phase, and (b) larger oocytes which have initiated the formation of the Balbiani body. Hematoxylin and eosin, 100x.

Figure 4: Ovarian section, in May, showing oocytes with Balbiani bodies at various stages of development. (a) Earlier stage showing the juxtanuclear position of the Balbiani body; (b) slightly larger oocyte in which the Balbiani body has started to expand and migrate to the periphery; and (c) the Balbiani body is completely dispersed. Hematoxylin and eosin, 100x.

FIGURE 3

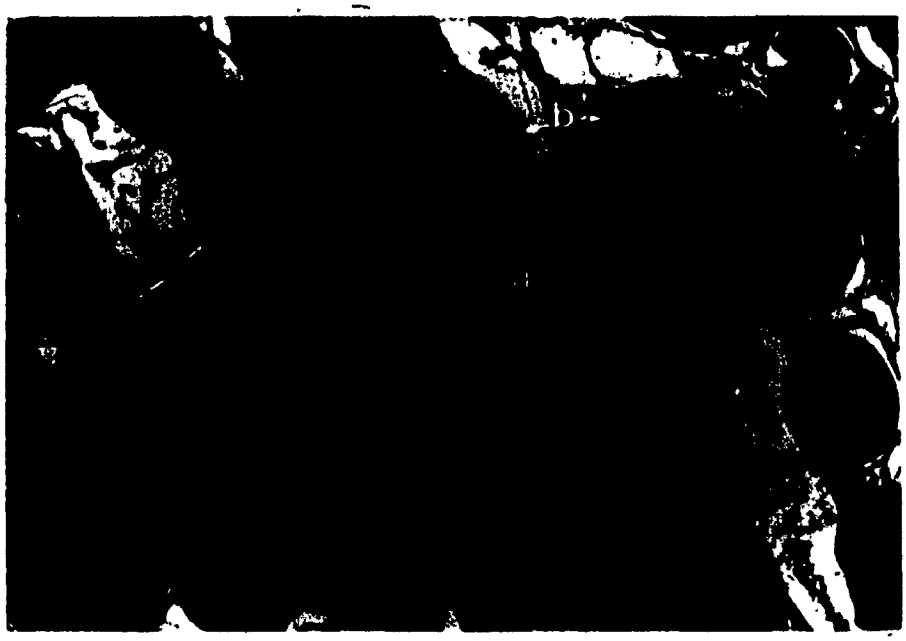

FIGURE 4

Figure 5: In May, some oocytes present are in the Balbiani body stage (a), while others (b) have initiated the accumulation of secondary yolk vesicles (yv). Hematoxylin and eosin, 100x.

Figure 6: In October, oocytes increase markedly in size and contain numerous secondary yolk vesicles (yv). Note that the nuclear membrane is absent and the nuclear contents have dispersed in the ooplasm. Hematoxylin and eosin, 100x.

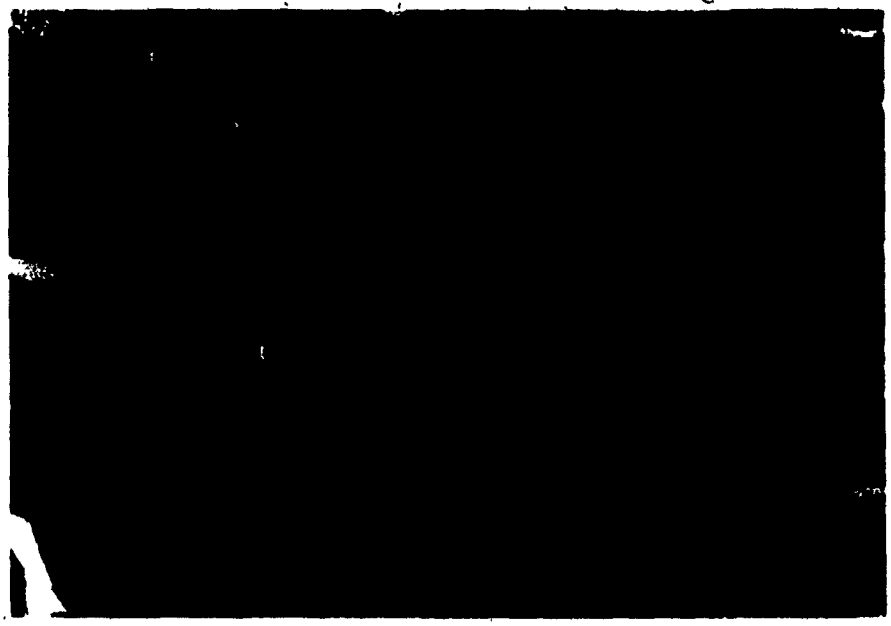

FIGURE 5

FIGURE 6

Figure 7: Eggs obtained by hand stripping a mature female rainbow trout in the laboratory, in December.

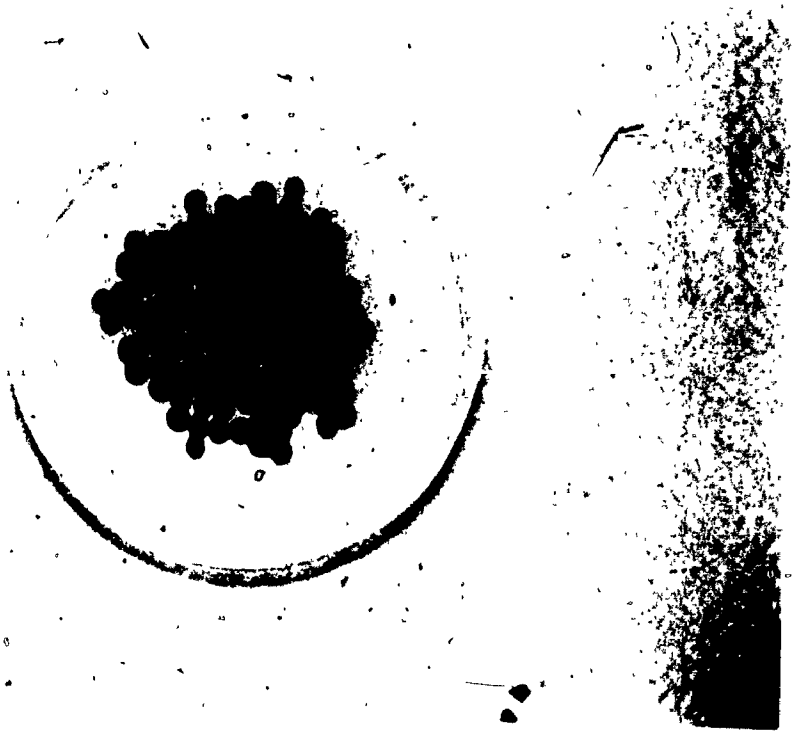

FIGURE 7

proportion of females that completed the reproductive cycle was approximately 30%. In the non-vitellogenic group the ovaries were undeveloped and appeared similar to those of fish sampled in April (Fig. 3). This was reflected in the lower levels obtained for all four parameters when compared to those of vitellogenic females.

B. Exposure to Sublethal Concentrations of Cyanide.

(i) Exposure to 0.01, 0.02 and 0.03 mg/L HCN for 7 days in October and May.

The first series of experiments was designed to examine the effects of exposure to sublethal concentrations of cyanide (0.01, 0.02 and 0.03 mg/L HCN) for 7 days, on HSI, GSI, TSCa and TSPP levels in female rainbow trout, All fish were sacrificed on Day 7. Experiments were performed in October and May, i.e., during the exogenous and endogenous vitellogenesis stages, respectively, of the reproductive cycle. Female rainbow trout had an average weight of 245.93 ± 10.48 grams in October, and 275.55 ± 10.06 grams in May.

In October, as seen in Table 4, the control values of the measured parameters remained constant over the 7 day experimental period. HSI values declined with increasing concentrations of HCN, from a control mean level of 1.407 ± 0.049% to 1.009 ± 0.130% recorded at the highest HCN concentration (Fig. 8A). The response of TSCa levels to HCN was nonlinear. The mean TSCa levels showed a sharp decline from control values of 18.879 ± 0.769 mg% to 7.024 ± 0.790 mg% (SNK test: p 0.01), following exposure to 0.01 mg/L HCN (Fig. 8C). Mean TSCa levels increased thereafter, with rising HCN concentration,

Table 4

Means ± SEM for the hepatosomatic (HSI) and gonadosomatic (GSI) indices, and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels in female rainbow trout, before and after exposure to 0.01, 0.02 and 0.03 mg/L of cyanide (HCN) for 7 days in October, 1982. Summary results for 1-factorial ANOVA are included.

[HCN], mg/l	n°	HSI, %	GSI, %	TSCa, mg%	TSPP, µg/ml
0.00 (Day 0)	5	1.452 ± 0.143	0.573 ± 0.204	22.054 ± 0.538	20.846 ± 3.085
0.00 (Day 7)	6	1.407 ± 0.049	0.777 ± 0.507	18.879 ± 0.769 (n=2)	21.513 ± 2.794 (n=2)
0.01 (Day 7)	7	1.361 ± 0.108	0.166 ± 0.046	7.024 ± 0.790 (n=4)	22.022 ± 4.415 (n=4)
0.02 (Day 7)	5	1.119 ± 0.115	0.238 ± 0.041	9.277 ± 1.011	33.058 ± 3.050
0.03 (Day 7)	5	1.009 ± 0.130	0.299 ± 0.084	14.327 ± 1.489	24.677 ± 4.789
F; df; p (Day 7)		3.297;3,19;p<0.005	n.s.	14.021;3,12;p<0.0005	4.084;3,12;p<0.05

Figure 8: The effect of exposure to 0.01, 0.02 and 0.03 mg/L of cyanide (HCN) for 7 days, on the hepatosomatic and gonadosomatic indices, and on the levels of total serum calcium and total serum phosphoprotein phosphorus in female rainbow trout during October, 1982. Each point represents a mean ± SEM. SEM is not shown when it is <0.1.

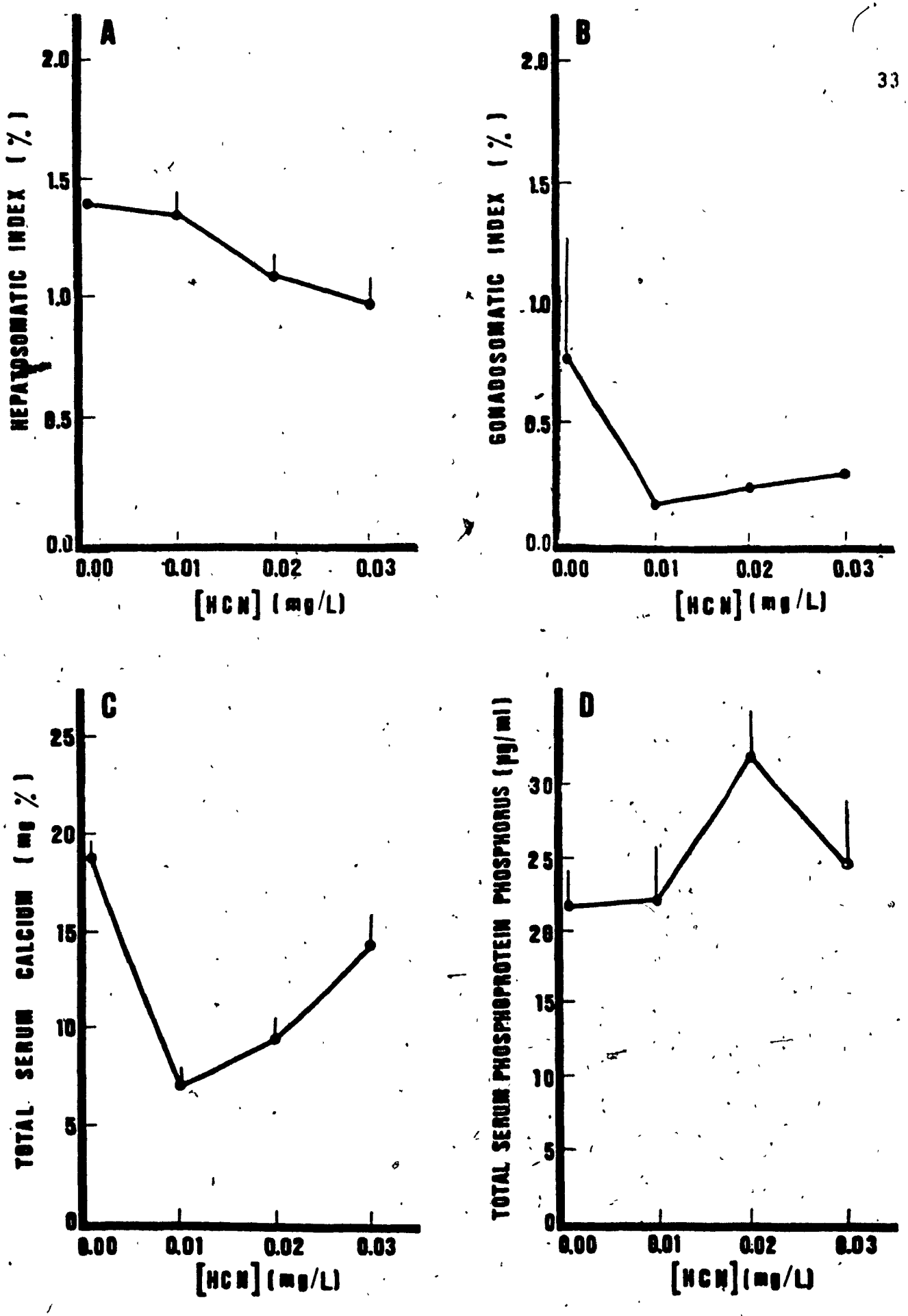
A
HEPATOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
B
GONADOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
C
TOTAL SERUM CALCIUM (mg %)
25
20
15
10
5
0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
D
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (µg/ml)
30
25
20
15
10
5
0
0.00
0.01
0.02
0.03
[HCN] (mg/L)

FIGURE 8

concentration, but at 0.03 mg/L HCN, TSCa values were still reduced (14.327 ± 1.489 mg%) although not significantly relative to controls. Exposure to 0.02 mg/L HCN elevated significantly mean TSPP levels to 33.058 ± 3.050 µg/ml from 21.513 ± 2.794 µg/ml reported for control females (SNK test: $p < 0.01$) (Fig. 8D). TSPP levels, then declined significantly to 24.677 ± 4.789 µg/ml at 0.03 mg/L HCN relative to 0.02 mg/L HCN (SNK test: $p < 0.05$), but were not statistically different from control levels.

The GSI demonstrated a trend towards lower mean values following exposure to all HCN concentrations, although the reduction was not significantly different when compared to controls (Fig. 8B). There was a large animal-to-animal variation in GSIs within the control group, which had a mean value of 0.777 ± 0.507%. The lowest GSI recorded was 0.166 ± 0.046% at the lowest concentration of HCN.

When the same experimental procedure was repeated in May, during early vitellogenesis, a different response was observed for the following parameters: TSCa, TSPP and GSI, after exposure to HCN (Table 5). Similar to that observed in October (Table 4) the measured parameters remained stable over the 7 day period. As seen in the previous experiment mean HSI values declined at all HCN concentrations (Fig. 9A). Mean HSI values for the control group were recorded at 1.416 ± 0.076%, then declined to 1.141 ± 0.093% at 0.01 mg/L HCN and stayed similar at the remaining HCN concentrations. Mean TSCa and TSPP levels increased simultaneously, with rising HCN concentrations from control values of 17.265 ± 0.515 mg% and 12.896 ± 2.792 µg/ml respectively, to maximal levels of 23.290 ± 1.126 mg% for TSCa and

Table 5

Means ± SEM for the hepatosomatic (HSI) and gonadosomatic (GSI) indices, and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels in female rainbow trout, before and after exposure to 0.01, 0.02 and 0.03 mg/L of cyanide (HCN) for 7 days in May, 1983. Summary results for 1-factorial ANOVA are included.

[HCN], mg/l	n	HSI, %	GSI, %	TSCa, mg%	TSPP, µg/ml
0.00 (Day 0)	4	1.398 ± 0.046	0.310 ± 0.029	17.332 ± 0.762	11.983 ± 1.745
0.00 (Day 7)	5	1.416 ± 0.076	0.358 ± 0.189	17.265 ± 0.515	12.896 ± 2.792
0.01 (Day 7)	6	1.141 ± 0.093	0.208 ± 0.027	16.630 ± 0.872	20.343 ± 1.185
0.02 (Day 7)	8	1.011 ± 0.557	0.436 ± 0.048	22.142 ± 0.455	21.175 ± 2.427
0.03 (Day 7)	9	1.149 ± 0.063	0.344 ± 0.048	23.290 ± 1.126	22.110 ± 1.561
F; df; p (Day 7)		4.877;3,24;p<0.01	n.s.	8.450;3,24;p<.001	5.815;3,24;p<0.005

Figure 9: The effect of exposure to 0.01, 0.02 and 0.03 mg/L of cyanide (HCN) for 7 days, on the hepatosomatic and gonadosomatic indices, and on the levels of total serum calcium and total serum phosphoprotein phosphorus in female rainbow trout, during May, 1983. Each point represents a mean ± SEM. SEM is not shown when it is <0.1.

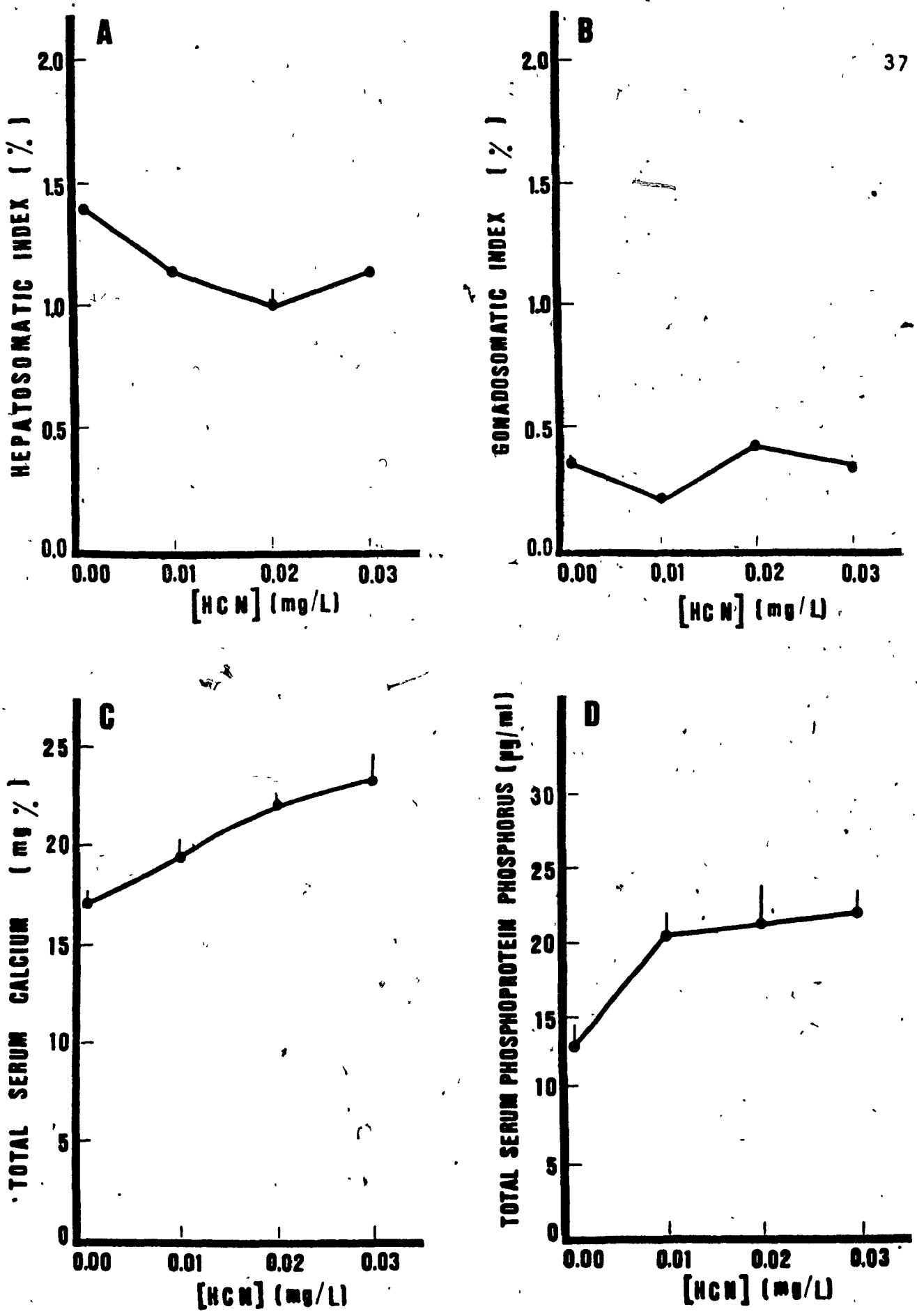
A
HEPATOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
B
GONADOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
C
TOTAL SERUM CALCIUM (mg %)
25
20
15
10
5
0
0.00
0.01
0.02
0.03
[HCN] (mg/L)
D
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (µg/ml)
30
25
20
15
10
5
0
0.00
0.01
0.02
0.03
[HCN] (mg/L)

FIGURE 9

22.110 ± 1.561 µg/ml for TSPP, at the highest HCN concentration (Figs. 9C and 9D). These changes represent a significant increase in the levels of these two parameters in the serum of female rainbow trout following exposure to HCN (Table 5). The GSI showed no significant change in mean values between HCN treated fish and controls (Fig. 9B).

(ii) Exposure to 0.01 mg/L HCN for 12 days in December.

The reduction in TSCa observed at 0.01 mg/L HCN in October after a 7 day exposure, led to the investigation of the effects of this sublethal concentration of HCN over a longer time period during active exogenous vitellogenesis. Fish were exposed to 0.01 mg/L HCN for 12 days in December and sampled on Days 1, 3, 6 and 12 of the experimental period. The average weight of females in this study was 255.25 ± 7.35 grams. The female population was divided after sampling into two groups based upon the level of reproductive development of the ovaries: non-vitellogenic and vitellogenic.

In the non-vitellogenic group (Table 6) control and HCN exposed females were similar for the four indirect indicators of vitellogenesis. Mean TSPP (Fig. 10D) and GSI (Fig. 10B) values remained similar between the two groups throughout the experimental period. Mean TSPP levels, on Day 12, were 10.995 ± 0.513 µg/ml in controls and 10.174 ± 1.221 µg/ml in HCN exposed fish. Mean GSI values were similar for control and HCN treated groups for the duration of the experiment. Changes in mean HSI values (Fig. 10A) followed an identical pattern for control and HCN treated fish, during

Table 6

Means ± SEM for the hepatosomatic (HSI) and gonadosomatic (GSI) indices, and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels in non-vitellogenic female rainbow trout, after exposure to 0.01 mg/L of cyanide (HCN) for 12 days in December, 1982.

Day	[HCN], mg/l	n	HSI, %	GSI, %	TSCa, mg%	TSPP, µg/ml
1	0.00	2	1.222 ± 0.186	0.224 ± 0.005	14.065 ± 0.545	10.739 ± 0.256
	0.01	3	1.303 ± 0.237	0.243 ± 0.069	15.649 ± 1.922	11.508 ± 1.777
3	0.00	2	1.723 ± 0.413	0.247 ± 0.002	16.969 ± 0.857	11.508 ± 0.793
	0.01	3	1.568 ± 0.077	0.248 ± 0.036	14.383 ± 0.224	11.680 ± 0.342
6	0.00	2	1.438 ± 0.070	0.248 ± 0.046	13.807 ± 1.300	10.483 ± 1.539
	0.01	3	1.323 ± 0.166	0.245 ± 0.029	14.597 ± 0.753	9.627 ± 0.452
12	0.00	2	1.455 ± 0.100	0.242 ± 0.002	20.468 ± 0.395	10.955 ± 0.513
	0.01	5	1.407 ± 0.171	0.292 ± 0.025	16.795 ± 1.640	10.174 ± 1.221

Figure 10: The effect of exposure to 0.01 mg/L of cyanide (HCN) for 12 days, on the hepatosomatic and gonadosomatic indices, and on the levels of total serum calcium and total serum phosphoprotein phosphorus in non-vitellogenic female rainbow trout during December, 1982. Each point represents a mean ± SEM. SEM is not shown when it is <0.1.

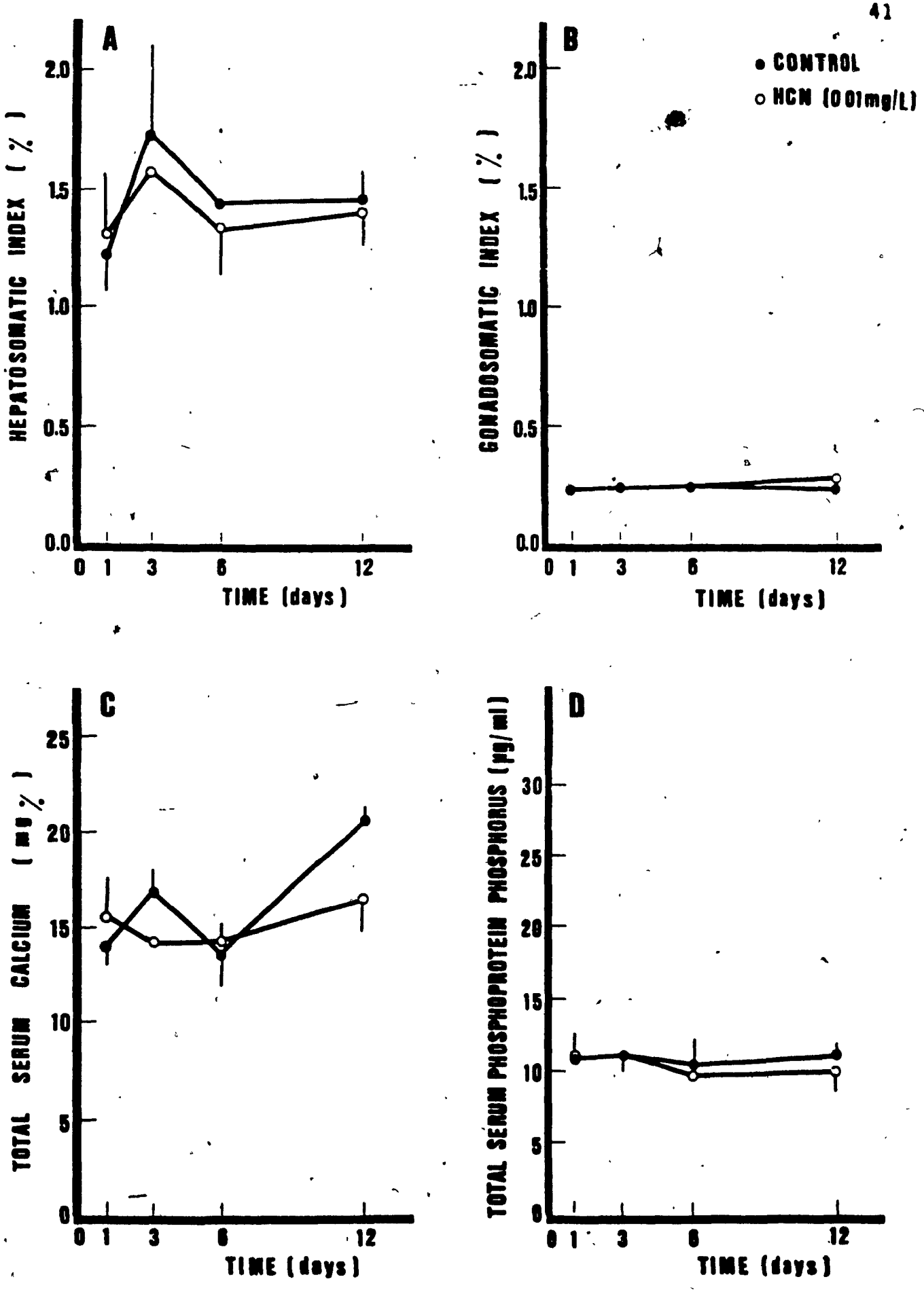
A
HEPATOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0 1 3 6 12
TIME (days)
B
GONADOSOMATIC INDEX (%)
CONTROL
HCN (0.01mg/L)
C
TOTAL SERUM CALCIUM (mg %)
25
20
15
10
5
0
D
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (µg/ml)
30

FIGURE 10

Table 7

Data for the hepatosomatic (HSI) and gonadosomatic (GSI) indices, and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels in vitellogenic female rainbow trout, after exposure to 0.01 mg/L of cyanide (HCN) for 12 days in December, 1982.

Day	[HCN], mg/l	n	HSI, %	GSI, %	TSCa, mg%	TSPP, µg/ml
1	0.00	1	1.252	9.066	25.212	97.193
	0.01	1	1.681	6.320	17.083	39.215
3	0.00	-	-	-	-	-
	0.01	-	-	-	-	-
6	0.00	2	1.610 ± 0.088	9.370 ± 1.287	24.317 ± 5.616	87.269 ± 3.897
	0.01	1	1.936	9.803	28.038	77.696
12	0.00	4	1.677 ± 0.556	11.695 ± 2.359	30.043 ± 4.078	92.448 ± 20.577
	0.01	2	1.640 ± 0.041	6.909 ± 0.188	17.751 ± 0.668	39.215 ± 0.952

Figure 11: The effect of exposure to 0.01 mg/L of cyanide (HCN) for 12 days, on the hepatosomatic and gonadosomatic indices, and on the levels of total serum calcium and total serum phosphoprotein phosphorus in vitellogenic female rainbow trout during December, 1982.

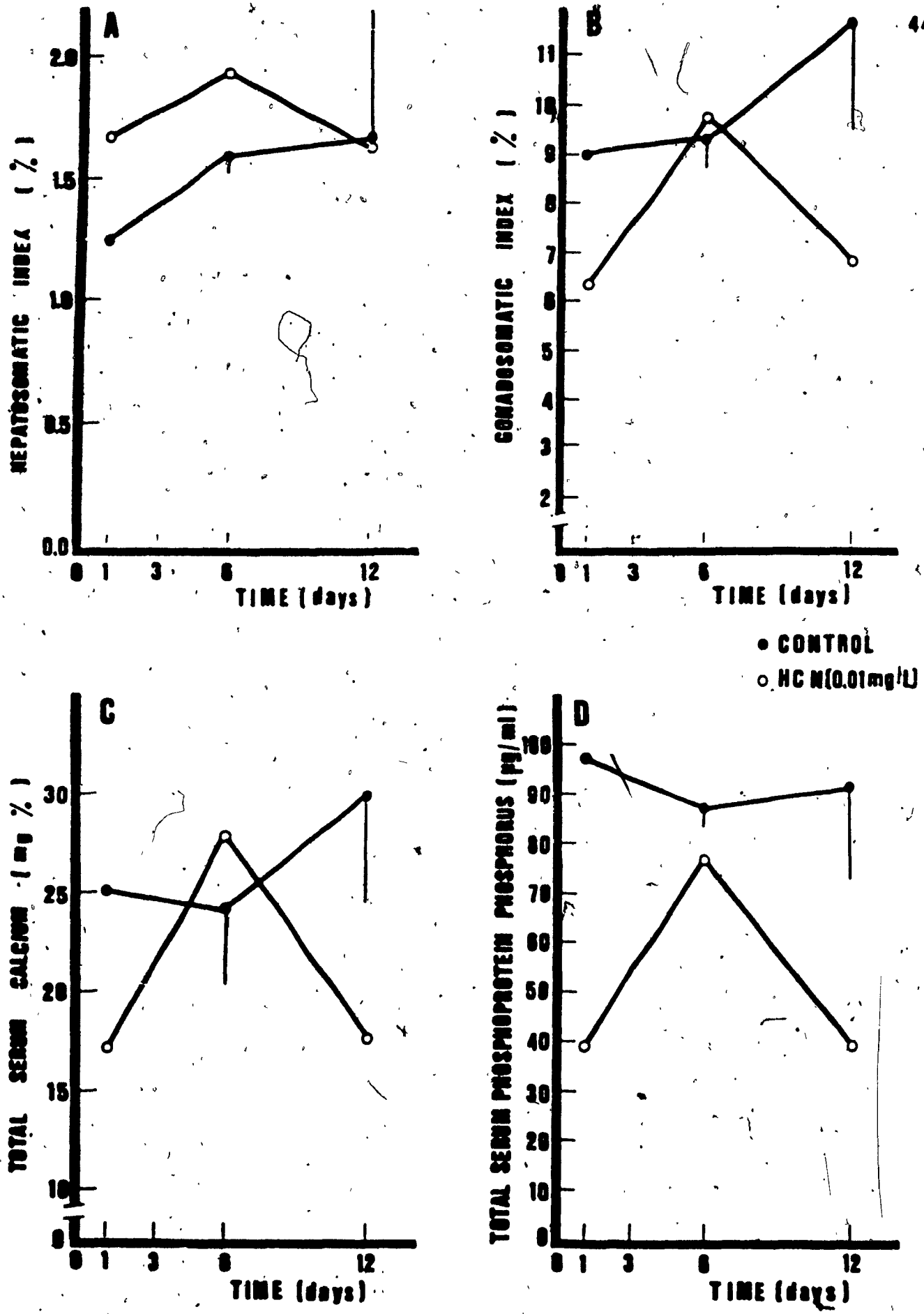
A
HEPATOSOMATIC INDEX (%)
2.0
1.5
1.0
0.5
0.0
0 1 3 6 12
TIME (days)
B
GONADOSOMATIC INDEX (%)
11
10
9
8
7
6
5
4
3
2
0 1 3 6 12
TIME (days)
• CONTROL
○ HC N(0.01mg/L)
C
TOTAL SERUM CALCIUM (mg %)
30
25
20
15
10
0
0 1 3 6 12
TIME (days)
D
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (µg/ml)
100
90
80
70
60
50
40
30
20
10
0
0 1 3 6 12
TIME (days)

FIGURE 11

the 12 day period. Both groups showed an elevation in mean HSI levels at Day 3 to 1.723 ± 0.413% for controls and 1.568 ± 0.077% for HCN exposed fish, when compared to the remaining sampling days. On Day 12, mean TSCa levels were 20.468 ± 0.395 mg% for controls and 16.795 ± 1.640 mg% in HCN treated fish but no significant differences occurred (Fig. 10C).

Exposure to sublethal HCN on vitellogenic fish (Table 7) showed a trend towards lower mean GSI, TSCa and TSPP levels although changes were not significant as a result of small sample sizes. Vitellogenic females represented approximately 30% of the total female population. At the end of the experimental period, mean GSI values in HCN treated fish (Fig. 11B) had declined from control values of 11.695 ± 2.359% to 6.909 ± 0.188%. Similarly, mean TSCa (Fig. 11C) and TSPP (Fig. 11D) levels dropped from 30.043 ± 4.078 mg% and 92.448 ± 20.577 µg/ml, respectively, reported for controls to 17.751 ± 0.688 mg% for TSCa and 39.215 ± 0.952 µg/ml for TSPP in HCN exposed mature females.

(iii) Exposure of E_2 injected rainbow trout to 0.01 mg/L HCN for 12 days in December.

The small proportion of females undergoing full vitellogenesis in the previous December 1982 experiment led to its artificial induction in the following year. Hence, in December 1983, the effect of sublethal cyanide exposure, on rainbow trout in which exogenous vitellogenesis was artificially induced by estradiol-17β was examined (Table 8). In this experiment both non-vitellogenic female and male rainbow trout were utilized, since males and females respond similarly

Table 8

Means ± SEM for the hepatosomatic (HSI) and gonadosomatic (GSI) indices, and for total serum calcium (TSCa) and total serum phosphoprotein phosphorus (TSPP) levels after estradiol-17β (E_2) and/or cyanide (HCN) treatment in rainbow trout, during December, 1983. Results for the 2-factorial ANOVA are included.

Day	Group	n	HSI, %	GSI, %	TSCa, mg%	TSPP, ug/ml
1	Control	5	0.832 ± 0.102	0.300 (n=1)	18.204 ± 1.602	16.850 ± 1.544
	E_2	5	0.931 ± 0.074	0.257 ± 0.026 (n=3)	22.718 ± 2.018	22.719 ± 2.018
	HCN	5	0.954 ± 0.065	0.244 ± 0.038 (n=2)	19.882 ± 1.649	19.250 ± 1.651
	E_2 & HCN	5	1.039 ± 0.088	0.509 (n=1)	19.906 ± 2.004	19.906 ± 2.004
7	Control	5	1.151 ± 0.119	0.278 ± 0.017 (n=2)	25.266 ± 1.764	20.166 ± 2.538
	E_2	5	1.990 ± 0.121	0.326 ± 0.023 (n=2)	30.199 ± 1.845	77.750 ± 7.850
	HCN	5	1.220 ± 0.082		18.868 ± 1.086	22.315 ± 3.146
	E_2 & HCN	5	1.347 ± 0.109	0.220 (n=1)	24.333 ± 2.315	85.950 ± 3.296
12	Control	5	1.237 ± 0.039	0.254 ± 0.009 (n=3)	18.823 ± 3.508	14.375 ± 2.633
	E_2	5	2.052 ± 0.286	0.206 ± 0.012 (n=2)	46.006 ± 4.107	144.250 ± 31.258
	HCN	5	1.299 ± 0.136	0.320 ± 0.020 (n=2)	17.778 ± 1.415	12.050 ± 2.518
	E_2 & HCN	3	2.212 ± 0.155	--	32.483 ± 6.259	101.750 ± 23.849
	Time		F = 35.940 df = 2,46;p < 0.01	n.s.	F = 11.513 df = 2,43;p < 0.01	F = 37.351 df = 2,43;p < 0.01
	Treatment		F = 14.502 df = 3,46;p < 0.01	n.s.	F = 18.521 df = 3,43;p < 0.01	F = 53.674 df = 3,43;p < 0.01
	Interaction		F = 5.076 df = 6,46;p < 0.01	n.s.	F = 6.465 df = 6,43;p < 0.01	F = 16.711 df = 6,43;p < 0.01

Figure 12: The effect of sublethal cyanide exposure (0.01 mg HCN/L) on the hepatosomatic and gonadosomatic indices, and on the levels of total serum calcium and total serum phosphoprotein phosphorus in rainbow trout treated with estradiol 17B (5 mg E_2/Kg body weight) during December, 1983. Each point represents a mean ± SEM.

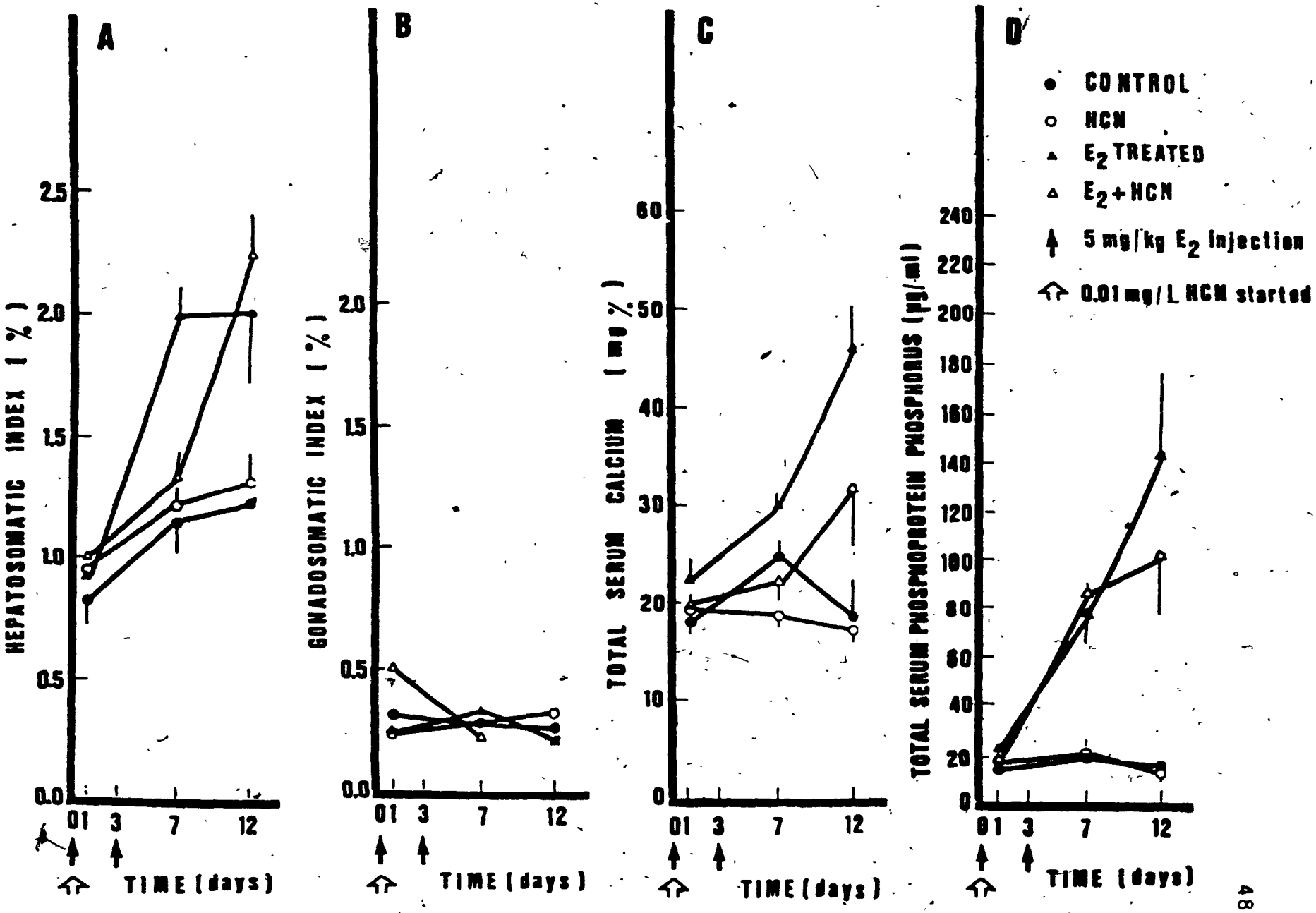
A
B
C
D
HEPATOSOMATIC INDEX (%)
GONADOSOMATIC INDEX (%)
TOTAL SERUM CALCIUM (mg %)
TOTAL SERUM PHOSPHOPROTEIN PHOSPHORUS (µg/ml)
TIME (days)
0 1 3 7 12
CONTROL
HCN
E2 TREATED
E2 + HCN
5 mg/kg E2 injection
0.01 mg/L HCN started

FIGURE 12

to E_2 treatment (Cyr, 1984). The average weight of fish was 226.37 ± 5.06 grams.

E_2 administration (5 mg E_2/Kg body weight) resulted in higher mean HSI, TSCa and TSPP levels when compared to the untreated groups. Sublethal HCN exposure (0.01 mg/L) for 12 days, resulted in significantly lower mean TSCa and TSPP levels in the E_2 treated HCN exposed group relative to the E_2 treated control group (Fig. 12). Mean TSCa levels in the E_2 control fish, at Day 12, were 46.006 ± 4.107% and in the E_2 & HCN group 32.483 ± 6.259 mg% (SNK: $p < 0.01$). The recorded mean TSPP levels, at Day 12 were 144.250 ± 31.258 and 101.750 ± 23.849 µg/ml for the E_2 and E_2 & HCN groups, respectively (SNK: $p < 0.05$). The GSI remained unchanged throughout the experimental period.

DISCUSSION

A) Annual reproductive cycle of female rainbow trout.

The present study provided profiles of GSI, HSI, TSCa and TSPP throughout the annual reproductive cycle of female rainbow trout (Table 3). This data also served as a baseline control for the toxicological experiments performed at various stages of the reproductive cycle.

The annual reproductive cycle of the female rainbow trout, Salmo gairdneri, is divided into three main stages: a pre-vitellogenic period (March-April), a vitellogenic period (May-December) which is subdivided into endogenous (May-July) and exogenous (August-December) vitellogenesis, and finally ovulation and spawning (January-February) (van Bohemen et al., 1981).

In the present study, during the pre-vitellogenic period, GSI, HSI, TSCa and TSPP values were the lowest recorded for the annual cycle. This stage is the quiescent period and the present data are supported by the low levels of sex steroids in the plasma reported by Scott and Sumpter (1983). At the ovarian level, this stage is characterized by the presence of small oocytes, with a high nucleus to cytoplasm ratio (de Vlaming, 1983) (Fig. 3). Scott and Sumpter (1983) suggested that the selection of oocytes that will develop into eggs during the oncoming reproductive cycle may occur during this stage.

During the endogenous phase of the vitellogenic period, there was an increase in all four parameters. In this phase, the oocytes synthesize a basophilic material which is stored within yolk vesicles

(Wallace and Selman, 1981). The Balbiani body is a possible source of endogenous yolk (Beams and Kessel, 1973 and Lesniak and Ruby, 1982) (Fig. 4). This endogenous yolk synthesis is accompanied by a rise in GSI. The gradual increase in TSCa and TSPP observed during this period suggests that small amounts of exogenous yolk are also being synthesized. Similarly, Ruby et al. (1986a) reported vitellogenin (Vg) in serum of rainbow trout using a direct radioimmunoassay (RIA) as early as June, indicating exogenous yolk precursors were synthesized. Histological observations of ovaries during this stage (Fig. 5) confirms the presence of exogenous yolk. Therefore, endogenous and exogenous processes overlap, the former predominating initially and the latter during the final stages of the vitellogenic period.

The exogenous vitellogenesis stage was marked by a sharp increase in all parameters investigated, between October and December. During this period, vitellogenin (Vg) is synthesized by the liver in response to estrogen produced from the ovary, released in the circulation, and sequestered, under gonadotropin control, by the oocytes (Bailey, 1957; Wallace and Selman, 1981). The increase observed in HSI in this study, during this period possibly represents synthesis of Vg, since electron microscope studies of hepatocytes by Emmersen and Petersen (1976), showed that HSI peaked concurrently with maximal levels of RNA in the liver and Vg in the serum. During exogenous vitellogenesis, the hepatocytes undergo dramatic structural changes: rough endoplasmic reticulum (rER) is strongly developed, Golgi bodies are enlarged with electron dense inclusions, and the nucleus and nuceolus present a

hypertrophied appearance, indicating active protein synthesis (Aida et al., 1973; van Bohemen et al., 1981).

Vg is subsequently released in the bloodstream and was detected, in this study, by a rapid increase in TSCa and TSPP levels between October and December. Whitehead et al. (1978a) observed an identical increase in these parameters, during these months, although the levels reported were higher. The same response was observed during these months with direct serum Vg measurements by van Bohemen et al. (1981) for rainbow trout, using gel electrophoresis, and by So et al. (1985) in landlocked salmon (Salmo salar Ouananiche) using the recently developed homologous RIA. Nagler (1985) using both indirect (TSCa and TSPP) and direct (RIA) Vg determinations showed a strong correlation between these parameters in rainbow trout during late vitellogenesis.

Vg is then sequestered into developing oocytes by micropinocytosis (Droller and Roth, 1966; Campbell, 1978), converted to the yolk proteins lipovitellin and phosvitin, and accumulated in yolk globules which later coalesce (Follet and Redshaw, 1974; Wallace, 1978; Wallace and Selman, 1981). This ovarian incorporation of large quantities of exogenous yolk was reflected in high GSI measurements recorded for this study during this stage. Figs. 6 and 7 show the oocyte growth that occurred between October and December. So et al. (1985) report similar changes in GSI in landlocked salmon and showed a strong correlation between this index and Vg levels.

It was observed that in December, at the peak of exogenous yolk production, only a small proportion (approximately 30%) of the female population had high GSI, HSI, TSCa and TSPP levels, i.e. was fully

mature at age 2+. Female rainbow trout spawn for the first time in either the second or third year (Scott and Sumpter, 1983) and after that, they do not necessarily undergo the reproductive cycle every year, which may account for the percentage of maturing females obtained in this study.

The final stage of the reproductive cycle comprises oocyte maturation, ovulation and spawning (de Vlaming, 1983) and is gonadotropin dependent (Ng and Idler, 1983; Scott et al., 1983). During this period, a sharp decline in the four parameters was observed in this study. Scott and Sumpter (1983) report that estradiol 17B levels are low, whereas gonadotropin and progesterone (17α-20βP and 17α-P) levels are elevated at this time. During this phase, oocytes are ovulated into the ovarian lumen and subsequently spawned under favorable environmental conditions (de Vlaming, 1983). However, spawning behaviour does not naturally occur in captive female rainbow trout and ova remain in the body cavity after ovulation (Bry, 1981). This was confirmed in the present study, when females sampled in February had the coelom full of atretic eggs.

The present study represents the first investigation when all four parameters were simultaneously monitored throughout one complete reproductive cycle. There was a marked rise in GSI, TSCa and TSPP between October and December, and these parameters were good indicators of the annual growth of the ovaries. Changes in HSI were less dramatic. Fluctuations in this indicator may reflect other metabolic processes. However, when used in combination with other parameters, it may provide additional information regarding the

process of vitellogenesis.

B - Exposure to sublethal concentrations of cyanide.

(i) Exposure to 0.01, 0.02 and 0.03 mg/L HCN for 7 days in October and May.

Exposure to sublethal concentrations of cyanide in October affects the process of exogenous vitellogenesis in female rainbow trout. Mean TSCa, GSI and HSI values were reduced whereas TSPP levels were higher when compared to controls. TSCa and TSPP should parallel each other when rising levels of these indirect indicators represent exogenous yolk precursors (section A; Whitehead et al., 1978a), but in this experiment they responded differently, TSCa levels were significantly reduced at 0.01 and 0.02 mg/L HCN while TSPP levels were elevated significantly at 0.02 mg/L HCN. TSPP levels appear to reflect Vg since Ruby (1986b) reports a similar rise using a highly sensitive homologous radioimmunoassay (RIA) for vitellogenin in landlocked salmon following exposure to sublethal HCN in October. The decline in TSCa levels under HCN exposure may reflect a disturbance in calcium regulation.

Several reports indicate that calcium regulation occurs mainly at the gill, which is also the site of action of calcium regulating hormones in many teleosts (Taylor, 1985; Shephard and Simkiss, 1978; Wendelaar Bonga et al., 1984; Milhaud et al., 1977). The reduction in TSCa levels that occurred in the present study at the lowest HCN concentration does not appear to reflect the direct effects of cyanide on the gill since Dixon and Leduc (1981) showed no histological damage

in gill tissue in rainbow trout after a 9 day exposure to 0.01 mg/L HCN. However, at higher concentrations the possibility of gill damage following HCN exposure cannot be excluded. The pituitary is the major source of calcium regulating hormones in freshwater teleosts (Pang, 1973). The hormones prolactin and ACTH, are effective in raising serum calcium levels by promoting calcium uptake at the gill (Pang, 1973; Wendelaar Bonga et al., 1984). HCN may affect production of prolactin and ACTH at the pituitary level, thus decreasing TSCa levels, although there is no evidence in the literature for this mechanism. There is, however, evidence that gonadotropin (GTH) is reduced following thiocyanate administration in the catfish (Heteropneustes fossilis) (Singh et al., 1977). Thiocyanate is a product of detoxification of cyanide (Westley, 1981).

In the present study, the rise in TSCa level beyond 0.01 mg/L HCN could be attributed to a mobilization of calcium from bony tissues to compensate for falling calcium levels in the blood. This mechanism is known to exist in humans (Nartey, 1981) where bone decalcification has been reported following cyanide/thiocyanate exposure.

This overall reduction of calcium levels under HCN exposure could have serious implications on ovarian growth and maturation. This is supported by the trend towards lower GSI values observed at all HCN concentrations. Vg is sequestered from the bloodstream into oocytes by pinocytosis (Whitehead et al., 1978a; Ng and Idler, 1985). Pinocytosis is calcium dependent (Brandt and Freeman, 1966). Fig. 13 shows a diagramatic representation of the sequence of events occurring

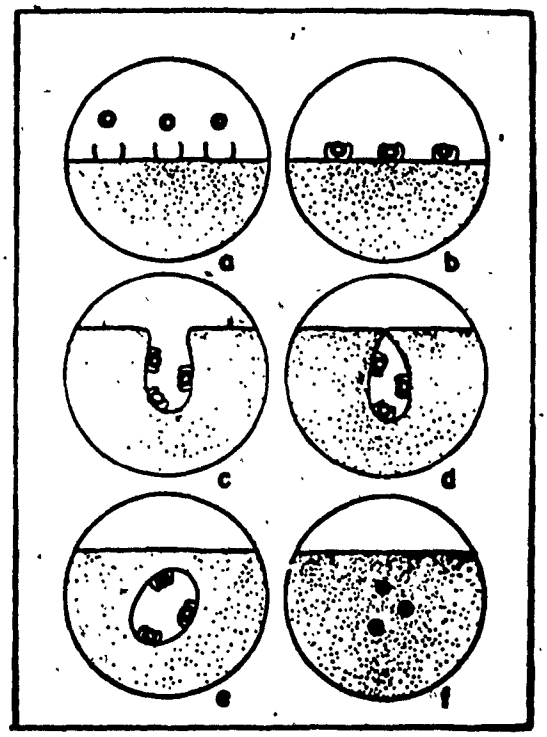

Figure 13: The sequence of events during pinocytosis: The approach (a) and attachment (b) of a particle, invagination of the cell membrane (c), formation of a pinocytotic vacuole (d, e), and incorporation of the material into the cytoplasm (f) (from Bennett, 1956).

during pinocytosis. Molecules must be positively charged, to bind with the negatively charged mucopolysaccharide cell membrane (a, b in Fig. 16), inducing pinocytosis (Rustad, 1961). It is possible that the calcium ion in the Vg molecule provides this positive charge for Vg and facilitates binding to the oocyte membrane. In addition, the invagination of the cell membrane associated with the formation of the pinocytotic vacuole (c, d in Fig. 16) requires extracellular calcium and ATP (de Terra and Rustad, 1959; Campbell, 1983). Therefore, HCN could produce its effect through a decline in TSCa levels and through inhibition of ATP synthesis, on this critical pinocytotic process.

Increased TSPP levels recorded with HCN exposure probably reflect rising levels of Vg in the serum. Two additional reports support this observation. Cyr (1984) recorded increased TSPP levels in estradiol treated rainbow trout after exposure to 0.02 mg/L HCN in October. Ruby (1986b) reported an elevation of plasma Vg levels, measured by a RIA, in female landlocked salmon following exposure to 0.005 mg/L HCN at the same time of the year. In the latter study, a decline in ovarian Vg was also reported, suggesting also the inhibition of Vg uptake by the ovary. In the present study, at the highest cyanide concentration (0.03 mg/L HCN), TSPP levels declined relative to 0.02 mg/L HCN probably indicating 1) a breakdown of the Vg molecule, to meet energy requirements, by the anaerobic catabolism of the lipid fraction of Vg (Nagler, 1985); or 2) a decrease in Vg synthesis, caused by the unavailability of lipid following HCN exposure. Kovacs and Leduc (1982) report a 70% reduction in dry weight gain, in juvenile rainbow trout exposed 0.03 mg/L HCN at 12°C, primarily due to

a decline in fat synthesis.

Cyanide exposure resulted in a significant decrease in HSI. In naturally reproducing females, the exogenous yolk production stage is usually reflected in the liver by an increase in HSI due to the active synthesis of Vg by the hepatocytes (van Bohemen et al., 1981). The decline in HSI in the present experiment may be associated with a depletion in liver glycogen levels rather than in Vg since Raymond (1984), using similar cyanide concentrations, observed that the decrease in HSI coincided with a reduction in liver glycogen, which was attributed to a shift from aerobic to anaerobic metabolism. Raymond (1984) also reported that glycogen levels and HSI declined with rising cyanide concentrations, a trend also observed in the present study. Decreased liver glycogen following cyanide exposure could stress reproductively active females, since Petersen and Emmersen (1977) report that hepatic glycogen and lipid represent important energy sources in the metabolic requirements for ovarian growth and spawning.

The response observed in May following cyanide exposure differed from that reported when females were exposed in October under identical conditions indicating that the reproductive response to sublethal cyanide can differ with season. In May, females are entering the vitellogenic phase of the cycle. Early vitellogenesis was confirmed by histological examination of the ovaries. TSCa and TSPP levels of cyanide exposed females increased with rising concentrations of cyanide relative to controls after 7 days. Similarly, Ruby et al. (1986a) using a direct RIA for Vg, reported an

increase in Vg levels on day 6 of a 12 day experimental period, where rainbow trout were exposed to low concentrations of cyanide (0.01 mg/L HCN) in May. At day 12 of that experiment, Vg had declined to non-detectable levels, suggesting that Vg synthesis had ceased. The length of time of the present experiment did not permit an assessment of effect of sublethal cyanide exposure over a longer period of time.

There are two possible explanations for the increase in TSCa and TSPP levels reported in the present study. A stimulatory effect on the synthesis of yolk precursors by the liver. The stimulation of biological processes by low levels of toxicants, a phenomenon called hormesis, has been previously reported in the literature (Stebbing, 1981, 1982). Stimulation of growth, for example, was reported by several authors after cyanide exposure (Cheng, 1978; Leduc, 1978; Dixon and Leduc, 1981), and has been associated with 1) a slight hyperthyroidism, or 2) an increase in water content and, hence, in wet weight, observed following thiocyanate/cyanide exposure (Leduc, 1982). Secondly, the lack of Vg uptake by the ovary as a result of the absence of vitellogenic gonadotropin at this time of year. Vitellogenic gonadotropin is the hormone responsible for the stimulation of ovarian Vg incorporation (Ng and Idler, 1983). Failure of the oocytes to sequester Vg is supported by similar GSI values in control and cyanide exposed females.

In summary, there is a seasonal difference in response to cyanide exposure between females exposed in May and October.

(ii) Exposure to 0.01 mg/L HCN for 12 days in December.

In December, in non-vitellogenic fish, no significant differences occurred between control and cyanide exposed females, for the four parameters of vitellogenesis. However, in the cyanide exposed vitellogenic group, there was a trend towards lower GSI, TSCa and TSPP levels at the termination of the experimental period. Changes in HSI did not reflect those occurring in the other parameters. The decline in TSCa and TSPP concurrent with a decrease in GSI among cyanide exposed fish at day 12, suggests a reduction in yolk precursor formation, and in its subsequent uptake from the blood by oocytes. Singh et al. (1977) demonstrated that gonadotropin production by the pituitary was decreased by thiocyanate in the catfish, Heteropneustes fossilis. Thiocyanate is the product of a detoxification mechanism of cyanide, in a reaction between cyanide and thiosulfate, mediated by the enzyme rhodanese (Westley, 1981). Raymond (1984) measured thiocyanate in cyanide exposed rainbow trout. Thiocyanate interferes with iodide uptake by the thyroid gland (Way, 1981) resulting in a reduction of thyroid hormone (TH). This is associated with an accelerated production of thyroid stimulating hormone (TSH) by the pituitary (negative feedback mechanism) at the expense of gonadotropin synthesis (Singh et al., 1977). A decline in gonadotropin output from the pituitary, could reduce estrogen synthesis by the ovary, which in turn would result in the decrease of exogenous yolk formation by the liver observed in cyanide exposed females. A reduction in circulating exogenous yolk would lead to decreased gonadal activity and a decline in GSI. Sing et al. (1977) similarly observed a reduction in gonadal

maturation in catfish following thiocyanate treatment.

These results suggest that (1) maturing vitellogenic females are more sensitive to low levels of sublethal cyanide than non-vitellogenic females, and (2) that vitellogenic females exposed to sublethal cyanide in December, at the peak of exogenous vitellogenesis show a trend towards reduced exogenous yolk formation, and in its uptake by the ovary. This would probably decrease the number of viable eggs available for the oncoming spawning time in January-February.

(iii) Exposure of E_2 injected rainbow trout to 0.01 mg/L HCN for 12 days in December.

Cyr (1984) developed a model of artificial induction of exogenous yolk production by E_2 injections in immature male and female rainbow trout. Since fish of both sexes respond similarly to E_2 administration (Elliott et al, 1979; Sundararaj and Nath, 1981; Cyr, 1984) both non-vitellogenic females and males were utilized in the present experiment. Statistical analysis of data further indicated that there were no significant differences between males and females in the response to E_2 treatment, and therefore data obtained for both sexes were combined.

Based on the previous December experiment, only a small proportion ($\simeq$30%) of females enter full vitellogenesis each year. Artificial induction of exogenous yolk production, using an identical model system, provided an opportunity to compare the response to sublethal cyanide exposure between naturally reproducing mature

females and induced rainbow trout.

Exposure of E_2 treated rainbow trout to 0.01 mg/L HCN for 12 days resulted in lower TSCa and TSPP levels when compared to the E_2 control group. This response was similar to that observed in naturally reproducing vitellogenic females exposed to cyanide, providing further support for the above results. Decreasing levels of TSCa and TSPP suggest a decline in yolk precursor formation caused by cyanide exposure.

In the present experiment, however, no significant changes were observed in GSI of non-vitellogenic females relative to the vitellogenic females. A possible explanation for this is the absence of vitellogenic gonadotropin in immature females. This hormone promotes Vg uptake by the ovary and is not affected by estradiol treatment.

In summary, in May, at the onset of vitellogenesis, exposure to a range of sublethal cyanide concentrations for 7 days had a stimulatory effect on the synthesis of yolk precursors by the liver. In October, during exogenous vitellogenesis, results indicate an inhibition of yolk uptake by the ovary caused by low levels of calcium. In December, during late vitellogenesis, exposure to 0.01 mg/L HCN for 12 days had an inhibitory effect on exogenous yolk production, therefore possibly decreasing the numbers of viable eggs available for the oncoming spawning time. This study demonstrated for the first time a seasonal difference in the response of naturally reproducing female rainbow trout to sublethal cyanide exposure. These results therefore indicated that during toxicological studies, the season in which tests

are being performed must be considered. This investigation has shown that vitellogenic mechanisms respond differently to sublethal cyanide with season. Future studies are required to ascertain if hormonal changes may explain the variation in response with season following sublethal cyanide exposure.

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