Alterations in Biochemistry and Morphology of Feral Fish Downstream from an Untreated Groundwood/Sulphite Pulp Mill Discharge and Characterization of the Effluent Induced Mixed-Function Oxygenase Response.

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

Department of Zoology
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THE UNIVERSITY OF MANITOBA

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CANDI LYNNE BEZTE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

White suckers captured downstream from the Pine Falls pulp and paper mill exhibited an increase in liver somatic index and an induction of the mixed-function oxygenase (MFO) system and decreases in plasma testosterone, fecundity and hepatic stores of vitamins A and E. The MFOs were positively correlated with liver somatic index and negatively correlated with hepatic vitamins, condition factor and most reproductive indices; hepatic vitamins were positively correlated with condition factor and reproductive indices. The majority of the differences between reference and downstream fish appears to be related to the presence of the pulp mill, because effects diminished with increasing distance from the effluent outfall. These effects may be caused by the current (1993-1994) release of effluent and/or to the habitat degradation of the area.

In a dose-response experiment the MFO enzyme system of rainbow trout was induced by an effluent concentration of 0.23%; less than one tenth of the estimated 96-hour LC50 value of 3.0%. The time-dependence of the MFO response was examined at an effluent concentration of 1% and was significantly induced after 2 days, remained at this induced level for the remaining 6 days of effluent exposure and declined within 2 days after the fish were moved to clean water.

Fish downstream from the Pine Falls pulp mill exhibited responses similar to fish captured downstream from bleaching kraft pulp mills. The MFO inducer(s) in this effluent behaved like polycyclic aromatic hydrocarbons, not highly chlorinated dioxins and/or furans. At the time of this study the effluent released from the mill was untreated; a secondary treatment facility which may alleviate some if not all of these impacts has since been installed.

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LIST OF ABBREVIATIONS

AHH	Aryl Hydrocarbon Hydroxylase
B[a]P	Benzo[a]pyrene
BKME	Bleached Kraft Mill Effluent
BSA	Bovine Serum Albumin
CE (room)	Controlled Environment room
CFAC	Condition Factor
D1	Site D1; sampling site located within 1 km of the effluent discharge
D2	Site D2; sampling site located within 6 to 8 km of the effluent discharge
DMSO	dimethylsulphoxide
EIA	Enzyme Immunoassay
EROD	7-ethoxyresorufin O-deethylase
GSI	Gonadosomatic Index
HPGL (axis)	Hypothalamus-Pituitary-Gonadal-Liver axis
LC50	Lethal Concentration 50; the concentration of a substance required to kill 50% of the exposed organisms within the specified time period, i.e. 96 hours
LSC	Liquid Scintillation Counter
LSI	Liver Somatic Index
MFO	Mixed-Function Oxygenase
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated Dibenzodioxin
PCDF	Polychlorinated Dibenzofuran
PHAH	Planar Halogenated Aromatic Hydrocarbon
QC	Quality Control
TCDD	Tetrachlorodibenzodioxin
U	Site U; the upstream reference site located above the pulp mill effluent discharge and isolated from the downstream sites (D1 and D2) by the Powerview dam.

GENERAL INTRODUCTION

Aquatic toxicology has been defined as the "qualitative and quantitative study of the adverse or toxic effects of chemicals and other anthropogenic materials or xenobiotics on aquatic organisms" (Rand and Petrocelli, 1985). At low concentrations or for short exposure periods, the normal homeostatic mechanisms of an organism may be able to cope with toxicant exposure and the substance may be excreted or detoxified with no permanent damage. Increasing concentrations and/or exposure times may lead to physiological changes which temporarily impair certain bodily functions. If the concentration or exposure period continues to increase, a critical point will be reached such that even if exposure were terminated, the damage to the organism would remain. Ultimately, there will be a concentration and/or exposure period at which disease and finally death ensue (Hellawell, 1986). Past toxicological research was focussed on the acute effects of relatively high toxicant concentrations. Such studies often used the end-point of death as an indication of toxicity. It is now recognized that sub-lethal responses, such as disturbed behavioural patterns, impaired physiology or induced sterility can ultimately have effects on populations similar to the rapid death of individuals (Hellawell, 1986).

The lowest level of biological organization is a biochemical reaction within an individual organism. Impacts must occur at this biochemical level before effects can be manifested in cells, tissues, organs, organ systems, organisms, populations or communities (Bucheli and Fent, 1995; Delorme, 1995). Effects should be detectable at each of the previous levels of biological organization before they can be manifested at the next higher level of biological organization. For this reason, the examination of biochemical parameters

is thought to function as an "early warning" of potentially more serious pathologies (Payne et al., 1987; Adams et al., 1989; Brouwer et al., 1990; Fox, 1993; Palace and Brown, 1994; Bucheli and Fent, 1995). However, some alterations in biochemistry may simply indicate exposure and may not be associated with any discernable adverse effects at higher biological levels (Kloepper-Sams and Benton, 1994; Chapman, 1995).

A biomarker may be broadly defined as a measurable response at any level of biological organization that can be related to contaminant exposure (Bucheli and Fent, 1995). These may include the following; induction of the stress response, induction of detoxification systems, inhibition of specific enzymes, metabolic impairments that detrimentally alter synthetic or degradative processes or that deplete energy, decreases in vitamin or substrate stores, impaired growth or failure to thrive, weight loss, genetic damage, impairment of immune system function, impaired or altered reproductive function and impaired organ or tissue function (Fox, 1993). More specifically, the term biomarker has been used to describe the generally rapid and highly sensitive responses at the biochemical and cellular level (Bucheli and Fent, 1995).

Effects of Pulp Mill Effluents on Fish

A wide array of impacts have been reported in fish exposed to effluents of the pulp and paper industry, with most research having been done on effluents from chlorine-bleaching kraft pulp mills. These effects range from the biochemical/cellular level to the population and community levels and include; an increase in mixed-function oxygenase activities, a reduction in plasma levels of steroid hormones, a reduction in hepatic stores of vitamins A and E, a

decrease in fecundity, a decrease in gonad size, an increase in liver size, an increase or decrease in condition factor, (a measure of the weight of a fish in relation to its length), a reduced rate of growth, an increase in the age of first sexual maturity, high larval mortality, a reduction in adult fish populations, and a reduction in fish species richness and community composition (Andersson et al., 1988; Rogers et al., 1989; McMaster et al., 1991; Munkittrick et al., 1992b; Hodson et al., 1992; Servos et al., 1992; Van Der Kraak et al., 1992; Adams et al., 1992; Munkittrick et al., 1994; Kloepper-Sams and Benton, 1994; Sandström, 1994; Barker et al., 1994; Gagnon et al., 1995; Brown and Vandenbyllaardt, 1996).

One of the most consistent effects of bleaching kraft pulp mill effluents is an increase in mixed-function oxygenase (MFO) activity (Hodson, 1996). In the past this response has been attributed to the presence of the chlorinated dioxin and furan compounds produced by this type of pulp and paper production (Rogers et al., 1989; Hodson et al., 1992; Kloepper-Sams and Benton, 1994; Servizi et al., 1994). These compounds are known to be potent inducers of this enzyme system and have been found in varying amounts in bleaching kraft pulp mill effluents (Parrott et al., 1995; Rogers et al., 1989; Hodson et al., 1992; Kloepper-Sams and Benton, 1994; Servizi et al., 1994).

There has been little research on feral fish downstream of pulp mills that do not use chlorine-bleaching or the kraft pulping process. In 1992, Finnish researchers (Pesonen and Andersson, 1992; Lindström-Seppä et al., 1992) provided the first evidence that non-chlorinated effluents were capable of affecting fish by inducing the MFO system. In Canada, field research on these types of effluents is limited to a survey of Ontario pulp mills

(Munkittrick et al., 1994). This survey included two mills that did not use chlorine bleaching or the kraft pulping process and fish downstream from these mills had an increase in MFO activity (only in males), smaller gonads, larger livers and lower levels of the steroid hormone, estradiol (females). These findings suggest that chlorinated dioxins and furans are not the only causative agents of deleterious effects in fish downstream of pulp mill effluent discharges and that further examinations of effluents which do not contain chlorine are warranted.

Pulp mill effluents contain hundreds of different compounds (Owens, 1991; Hodson et al,. 1992) and the individual chemical components of pulp mill effluents responsible for the biochemical and morphological effects in fish are presently unknown. There is a strong correlation between the molecular size and shape of a compound capable of inducing the MFO system and the nature of the induction (Hodson, 1996). MFO induction tends to be very persistent when caused by highly chlorinated compounds and rapidly depleted when caused by nonhalogenated compounds (Bucheli and Fent, 1995; Parrott et al., 1995). Recent work indicates that the compound(s) responsible for MFO induction in a secondary treated bleached kraft mill effluent (BKME) can be readily cleared from fish. MFO activity was reduced after 4 days in clean water and fish captured downstream from this mill after a 2 week shutdown also had lower MFO activities (Munkittrick et al., 1992b). This, together with the finding of similar impacts in fish downstream from pulp mills which use no chlorine, further contradicts the hypothesis that the inducers are highly chlorinated dioxin and/or furan compounds. If induction were caused by chlorinated dioxins and furans then it would persist for much more than a few days (Muir et al., 1990; Parrot et al., 1995, Delorme, 1995).

Recent laboratory work with a variety of pulp mill effluents has shown that non-

bleaching sulphite/groundwood effluents (similar to those produced at the Pine Falls pulp mill) are capable of inducing the MFO response (Gagne and Blaise, 1993). Gagne and Blaise (1993) indicated that there was a need to determine the time-course of the MFO response, i.e. how long it takes for induction to occur and how long it takes for it to decline after removal of the fish to clean water. They also pointed out the need to determine the threshold effluent concentration for MFO induction, i.e. the lowest concentration of exposure which results in a significant response. The threshold value will allow comparisons of different effluents for their potency as inducers, and time-course information would give some indication of the stability of the inducer(s) in the fish. Laboratory experiments are also a confirmatory measure because they can examine the cause/effect relationship between effluent exposure and a measurable response, such as MFO induction. This ensures that at least one of the effects noted in fish captured in the field can be caused in laboratory fish that are exposed only to the effluent. Experimental work with the effluent also offers a way to discriminate between the effects of current loadings and those that may be derived from historical sediment contamination.

Characteristics of the Pine Falls Pulp and Paper Mill

The pulp mill is located in the town of Pine Falls (50°34'N, 96°13'W) approximately 100 km northeast of Winnipeg and produces newsprint using sulphite (~25%) and groundwood (~75%) pulping processes. The wood supply is 80% spruce, 15% jack pine and 5% balsam fir. Where necessary (approximately 50% of the time) the groundwood pulp is brightened with sodium hydrosulphite; the sulphite pulp is never brightened and no chlorine

is used in these pulping processes. The maximum production capacity of the mill is 175 000 tonnes per year (~480 tonnes/day, although operation is usually somewhat lower than this) and the average daily effluent discharge during the months of sample collection ranged from 21 691 to 24 492 $m^3 d^{-1}$. The mill has been discharging untreated effluent (various streams within the mill pass through a clarifier to reduce suspended solids, but the whole effluent receives no other treatment prior to discharge) directly to the Winnipeg River since 1927. The effluent enters the river at the southern shore through a 1.2 m (i.d.) pipe which is suspended above the water surface. The discharge point on the Winnipeg River is located between the Powerview dam (a few kilometres upstream) and Traverse Bay on Lake Winnipeg (approximately ten kilometres downstream). Past mill operations have significantly impacted the nature of the benthic substrate and the invertebrate populations in the area. The sediments are highly contaminated with wood fibres and are dominated by pollution tolerant chironomid and oligochaete species (Friesen et al., 1994; Wong et al., 1996). The effluent is acutely toxic to fish; the rainbow trout 96-hour LC50 in 1993 and 1994 ranged from three to four percent effluent by volume (T. Youmans, Environmental Protection, personal communication). These conditions were prevalent for the duration of this study and continued until late 1995, when the newly installed secondary treatment facility (activated sludge and aeration) began operation. The following field and laboratory studies provide background information which may be used to monitor the efficacy of this new treatment facility.

Thesis Objectives

It was hypothesized that discharges from the Pine Falls pulp mill could cause effects in fish similar to those being detected elsewhere. For these reasons fish from the Winnipeg River from one site upstream and two sites downstream of the Pine Falls mill were examined for the following biochemical and morphological parameters: liver MFO activities determined by EROD (7-ethoxyresorufin O-deethylase) and AHH (aryl hydrocarbon hydroxylase), plasma concentrations of testosterone and 17β-estradiol, liver concentrations of vitamins A and E, maturity index, fecundity, egg size, egg weight, gonadosomatic index (GSI, the weight of the gonad in relation to the body weight), liver somatic index (LSI, the weight of the liver in relation to body weight), condition factor (CFAC, weight in relation to length), length, weight and age.

Objective: To determine if the untreated discharges from the Pine Falls Pulp Mill are affecting any of the above mentioned parameters in feral fish of the Winnipeg River.

Laboratory experiments were carried out with rainbow trout to assess the toxicity of the effluent and its potential to induce the MFO system. The threshold and time-course of the induction were examined to indicate the strength and stability of the inducer(s).

The effluent was known to be highly toxic to fish (T. Youmans, Environmental Protection, personal communication), but this toxicity was further confirmed and characterized. Aside from examining the toxicological properties of the effluent these preliminary semi-static experiments were done to determine appropriate effluent concentrations and handling procedures for the effluent in the more labour-intensive experiments used to characterize the EROD response. The objectives of these preliminary

experiments were to:

1. Determine whether the estimated rainbow trout 96-hour LC50 differs with effluent storage time

2. Determine if the toxicity of the effluent is entirely within the liquid/small particulate fraction

3. Determine whether the toxic compounds in the effluent are volatile

4. Determine whether tank aeration will alter effluent toxicity.

Flow-through experiments were carried out to assess the MFO response of rainbow trout to the effluent. The objectives of these experiments were to:

1. Determine whether effluent exposure induces the MFO system of rainbow trout as assessed using EROD

2. Estimate the threshold effluent concentration required to increase the EROD response

3. Determine the length of effluent exposure time required to induce the EROD response

4. Determine the length of time required to eliminate the EROD response once previously exposed fish are moved to clean water.

Chapter 1: Biochemical and morphological changes in feral fish downstream from the groundwood/sulphite pulp mill in Pine Falls, Manitoba.

INTRODUCTION

The homeostatic mechanisms of fish are continuously challenged by the demands of the aquatic environment and exposure to xenobiotics further increases this challenge (Adams et al., 1989; Fox, 1993). Chemical monitoring of environmental xenobiotics may identify potential environmental problems, but does not detect or describe effects and does not assure that biota are protected from the potential impacts of biomagnification, chemical mixtures, unidentified compounds or additional environmental stressors (Fox, 1993). It also does not determine the bioavailability of the compounds. Contaminants may be present in the water column or sediments, but if they are not able to be taken up by organisms then their relevance to biotic communities is likely to be negligible. The use of biomarkers to assess environmental contamination has many advantages. Biomarkers only assess the effects of biologically available pollutants; integrate exposure to all pollutants present in the environment thereby reflecting the cumulative, synergistic or antagonistic effects of complex mixtures; integrate the effects of chemical speciation of the contaminant(s) within the environment as well as their environmental distribution and biomagnification; reflect all of the other types of stressors which may be present in the native environment, such as fluctuations in temperature, oxygen availability, photoperiod, food availability and current velocity (Adams et al., 1989; Mayer et al., 1992; Bucheli and Fent, 1995). All of these variables may alter the biological impact of a contaminant.

Mixed-Function Oxygenase

Mixed-function oxygenases belong to the P-450 family of heme proteins and certain members of this protein family can be induced by a number of xenobiotics including polycyclic aromatic hydrocarbons (PAH), polychlorinated dibenzodioxins (PCDD), polychlorinated dibenzofurans (PCDF) and planar polychlorinated biphenyls (PCB) (Bucheli and Fent, 1995). Induction involves the binding of the contaminant to the aryl hydrocarbon (Ah) receptor within the cell and the translocation of this contaminant/receptor complex into the cell nucleus. The complex combines with the cell's DNA and through transcription and translation results in the production of a P-450 protein with measurable catalytic activity (Bucheli and Fent, 1995; Figure 1). MFOs catalyze the phase I reactions of xenobiotic transformation (detoxification) and involve the addition of one atom of oxygen into the substrate. The oxidized xenobiotic is often further modified by phase II reactions which may involve conjugation of the oxidized xenobiotic compound with an endogenous molecule such as a sugar or an amino acid (Andersson and Förlin, 1992). These conjugated products are generally less toxic and more readily excreted. The EROD and AHH assays measure the rate of ethoxyresorufin dealkylation and benzo[a]pyrene (B[a]P) hydroxylation, respectively, and appear to be the most sensitive catalytic activities used to determine the inductive response in fish (Andersson and Förlin, 1992).

Steroid Hormones and other Reproductive Indices

The reproductive endocrine system consists of the hypothalamus-pituitary-gonadal-liver (HPGL) axis and contaminant impacts at any level in this axis can affect the other levels

in the axis through a series of biochemical reactions or biochemical feedback mechanisms Figure 2). The hypothalamus releases gonadotropin releasing hormone or gonadotropin release inhibiting factor which function to either stimulate or inhibit the release of gonadotropin from the pituitary gland. The pituitary gland produces and releases gonadotropin in response to signals from the hypothalamus. The gonadotropin acts on the gonads, stimulating the production of steroid hormones such as testosterone and estradiol. The liver functions to metabolize and excrete hormones (whose levels may feed back to other areas within the axis) and in females also produces vitellogenin which will be taken up into developing oocytes and converted into yolk. (Thomas, 1990; Kime, 1995).

A wide variety of chemicals have been found to interfere with vertebrate reproduction (Thomas, 1990; Kime, 1995; McMaster et al., 1996). Impacts may originate at any level within the HPGL axis and involve reductions in plasma levels of steroid hormones, reductions in the size and number of eggs, reductions in the number and motility of sperm and reductions in gonadosomatic indices (the gonad size in relation to body size), (Thomas, 1990; McMaster et al., 1992; Van Der Kraak et al., 1992; Kime, 1995). Changes in hormonal levels often precede physiological disturbances and thus may function as sensitive indicators of chemical toxicity (Brouwer et al., 1990). "Reproduction is the most sensitive, consistent, and relevant end point tested to date in the laboratory, in mesocosms and experimental streams, and in field situations near some pulping discharges." (Owens, 1991).

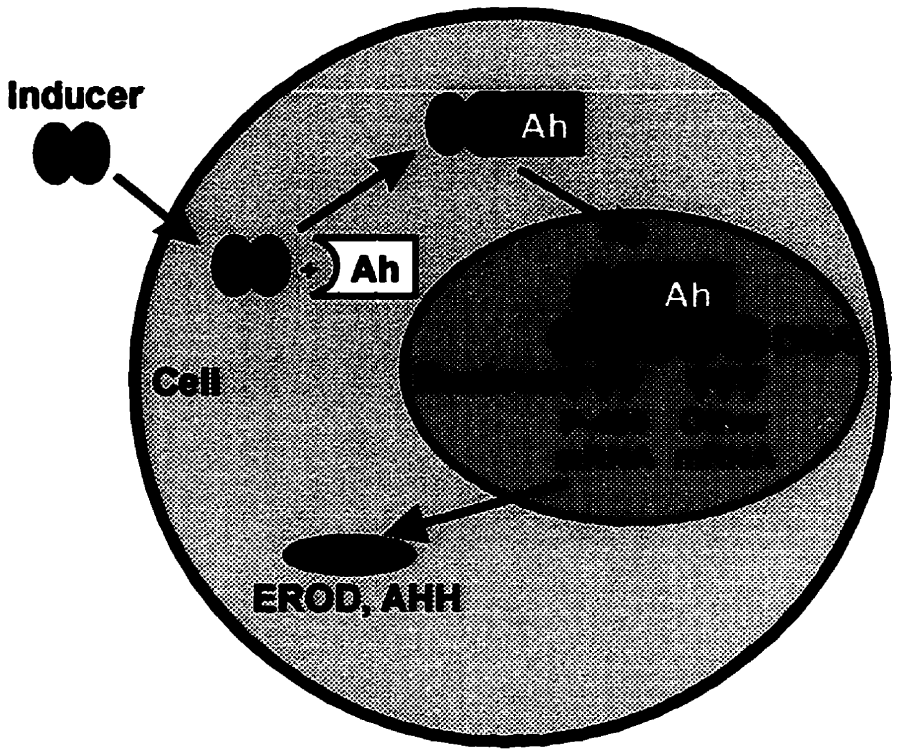


Figure 1: Schematic representation of the MFO response. The inducing compound enters the cell and binds to the aryl hydrocarbon (Ah) receptor The inducer/receptor complex then enters the nucleus to interact with the cell's DNA. This results in the production of P-450 mRNA. The mRNA codes for the production of a P-450 protein with catalytic activity that can be quantified using EROD and AHH assays.

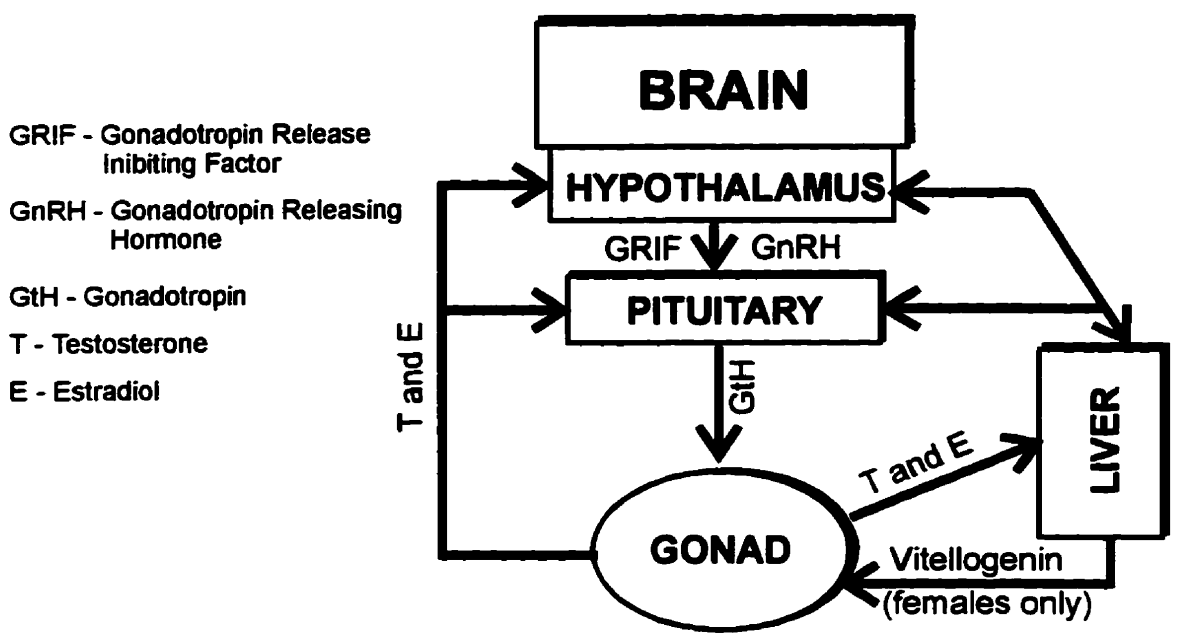


Figure 2: Schematic diagram of the hypothalamus-pituitary-gonadal-liver axis. The arrows indicate potential feedback routes within the axis.

Hepatic Retinoids and Tocopherol

Vitamins A and E, otherwise referred to as retinoids and tocopherol, are fat-soluble vitamins and their depletion has been shown to indicate exposure to a variety of environmental contaminants (Peakall, 1992). Fish obtain these vitamins directly from their diet by consuming other plants and/or animals. Vitamin A cannot be obtained directly from plants, but plants do contain carotenoid pigments which function as pro-vitamins and can be converted into vitamin A by the fish (Halver, 1982). Vitamin A has a variety of functions within the body including roles in vision, growth and differentiation of epithelial cells, general growth, reproduction, immunocompetence, hepatic pathology and bone metabolism (Halver, 1982; Taveekijakarn et al., 1994). Tocopherol's primary function is as an antioxidant, where it functions as a part of the cellular defence against the damaging effects of free radicals, although it also plays a role in reproduction and immunocompetence (Blazer and Wolke, 1984; Serbinova et al., 1991, Combs, 1992; Roberfroid and Calderon, 1995). More recently, vitamin A has also been recognized as having antioxidant activity (Palozza and Krinsky, 1991, Ribera et al., 1991 and Roberfroid and Calderon, 1995). Dietary exposure to chemicals known to induce the MFO system such as PCBs, PCDDs and PCDFs have been shown to cause changes in vitamin A metabolism. Zile (1992) reported severely depleted body stores of vitamin A after chronic exposure to planar halogenated aromatic hydrocarbons (PHAH). Trout deficient in vitamin E have been shown to be more susceptible to contaminant toxicity (Williams et al., 1992) and vitamin E concentrations have been shown to be reduced after exposure to a coplanar PCB (Palace and Brown, 1994; Palace et al., 1996).

Morphological Parameters

Liver somatic index, gonadosomatic index and assessments of energy reserves are crude measures of animal condition and may show signs of contaminant exposure (Mayer et al., 1992). During periods of high energy intake fish can store excess energy in the liver as glycogen. Thus an increase in the LSI may indicate an increase in the nutritional status and overall condition of the fish (Busacker et al., 1990). Liver somatic indices may also increase in direct response to contaminant exposure, possibly due to the increased production of proteins which function in the detoxification process of the liver (Andersson et al., 1988; Kumar and Mukherjee, 1988). An increase in nutritional status may also be seen with increased development of reproductive organs and be measurable as an increase in the gonadosomatic index (GSI) (Scott, 1962; Bagenal, 1969; Mayer et al., 1992). Condition factor compares the weight of a fish with its length. Under conditions of increased caloric intake the storage of energy in somatic tissues, like liver and muscle, can increase the weight of a fish in relation to its length, thus the fish is more plump (Busacker et al,. 1990). Contaminants can affect these morphological parameters by altering the diet of the fish, by altering their metabolism or by increasing their level of stress, thereby using up their energy stores. Exposure to a BKME was shown to increase condition factor and decrease gonadosomatic indices in a white sucker (*Catostomus commersoni*) population. It was hypothesized that effluent exposure caused an altered energy allocation from the development of reproductive tissues to the development of somatic tissues (McMaster et al., 1991).

Lengths and weights of fish are commonly used to assess whole-body growth (Busacker et al., 1990). Fish of the same age from the same population should not differ

dramatically in length or weight. If contaminant exposure effects the growth of fish this may be indicated by reductions in fish size at a given age (Munkittrick and Dixon, 1988; McMaster et al., 1991; Gagnon et al., 1995).

Biomarkers and Pulp Mill Effluents

Research has shown that effluents from the pulp and paper industry have the potential to affect biomarkers at all levels of biological organization, although the most commonly reported effects are at the biochemical level (Andersson et al., 1988; Rogers et al., 1989; McMaster et al., 1991; Adams et al., 1992; Munkittrick et al., 1992b; Hodson et al., 1992; Swanson et al., 1992; Van Der Kraak et al,. 1992; Kloepper-Sams and Benton, 1994; Gagnon et al., 1995). A range of biomarkers was examined in fish from the Winnipeg River, to assess the impact of effluent discharges from the Pine Falls pulp mill. The biomarkers examined ranged from those at the biochemical level to those at the level of the whole organism, including: liver MFO activities determined by EROD and AHH, plasma concentrations of testosterone and 17β-estradiol, liver concentrations of vitamins A and E, maturity index, fecundity, egg size, egg weight, gonadosomatic index (GSI, the size of the gonad in relation to the body size), liver somatic index (LSI, the size of the liver in relation to body size), condition factor (CFAC, weight in relation to length), length, weight and age. Liver tissue was used for the vitamin analyses because most vitamin A (90%) is stored in the liver (Brewster, 1984) and vitamin E has been reported to be reduced in liver tissues of fish exposed to PCBs (Palace et al., 1996). This was the first time that retinoids and tocopherol have been analyzed in wild fish exposed to non-chlorinating pulp mill effluents.

Two species were chosen for study; white sucker (*Catostomus commersoni*) and northern pike (*Esox lucius*). White sucker were chosen because they are relatively low in the food chain, often used in Canadian studies on the effects of pulping effluents, common throughout Canada, not valued by commercial or sports fishermen and present at both upstream and downstream sites in the Winnipeg River. Northern pike were chosen because they are relatively high in the food chain, not highly valued by commercial or sports fishermen and present at both upstream and downstream sites in the Winnipeg River.

MATERIALS AND METHODS

Study sites

Fish were sampled from three sites (U, D1 and D2) on the Winnipeg River in August of 1993 and 1994 and from two sites (U and D1) in the spring of 1994 (Figure 3). Sample site U was located upstream of the Pine Falls mill and also upstream of the Powerview Dam, site D1 (near downstream) was located within 1 km downstream of the effluent discharge and site D2 (far downstream) was located within 6 to 8 km downstream of the effluent discharge a short distance above the entrance to Traverse Bay. The fish from the upstream reference location were isolated from those at the downstream sites by the Powerview Dam and as such could not be exposed to the effluent from the Pine Falls mill. In 1993, fish from site D1 were obtained immediately below the effluent outfall to approximately 500 m downstream of the effluent discharge, in 1994 this area was extended to within 1 km of the effluent discharge to reduce the amount of time required to obtain samples.

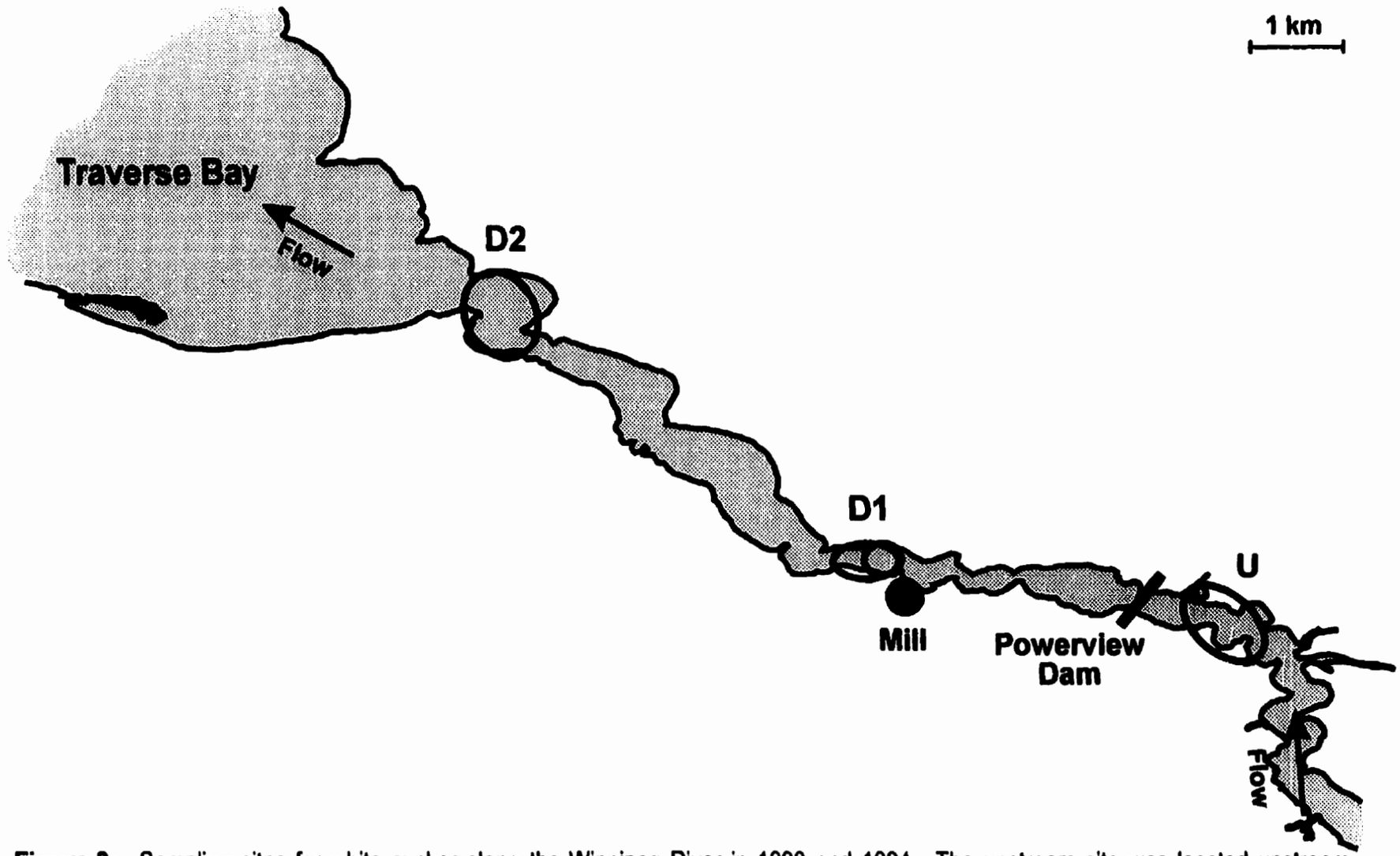


Figure 3: Sampling sites for white sucker along the Winnipeg River in 1993 and 1994. The upstream site was located upstream of the mill and upstream of the Powerview dam and is labelled U on the map. Site D1 was the near downstream site and was located within 1 km of the effluent outfall. The smaller circle at site D1 indicates the sampling area at this site in August, 1993 and May, 1994 and the larger circle indicates the size of the sampling area in August, 1994. Site D2, the far downstream site, was located approximately 6 to 8 km downstream of the effluent discharge.

The dilution of the effluent in the river, assuming that the effluent was diluted by the entire discharge of the river immediately (i.e. complete mixing), was calculated by dividing the average daily discharge of effluent by the average daily discharge of the river at the Powerview dam (consecutive daily values from one week prior to sampling and up to the completion of sampling were used; effluent discharge volumes were supplied by the mill and river discharge volumes were supplied by Manitoba Hydro). The effluent dilution ratios determined by dividing effluent discharge by river discharge were 1:5302 in August, 1993, 1:2337 in May, 1994 and 1:3208 in August, 1994. The effluent concentration that fish near the mill could have experienced has been estimated from these complete mixing dilution ratios and counts of total coliform bacteria which were used as an effluent trace. The bacteriology indicated the amount of horizontal mixing across the river at several distances downstream (Friesen et al., 1994). 250 m downstream from the effluent outfall the bacterial trace was lost within less than one-fifth of the river's width, meaning that the dilution available at this location was only about one-fifth that available at the zone of complete mixing. For example, in August, 1994, the dilution ratio at the zone of complete mixing was 1:3208, however, the amount of river water available for dilution at this site was only about 1/5 because the effluent trace was lost within the first fifth of the river's width. Thus, the effluent concentration at 250 m downstream can be roughly estimated as 5-times the concentration at the zone of complete mixing, or in August, 1994, approximately ((1/3208) * 100) * 5 = 0.16%. Using this same method of calculation the approximate effluent concentration 50 m downstream from the effluent discharge in August, 1994 was 0.66%. These calculations provide a rough estimate of potential effluent concentrations at site D1 and suggest that fish from this site

were exposed to effluent concentrations of less than 1%.

Fish Sampling

Northern pike could not be captured in sufficient numbers for statistical comparison and so the following analyses, results and discussions apply only to white sucker. Mature fish were captured using gill nets with mesh sizes ranging from 8.75 to 11.25 cm. A majority of the summer samples were obtained with the nets being run every hour; in the spring the nets were run after a period of approximately three hours and in August, 1994 some of the samples were obtained using overnight sets. At each sampling time only live fish were sampled. Once removed from the nets, fish were anaesthetized (until equilibrium was lost) in potassium phosphate buffered (K_2HPO_4, 0.2 g/L) tricaine methanesulfonate (MS 222, 0.4 g/L) and blood was obtained by caudal puncture with heparinized syringes. The blood was immediately placed on ice and once received on shore it was centrifuged to isolate the plasma. Plasma was transferred into 1.5- mL microcentrifuge tubes and frozen on dry ice. Total fish weight (to the nearest g, excluding blood already drawn), was determined using an A and D electronic balance (model EK-12 KA, 12000 g x 1 g) and fork length was determined to the nearest mm. Liver (minus the gall bladder) and gonad weights were determined to the nearest 0.1 g on an A and D electronic balance (model EK-1200A, 1200 g x 0.1 g). Whole livers were placed in Whirl-Pak® bags and quick frozen between slabs of dry ice. Subsamples of gonad were placed in histological tissue capsules and preserved in Davidson's fixative (95% ethyl alcohol, 30 mL; formalin, 20 mL; glacial acetic acid, 10 mL; distilled water, 30 mL; glycerine, 10 mL) and phosphate buffered 10% formalin. The left pectoral fin was

removed for aging purposes and placed in an envelope to dry. Samples were returned to the Freshwater Institute for analysis. Liver and plasma samples were stored at -80°C and the remaining carcasses were stored at -20°C.

Determination of Mixed Function Oxygenase Activity

Microsome Preparation

All equipment and solutions used in the preparation of microsomes were prechilled in a controlled environment room at 2°C and all work was either completed in this room or the samples were maintained on ice. Frozen livers were subsampled to obtain 2 to 4 grams of tissue; any remaining liver was immediately returned to the -80°C freezer. The tissue was weighed (Metler P1200) and allowed to partially thaw, at which time it was minced with scissors and placed in 15-mL conical glass tissue homogenizers. The tissue was homogenized in 4 mL KCl-HEPES buffer (0.02M (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid], BDH; 0.15M potassium chloride, BDH; pH adjusted to 7.5 with potassium hydroxide, BDH) per gram of tissue, using 5 to 7 passes of a motorized Teflon® pestle. The homogenate was transferred to polycarbonate tubes and centrifuged at 12,000 x g for 20 minutes at 2°C (10,000 rpm in a Sorvall Superspeed RC2-B refrigerated centrifuge using an SM-24 rotor). The post-mitochondrial supernatant produced was transferred to ultracentrifuge tubes and centrifuged at 105,000 x g for 75 minutes at 2°C (39,000 rpm in a Sorvall RC-60 ultracentrifuge using a T1270 rotor). The supernatant was discarded and the pellet was carefully rinsed 3 times with Tris resuspension buffer (0.5M Tris-(hydroxymethyl)methylamine, BDH; 1mM dithiothreitol, Sigma; 1mM

Ethylenediametetraacetic acid, Sigma; 20% glycerol by volume, Sigma), prior to resuspension in enough Tris buffer to yield a final protein concentration of 5 to 10 mg/mL. Three 20-μL portions of the microsome preparation were added to three tubes containing 1 mL of redistilled water (dilutions for the protein analysis) and the remainder was transferred to cryovials for immediate freezing and storage in liquid nitrogen.

Protein Analysis

Microsome preparations were diluted 1 to 51 with redistilled water and were analyzed in triplicate on the same day or one day following microsome preparation (stored samples were covered with film and left in the 2°C CE room). A series of bovine serum albumin (BSA, Sigma) standards with concentrations ranging from 0.05 to 0.3 mg/mL, Tris buffer blanks and 4 Quality Control (QC) BSA samples (also in triplicate) were run with each batch of samples. The protein QCs were samples of known BSA concentration which were reanalyzed with each batch of samples. The QCs were prepared in a 0.1% solution of sodium azide which prevented the degradation of the protein. Three of the QCs were prepared in the lab from BSA with concentrations of 0.0784, 0.101 and 0.201 mg/mL and one was purchased from Fisher with a protein concentration of 0.252 mg/mL. Protein was determined as described by Markwell et al. (1981). The assay reagent was prepared by mixing together 100 parts of reagent A (2% sodium carbonate, BDH; 0.4% sodium hydroxide, BDH; 0.16% sodium tartrate, BDH; 1.0% sodium dodecyl sulphate, BDH) with 1 part of reagent B (4% cupric sulphate-5-hydrate, BDH). Aliquots (60 μL) of sample (blank, standard, QC or microsome dilution) were added to 1 mL of the assay reagent and the tubes were then

incubated at room temperature for at least 10 (but less than 30) minutes. After the 10 or so minutes, 100μL of phenol (BDH) reagent (2 parts phenol to 1 part distilled water) were added to the tubes as they were being vortexed, and they were again incubated at room temperature for a minimum of 30 minutes. After the 30 or so minutes, the absorbance of the samples (against redistilled water) was read using a Beckman DU-7 spectrophotometer. Blanks were subtracted from the readings and microsomal protein concentrations were determined using the BSA standard curve. Protein samples were analyzed for cytochrome P-450 content by running carbon monoxide difference spectra (Omura and Sato, 1964a; Omura and Sato, 1964b).

Determination of Ethoxyresorufin *O*-Deethylase (EROD) Activity

EROD activity was determined as described in Pohl and Fouts (1980). One reagent blank and triplicate microsomal protein samples were run for each fish sample. Two resorufin (0.0006 and 0.01 mg/mL in DMSO = dimethylsulphoxide, Caledon; resorufin = 7-hydroxy-3H-phenoxazine-3-one, Peirce) standards (QCs) diluted in 3 mL of methanol (HPLC grade, Caledon) and 2 QC microsome preparations (one with low and one with high activity) were run in triplicate with each batch of samples. The reaction was carried out in 16 x 100 mm borosilicate glass culture tubes, to which 1100 μL HEPES buffer (0.1M HEPES, pH adjusted to 7.8 with KOH), 10 μL magnesium sulfate (154 mg/mL, Fisher), 50 μL BSA (40 mg/mL) and an NADPH generating system; 10 μL NADP (98.4 mg β-Nicotinamide Adenine Dinucleotide Phosphate/mL, prepared fresh, Sigma), 10 μL isocitric acid - trisodium salt (193.58 mg/mL, prepared fresh; Sigma) and 10 μL of isocitrate dehydrogenase (made by

dissolving 1000 I.U. in 9.5 mL of 0.005M HEPES in 50% glycerol and pH adjusted to 6.0 with HCl, BDH) were added. The tubes sat at room temperature for a minimum of 10 minutes (to allow generation of NADPH) after which time 50μL of the thawed microsome samples were added (microsomal preparations were thawed on ice). Then 2.5 mL of methanol (HPLC grade, Caledon) were added to the blank tubes and all tubes were transferred to a 25°C water bath for a minimum of five minutes. Under reduced laboratory lighting, 10 μL of 7-ethoxyresorufin (1.33 mg/mL dissolved in DMSO, Sigma) were added to each tube at 10 second intervals and the tubes were incubated at 25°C for 2 minutes (± 2 seconds). After the two minutes, 2.5 mL of methanol were added to each of the non-blank tubes to stop the reaction. Methanol addition precipitated the protein which was pelleted by centrifugation at 28 000 x g for 15 minutes (3500 rpm in a Beckman GS-6R centrifuge using a GH-3.7 rotor with tube adapters). Fluorescence of all samples (including QCs) was determined (against methanol) on a Perkin-Elmer LS50 luminescence spectrofluorometer with excitation and emission wavelengths of 530 and 585 nm respectively and slit widths of 9 nm. To further ensure the proper functioning of the fluorometer 2 solid QCs were analyzed. The solid QCs consisted of the #2 (excitation 342 nm; emission 482 nm; slits 10.0 nm) and the #6 (excitation 562 nm; emission 573 nm; slits 3.5 nm) fluorescence standards from Wilmad Glass.

All blanks were averaged and this value was subtracted from each of the samples prior to determining their enzyme activities. Enzyme activity was calculated using a response factor obtained by running 7 known resorufin standards (0.0005 to 0.1 mg/mL in DMSO) spiked into killed reaction mixtures. The results were expressed in nmol of resorufin produced per mg of protein per minute of incubation time (nmol/mg protein/min.).

Determination of Aryl Hydrocarbon Hydroxylase Activity

The aryl hydrocarbon hydroxylase assay was based on methods described in van Cantfort et al., 1977. Each batch of samples was run with one low and one high QC and samples were run in triplicate with triplicate blanks. The reaction was carried out in 16 x 125 mm borosilicate tubes (with screw caps) to which 800 μL Tris-HCl buffer (55.5 mM Tris with pH adjusted to 7.5 with HCl), 10 μL magnesium chloride (0.5M, BDH) and an NADPH generating system; 10 μL NADP (78.7 mg/mL, prepared fresh) 10 μL isocitric acid - trisodium salt (154.86 mg/mL, prepared fresh) and 10μL isocitrate dehydrogenase (prepared as described above) were added. The tubes sat at room temperature for a minimum of 10 minutes after which time 150 μL of thawed microsomal preparation was added to each tube. 2 mL of KOH-DMSO (0.15M KOH in 85% DMSO) were added to all blank tubes prior to the addition of 20μL of tritiated B[a]P (specific activity of 15 - 20 μCi/μmol, prepared by diluting the commercial preparation of tritiated B[a]P with unlabelled B[a]P) to all of the tubes. All (uncapped) tubes were placed in a 25°C water bath for 30 minutes, at which time the reaction in the sample tubes was stopped by addition of 2.5 mL KOH-DMSO. Two hexane extractions were done on all tubes to remove unreacted B[a]P. Each extraction involved addition of 3 mL of hexane (HPLC grade, Caledon), vortexing and shaking the tubes on a Burrell wrist action shaker for 5 minutes then centrifuging in a GS-6R centrifuge at 3500 rpm for 5 minutes and aspirating off the hexane layer. The extracts were again centrifuged to ensure separation of any protein from the DMSO layer, and 200μL of the clear supernatant were transferred to mini scintillation vials to which 5 mL of Atomlight scintillation solution were added. Samples were counted on a liquid scintillation counter (Beckman LS-7500) and

AHH activity was determined using a quench curve stored in the LSC memory from readings on a series of quenched standards. A background and an unquenched standard (Beckman) were included with each batch of samples to ensure proper LSC function. Prior to the calculations blanks (for each sample) were averaged and subtracted from the sample readings.

Steroid Hormone Analysis

Plasma steroid hormone levels (testosterone and 17β-estradiol in females and testosterone in males) were determined in white sucker by means of an enzyme immunoassay technique which has been validated for use in fish; the assay kit was purchased from Cayman Chemical Company (Brown et al., 1993). In brief, plasma samples were extracted in ethyl acetate:hexane (3:2 v/v) and the dried extracts were redissolved in assay buffer, which, after appropriate dilution, was used in the EIAs. Extraction efficiency, as determined for each sample by means of addition of a small quantity of radioactive tracer (either testosterone or estradiol, depending on the analysis) averaged 78.4 ± 1.4%. The percentage of radioactivity recovered is assumed to be identical to the percentage of the unlabelled hormone recovered in the extraction. Assay recovery was also monitored to ensure that the assays were specific to the appropriate hormone. This was accomplished by adding a large quantity of unlabelled hormone to samples which were previously extracted and measured. After addition of the spike the sample was reanalyzed. The amount of hormone expected is equal to the amount of hormone added plus the amount of hormone measured in the sample previously. Recovery was calculated by subtracting the expected value from the actual value and multiplying by 100. Recoveries near 100% indicate that the assay is specific and appropriate for quantifying

the hormone in question. Recoveries of spiked estradiol in the EIA were 101.2 ± 2.6% (mean ± S.E.M.) and the assay provided estimates of 17β-estradiol within 7%. Recoveries of spiked testosterone in the EIA ranged from 91.7 to 103.8% and gave estimates of testosterone concentration within 5%. In calculating the final hormone concentration extraction efficiency was used to correct for losses.

Histology of Reproductive Organs

Maturity indices, egg diameters, egg weights, absolute and relative fecundities were assessed using the methods of Brown et al. (1993).

Maturity indices were determined on Davidson's fixed tissues which were dehydrated in n-butanol and embedded in paraffin. Tissue sections of 8 μm were stained with Harris' hematoxylin and eosin and examined under a light microscope. Maturity index provided an indication as to the stage of sexual development of ovaries and testes; each ovary was placed into one of 11 possible groups and each testis placed into one of seven groups (the higher the group number the nearer the fish is to spawning). A description of these groups is provided in the Appendix (Table A1).

One-hundred vitellogenic (buffered formalin fixed) eggs were picked out of each ovary, lightly blotted and weighed to obtain an average egg weight (vitellogenic eggs are those in the process of accumulating yolk in preparation for release at the next spawning period). Egg diameters were determined on 25 of these eggs by use of calipers. Due to shrinkage (from dehydration) in formalin, egg diameters presented here are about 94% of the values which would be obtained from fresh eggs (B. Evans, personal communication).

Liver Vitamin Analysis

Retinol, retinyl palmitate (vitamers of vitamin A) and tocopherol (vitamin E) were measured in liver tissue by isocratic reversed phase HPLC (Brown and Vandenbyllaardt, 1996; Palace and Brown, 1994). In brief, a subsample of liver was homogenized in distilled deionized water and ethanol was added to precipitate the protein which was pelleted. The homogenate was then extracted with ethyl acetate/hexane (3:2, v/v) and residues were redissolved in the mobile phase, acetonitrile/methanol/water (70:20:10, v/v/v). Twenty μL of the mobile phase were injected onto a 3-μm bead size Adsorbosphere HS C_{18} column 4.6 mm i.d., 150 mm in length, with attached 10 mm Adsorbosphere guard column, thermostated at 26°C and with a flow rate 1.0 mL/minute. Retinol and retinyl palmitate were detected and quantified fluorometrically with excitation and emission wavelengths of 330 and 480 nm and tocopherol was detected and quantified spectrophotometrically with a UV wavelength of 292 nm. Recoveries of known spikes were used to correct for extraction efficiency.

Calculation of GSI, LSI and CFAC

These physiological parameters were calculated as follows:

Gonadosomatic index (GSI) = (gonad weight / (total body weight - gonad weight)) x 100

Liver somatic index (LSI) = (liver weight / (total body weight - liver weight)) x 100

Condition Factor (CFAC) = (weight (g) / length^3 (cm)) x 100

Liver somatic index and condition factor were not corrected for gonad weight because gonad weights were unavailable for male suckers in August, 1993 and the calculations had to be the same for all fish to facilitate statistical comparisons. Aside from this difference the

formulae for the calculations were taken from Hodson et al. (1992).

Fish Aging

Fish ages were determined using dried pectoral fins, by counting annuli in paraffin-embedded fin ray cross sections (Chalanchuk, 1984).

Statistical Analyses

Due to differences in some of the measured variables between the sexes, data for males and females have been analyzed separately. Homogeneity of variance was assessed using Bartlett's test, and where necessary ($p < 0.01$) data were transformed to obtain a more uniform variance by a $\log_{10}$ or Taylor's power law transformation. In instances where the variances could not be made more uniform by transformation, the Kruskal-Wallis nonparametric statistic was used to compare the means. Comparisons between sample sites for length and weight were done using ANCOVA with age as a covariate, gonad weight and liver weight were compared using ANCOVA with body weight as a covariate, and other parameters were analyzed using ANOVA, following the general linear models function of Systat (Wilkinson et al., 1992). Weight was examined as a covariate for all parameters analyzed by ANOVA (except GSI, LSI and CFAC, as weight is included in their calculations) and was never significant for any parameter at all sampling times and did not alter the outcome of the statistics. Samples were generally collected over a period of a few weeks which may be enough time to affect some of the results, especially those that fluctuate with reproductive development. For this reason year day was analyzed as a covariate, but this was

rarely significant and never altered the outcome of the statistics. Growth was examined using ANCOVA by comparing the slopes of length versus age plots, however, sample sizes proved to be too small for this type of analysis. Pairwise comparisons were conducted by applying Fishers Least Significant Differences (LSD) test. Correlations between variables were examined using Pearson's procedure. A probability level of <0.05 was considered to be significant. For clarity of presentation, arithmetic means with standard errors have been used in the figures .

RESULTS AND DISCUSSION

The primary focus of the field research was to determine whether there were measurable differences in fish downstream from the Pine Falls pulp mill relative to those caught upstream (i.e. site differences, Figure 3), however, during the statistical analysis differences between sampling times have also been examined. The most likely reason for differences between upstream and downstream sampling sites would be the presence of the mill, however, the presence of the dam and proximity to Lake Winnipeg can not be ignored as potential sources of variation among sites.

Differences between sampling times do little to accomplish the goal of defining whether the mill impacts the fish downstream and so will not be discussed unless warranted by affecting the outcome or enhancing the understanding of site differences.

Site differences for all variables will be discussed for each sex. To simplify presentation, the graphics will only show site differences for female and male white suckers and time differences will be dealt with solely in the text.

Sampling and Analysis of Feral White Suckers

A total of 138 mature white suckers (85 females and 53 males) were obtained from the Winnipeg River during August 1993, May 1994, and August 1994. Table 1 provides a breakdown of samples with respect to time, sex and site and Table 2 indicates the overall weights, lengths and ages of the fish.

Table 1: Summary of white sucker catch data for the Winnipeg River by sampling time, site and sex.

Sampling Time	August, 1993		May, 1994		August, 1994	
Sex	Male	Female	Male	Female	Male	Female
Upstream Site	6	9	3	8	4	15
D1 (Immediately downstream of mill)	9	15	13	9	7	15
D2 (6 to 8 km downstream)	6	7	-	-	5	7

Table 2: Weights, lengths and ages for white sucker taken from the Winnipeg River in 1993 and 1994.

Sex	N	Weight Range (g)	Average Weight (g)	Length Range (cm)	Average Length (cm)	Age Range (years)	Average Age(years)
Male	53	428 - 1719	949	32.0 - 47.8	40.2	3 - 13	6.4
Female	85	449 - 2082	1147	31.6 - 50.2	43.0	3 - 17	6.2

Summary statistics as categorized by sampling time, site and sex are provided in the Appendix (Table A2).

MFO Activity (Figures 4 and 5)

The carbon monoxide difference spectra of the hepatic microsome preparations indicated little degradation of P-450. Peaks at the 420 nm wavelength were always small relative to those at 450 nm (data not shown), indicating that little or no degradation of P-450

occurred and that the samples were appropriate for use in the enzyme activity assays (Omura and Sato, 1964a; Omura and Sato, 1964b).

FEMALES Hepatic EROD (Figure 4) and AHH (Figure 5) activities were higher in fish from site D1 than those from upstream, with EROD being induced by 8.6 and 4.1-fold in August, 1993 and 1994, respectively. There were no site differences in May. In August, 1994 fish from site D2 also showed an increase in EROD activity, being induced by 2.6-fold.

MALES The trends in EROD (Figure 4) and AHH (Figure 5) data for males were similar to those of the females, however, the only significant difference was an elevation in AHH activity at site D1 in August, 1993.

Numerous field studies have documented similar increases in MFO activities in a variety of fish species captured downstream from a variety of different pulp mill effluent discharges (Andersson et al., 1988; Larsson et al., 1988; McMaster et al., 1991; Hodson et al., 1992; Kloepper-Sams and Benton, 1994; Munkittrick et al., 1994). A majority of this research has focussed on pulp mills that use chlorine bleaching. Munkittrick et al. (1994) reported a low level of MFO induction downstream from two non-chlorinating pulp mills, although, different from our data, only male white suckers were induced.

MFO activity was increased at site D1 at both August sampling times, but was not increased when the fish were sampled in the spring. It has been documented that the MFO system of fish may (Boychuk, 1994; van den Heuvel, 1995) or may not (Förlin and Haux, 1990; McMaster et al., 1991; Munkittrick et al., 1991) be readily inducible in fish that are near spawning. Reasons for the lack of MFO induction in the spring may include one or more of the following: the possibility that the MFO system can respond differently at different

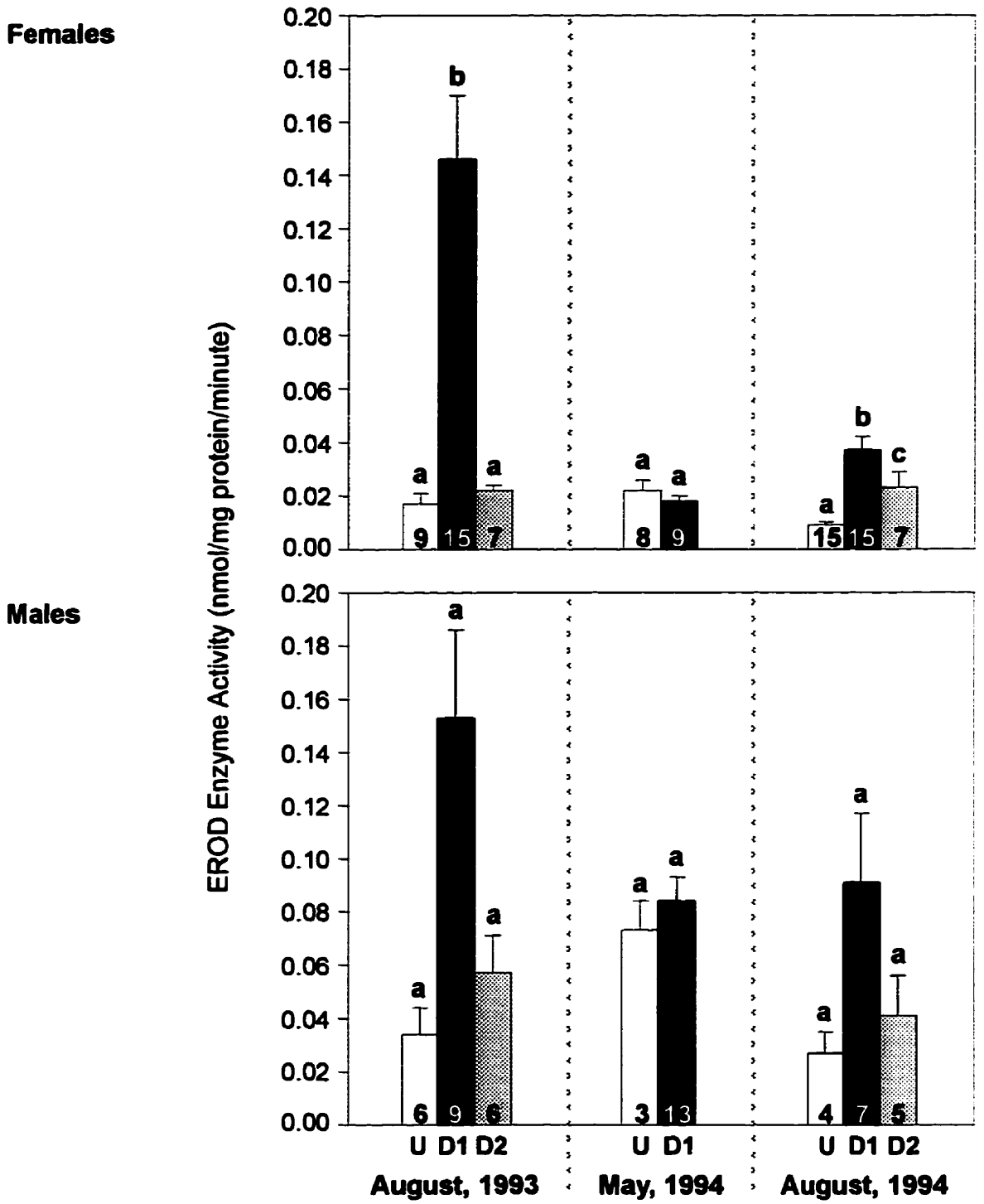


Figure 4: Mean 7-ethoxyresorufin O-deethyase (EROD) enzyme activity of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

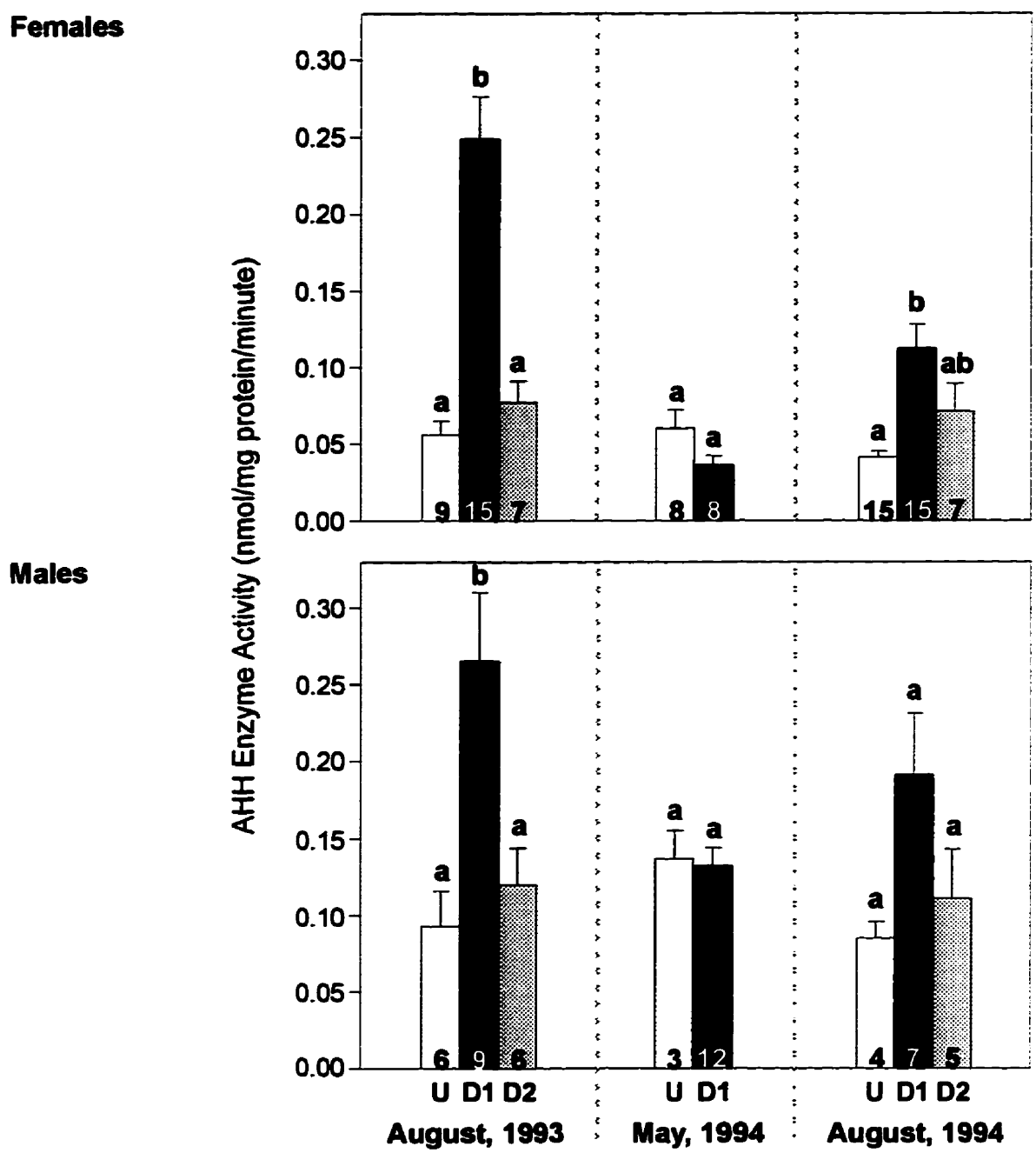


Figure 5: Mean aryl hydrocarbon hydroxylase enzyme activity of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

times in the reproductive cycle; the potential movement of the fish into the lake in the Winter (removing the fish from effluent exposure for a period of time, as proposed by some members of Fort Alexander); the possibility of catching fish non-native to the Winnipeg River at spawning time; and/or the potential for increased mobility of the fish in the spring relative to the summer. The EROD induction in females at both downstream sites in August, 1994, may be due to the increased effluent concentration in 1994 relative to 1993. This increased impact in August, 1994 conflicts with the reduction in EROD activity at site D1 between August, 1993 and August, 1994. This decrease could be due to the increased size of the sampling area in August, 1994 or to the storage of logs on the river in August, 1993; a practice which ceased prior to sampling in August, 1994. Log storage may have affected MFO activity at site D1 because some of the compounds causing the induction may have come directly from the logs (Bezte and Farmer, unpublished data).

Steroid Hormones (Figures 6 and 7A)

In August, 1994 some samples were obtained using overnight gill-net sets. When testosterone levels were compared between nets cleared hourly and those left overnight, fish obtained in overnight sets were found to have significantly lower levels of testosterone than those obtained with the hourly sets (data not shown). For this reason, all testosterone values for fish captured in overnight sets were deleted. McMaster et al. (1994) reported similar depressions in testosterone levels with increasing time in nets. It is well known that testosterone levels are sensitive to physical stress (Pickering et al., 1987). The results for other parameters did not differ between the hourly and overnight sets.

FEMALES Female suckers from site D1 had lower plasma testosterone concentrations than fish from upstream during both August sampling times, but there were no significant site differences in the spring (Figure 6). Testosterone levels at D1 were reduced to 21% those at the reference site in both summers. Estradiol was never significantly reduced downstream, although it was significantly reduced at site D1 relative to site D2 in August, 1993 (Figure 7A).

MALES Plasma testosterone levels in males followed a similar trend to those of the females, being reduced to 30% of the reference levels in the summers, however, testosterone was not significantly different between the upstream and downstream sites at any sampling time (Figure 6). Testosterone levels were lower at site D1 relative to site D2 in August, 1993.

White suckers caught immediately downstream of the Pine Falls mill exhibited reductions in plasma steroid hormones similar to those previously reported by others working on different pulp mills (McMaster et al., 1991; Hodson et al., 1992; Munkittrick et al., 1994). Female plasma estradiol was less sensitive to mill effects/effluent exposure than testosterone, as levels of estradiol were not lower at the downstream sites. McMaster et al. (1991) found reduced levels of testosterone and estradiol in female suckers downstream from a bleached kraft mill with primary effluent treatment, and they also had one sampling time when testosterone levels were significantly lower, but estradiol levels were not. Hormone levels at site D2 were similar to those at the upstream site at all sampling times indicating that hormone metabolism was not affected by the effluent/mill at this site. Reductions in steroid hormones noted at site D1 may be due to the inability of animals to produce them or to an

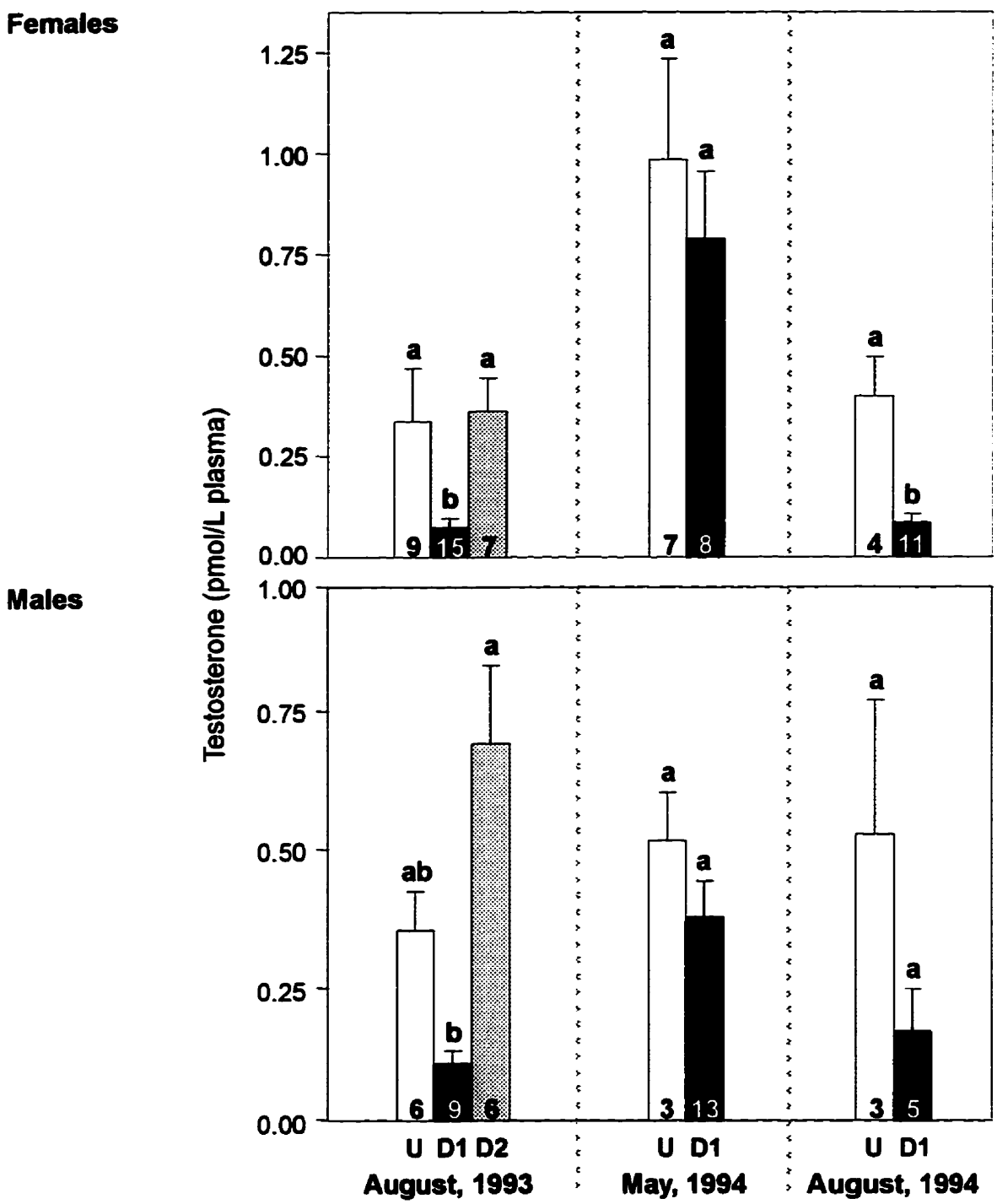


Figure 6: Mean plasma testosterone concentration of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

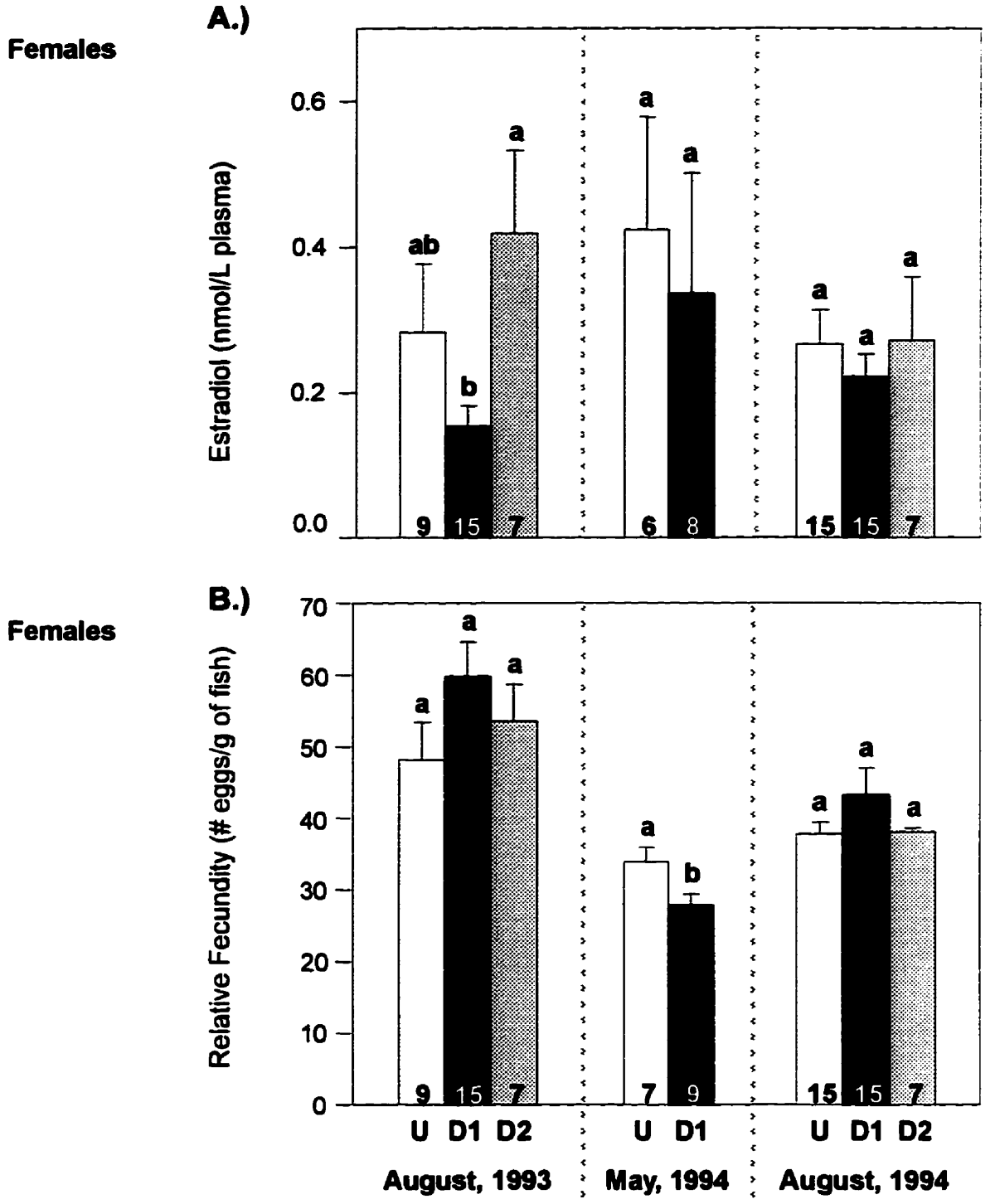


Figure 7: Mean plasma estradiol (A) and relative fecundity (B) of female white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size

increase in their rate of excretion (Kime, 1995). The production of steroid hormones has been shown to be inhibited by exposure to bleached kraft pulp mill effluents (Van Der Kraak et al., 1992; McMaster et al., 1993) and in August, 1994 McMaster et al. (1996) showed that the ovaries of fish from site D1 did have a reduced ability to synthesize testosterone (*in vitro*) relative to ovaries of fish from the upstream site.

McMaster et al. (1991) reported a similar if not greater decrease in testosterone levels in BKME exposed prespawning and spawning female white sucker when compared to those caught in the summer from the same sampling location. This is contradictory to these results which show a significant hormone reduction only in the summer. The reason for this discrepancy may be due to the overwintering of the fish in Lake Winnipeg, or to the possibility that the fish population in the spring is more mobile and/or non-resident to the area near the pulp mill; being present only in the spring for spawning.

Histology of Reproductive Organs (Figures 7B and 8)

There were no site differences in maturity indices for either sex at any time. This indicates that all fish examined were at the same stage of sexual maturity, thus validating comparisons of fecundity and egg size.

FEMALES Significant differences were noted in relative fecundity only in May, 1994 when the fecundity of fish from D1 was reduced by 17.4% (Figure 7B). The only difference in egg size occurred in August, 1994, when females from site D1 had smaller, lighter eggs relative to those of fish from upstream (Figure 8). Fish from all three sites had lower relative fecundities in August, 1994 than August, 1993, but only fish from the upstream

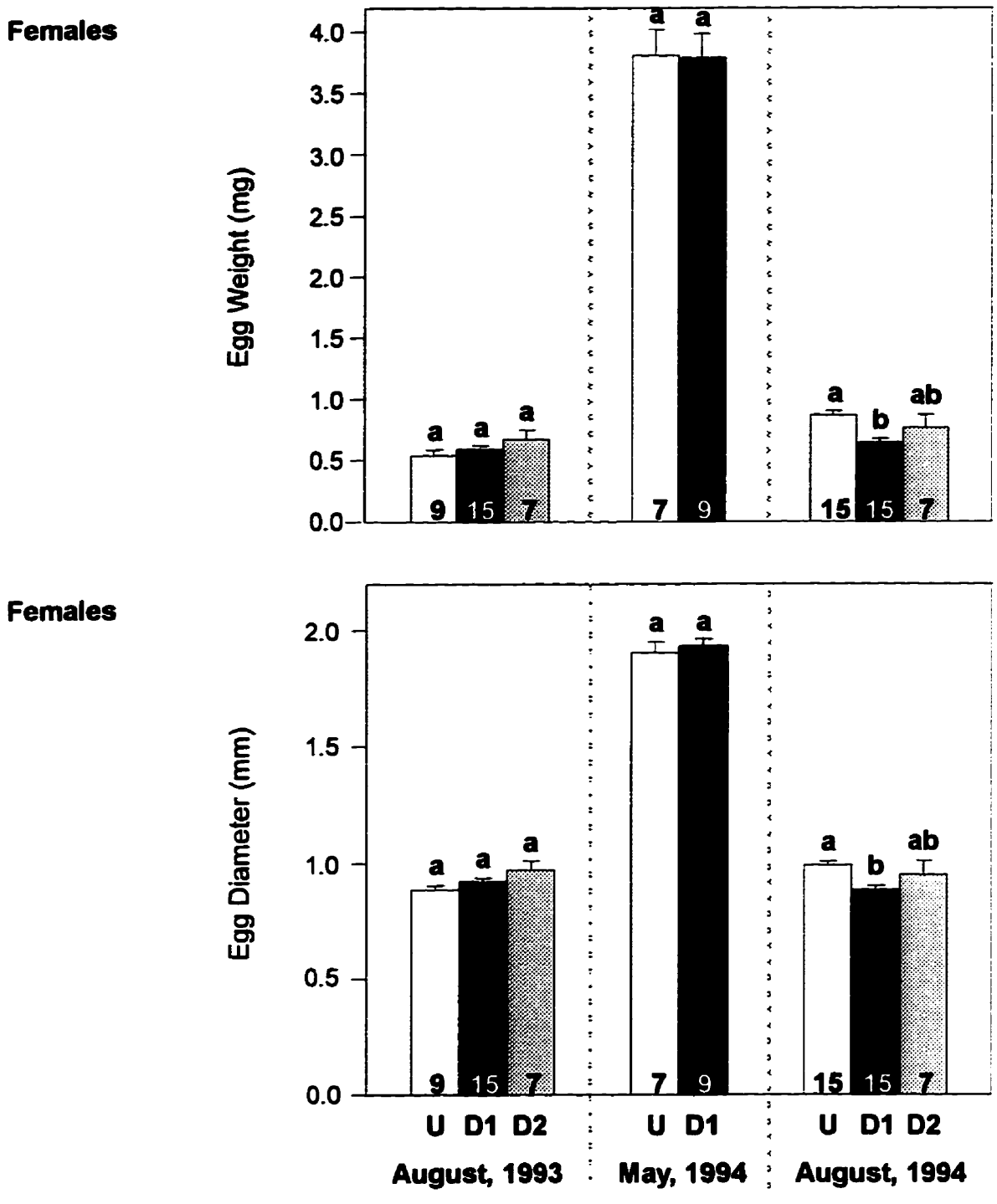


Figure 8: Mean egg weight (top) and egg diameter (bottom) of female white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

site had larger, heavier eggs in August, 1994 relative to August, 1993. White sucker ovaries contained fewer, larger eggs in May than at either of the August sampling times.

As the ovaries of a fish mature, the size of the eggs increases, but the number of eggs tends to decrease. The number of eggs decreases with ovarian development because a certain percentage of the developing eggs are lost during development (this process is referred to as oocyte atresia) (Scott, 1962). Thus, fish sampled early in egg development should have a large number of small eggs, while those same fish sampled near spawning should have fewer but larger eggs. The white sucker of the Winnipeg River did have fewer, larger eggs in the spring relative to those in the summer. The smaller eggs at site D1 relative to those from upstream or further downstream in August, 1994, suggests that ovarian development at this site was occurring at a slower rate. That a decrease in egg size was noted in 1994 and not 1993 may suggest that the increased effluent concentration in 1994 was having a greater effect on ovarian development. An examination of the site and time differences together revealed that egg sizes at D1 were the same in August, 1993 and August, 1994, but that egg sizes at the upstream site were larger in 1994 than they were in 1993. The larger egg sizes at site U in 1994, accompanied by the decrease in fecundity, indicated that the fish at this site were developing faster in 1994 than they had in the previous year. The reduced fecundity at the downstream sites in 1994 relative to 1993 is also an indication of an increase in developmental rate in 1994, however, it was unaccompanied by an increase in egg size; indicating that development at the downstream sites was not keeping pace with that at site U.

Testosterone levels were reduced in the summers when gonad maturation would be

taking place, however, there was no difference in fecundity estimates. This indicated that the lower testosterone may be insufficient to affect ovarian development at this time. After the first sampling season in August, 1993, it was hypothesized that although there appeared to be no effects on gonad development at the time, the downstream fish may not be able to maintain this level of gonad development through to spawning. Because of this, samples were also obtained just prior to spawning in May, 1994. The spring results support this hypothesis; female white sucker immediately downstream from the mill with lower hormone levels, were not able to attain a similar level of egg production as those from upstream, with higher hormone levels, although there is also the possibility that the population sampled in the spring was different from that sampled in the summer. Gagnon et al. (1994) reported a similar finding in white suckers exposed to bleached kraft mill effluent in the St. Maurice River. GSI was similar at all sites in the summer when hormone levels were reduced, but GSI was lower in the spring. In this study and that of Gagnon et al. (1995) significant effects on fecundity were not detected early in gonad development, but were found near gonad maturity, indicating the need to assess reproductive indices at different stages of the reproductive cycle.

There are several pulp mill studies specifically examining fecundity and egg size:

1) McMaster et al., 1991, reported that fish of the same age exposed to primary treated bleached kraft mill effluent were less fecund than reference fish; 2) Munkittrick et al. (1992a) showed that whitefish (*Prosopium williamsoni*) exposed to this same effluent had higher relative fecundities and lower egg weights than those from a reference site (indicative of a reduced rate of ovarian development); 3) Gagnon et al. (1995) reported alterations in fecundity of white suckers exposed to pulp mill effluent. The results presented here concur

with the others and suggest that the effects on fecundity and egg size reported to occur downstream from chlorine bleaching kraft mills also occur downstream from the Pine Falls mill.

At spawning time, fish from D1 produced 17.4% fewer eggs (but of similar size) than those from upstream. Although this was a significant decrease in fecundity it is important to note that all female fish obtained from the Winnipeg River had very high levels of fecundity. Relative fecundity estimates of approximately 20 eggs/g of fish have been found in white sucker from relatively pristine lakes in the Experimental Lakes Area (R. Evans, personal communication) and Scott and Crossman (1973) report a value of 25 eggs/g of fish. In contrast, the lowest relative fecundity of Winnipeg River white suckers occurred at site D1 in the spring and was greater than 30 eggs/g of fish. This indicates an overall high fecundity of the white sucker in this reach of the Winnipeg River, regardless of the mill inputs. Because Winnipeg River fish were so fecund, reduction at the D1 site is unlikely to have much impact on the white sucker population in the area. The free movement of the fish, their proximity to the lake and the possibility that the fish overwinter in the lake, all make it difficult to determine if effects were occurring at the population level.

While maturity and gonadosomatic indices (Table 3 and A2) were not different, fecundity estimates and examinations of egg sizes indicated that the fish at site D1 were somewhat less productive and that they developed somewhat slower than the fish upstream. Examinations of fecundity and egg size appear to be more sensitive indicators of potential reproductive effects than maturity or gonadosomatic indices.

Liver Vitamins (Figures 9, 10 and 11)

Hepatic concentrations of retinol, retinyl palmitate and tocopherol (α-tocopherol) could not be determined for every sample, because some liver samples were too small to provide sufficient tissue for all analyses.

FEMALES Hepatic concentrations of retinol, retinyl palmitate and tocopherol were reduced at site D1 during both of the August sampling times, but were unaffected in the spring. In August, 1994, tocopherol levels were also lower at site D2 relative to those at the upstream site (tocopherol in D2 fish was down to 63% of that in reference fish). At site D1 hepatic retinol levels were 13 and 26% of those at site U in August, 1993 and August, 1994 respectively (Figure 9); retinyl palmitate levels were 17 and 23% those of upstream fish in 1993 and 1994 respectively (Figure 10); and tocopherol levels were 36 and 45% those at site U in 1993 and 1994, respectively (Figure 11). The most notable time differences pertain to the increased retinyl palmitate levels at all sites in August, 1994 compared to those in August, 1993. During this time retinyl palmitate levels at site U increased from 179 to 459 μg/g, levels at site D1 increased from 31 to 107 μg/g and those at site D2 increased from 127 to 477 μg/g.

MALES Hepatic retinol levels were reduced at site D1 in May and at both the D1 and D2 sites in August, 1994, but were not reduced at either of the downstream sites in August, 1993 (Figure 9). In May, 1994, retinol levels at site D1 were reduced to 31% of the reference levels and in August, 1994, retinol levels were reduced to 39% and 23% at sites D1 and D2 respectively. Retinyl palmitate (Figure 10) and tocopherol (Figure 11) levels were significantly reduced at site D1 in August, 1993 and May, 1994, but there were no site

differences in August, 1994, and retinyl palmitate and tocopherol levels were never reduced at site D2. In August, 1993, retinyl palmitate and tocopherol levels at site D1 were 32% and 26% of the reference levels respectively. Retinyl palmitate levels were greater at sites U and D1 in August, 1994, relative to August, 1993.

The significance of lower hepatic retinol, retinyl palmitate and tocopherol in male suckers from site D1 in the spring should be interpreted cautiously, because the upstream sample size for these parameters was limited (n=3) and was accompanied by a high degree of variability.

Retinyl palmitate is the predominant storage form of vitamin A in white sucker (Branchaud et al., 1995). An examination of retinyl palmitate indicates the amount of vitamin A available to the fish. Because it is the storage form, retinyl palmitate level also provide information into the past uptake and dietary availability of this vitamin. Retinol levels indicate the amount of readily usable vitamin A and retinyl palmitate can be readily converted to retinol as required.

Retinol appeared to be somewhat more sensitive than retinyl palmitate, as retinyl palmitate was never significantly reduced at site D2 but retinol was reduced at site D2 in male suckers in August, 1994. Vitamin E (tocopherol) was also lower in female suckers from D1 in the summers and at site D2 in August, 1994. Retinol and tocopherol levels were more affected in August, 1994 as there were significant reductions at site D2 that did not occur in August, 1993. The greater impact in August, 1994 was not noted at site D1, but this may have been due to the larger area sampled at site D1 in August, 1994 (Figure 3). However, the sample sizes for female suckers were smaller in August, 1993 relative to August, 1994,

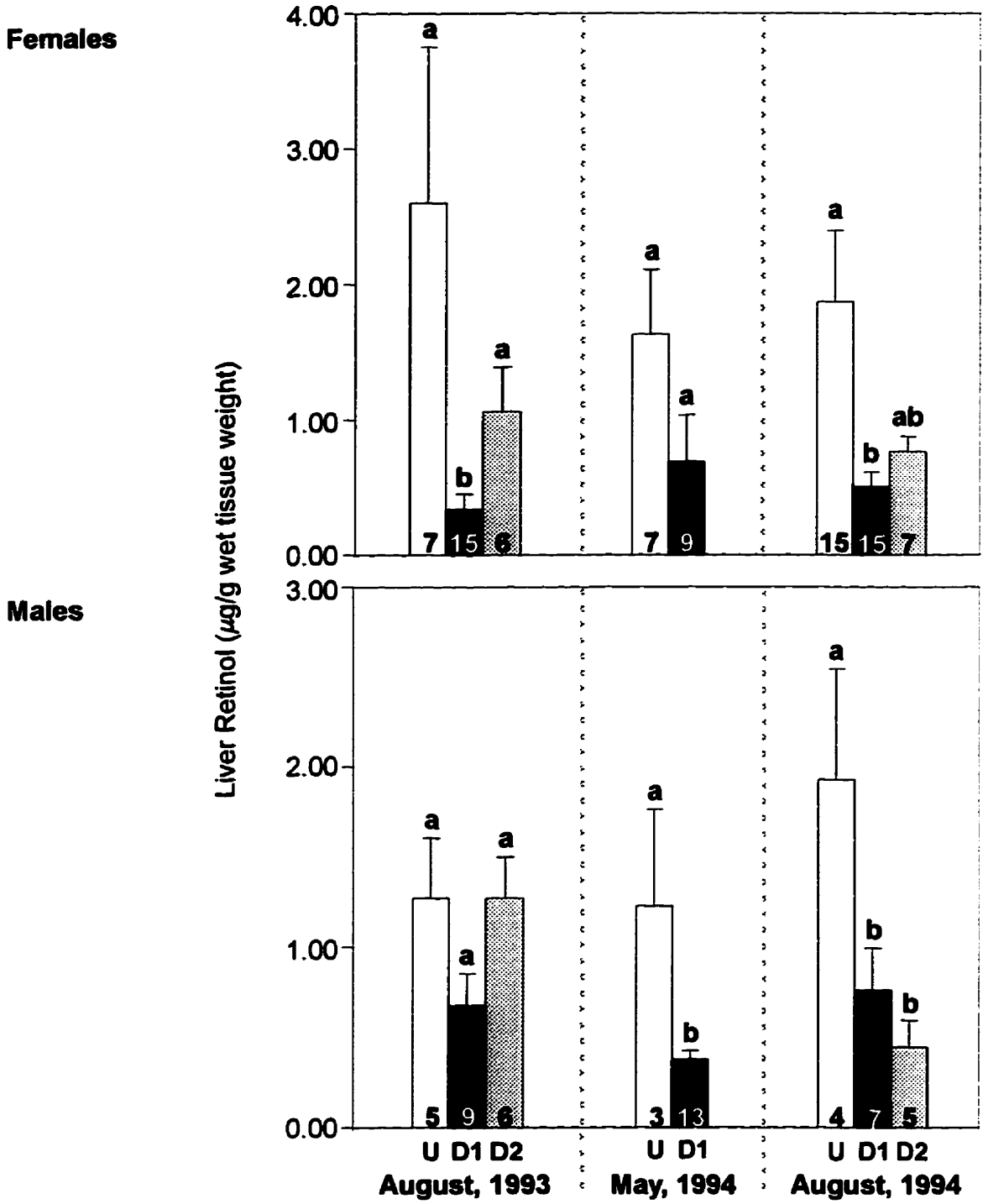


Figure 9: Mean hepatic retinol concentration of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars wth the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

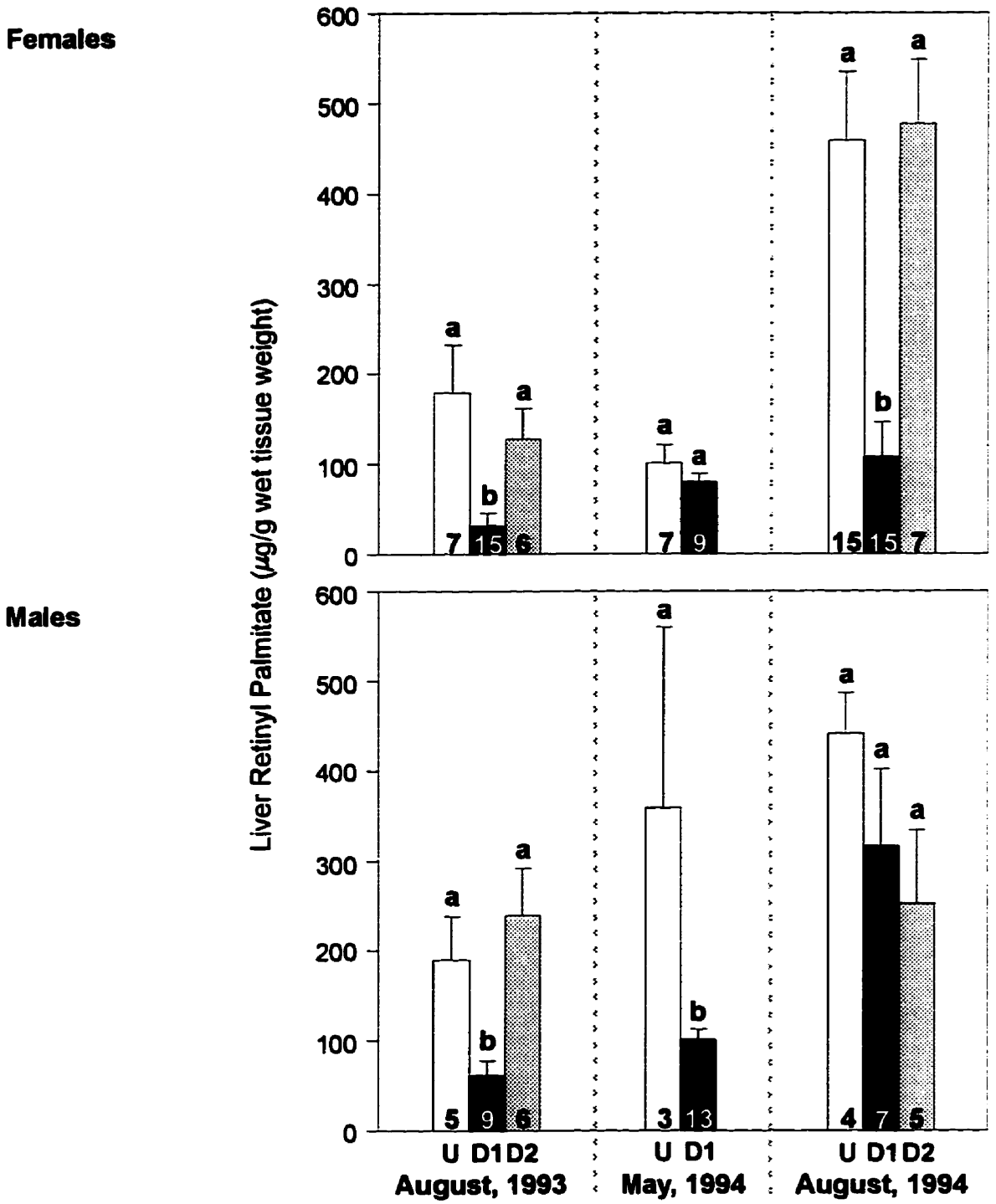


Figure 10: Mean hepatic retinyl palmitate concentration of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars wth the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

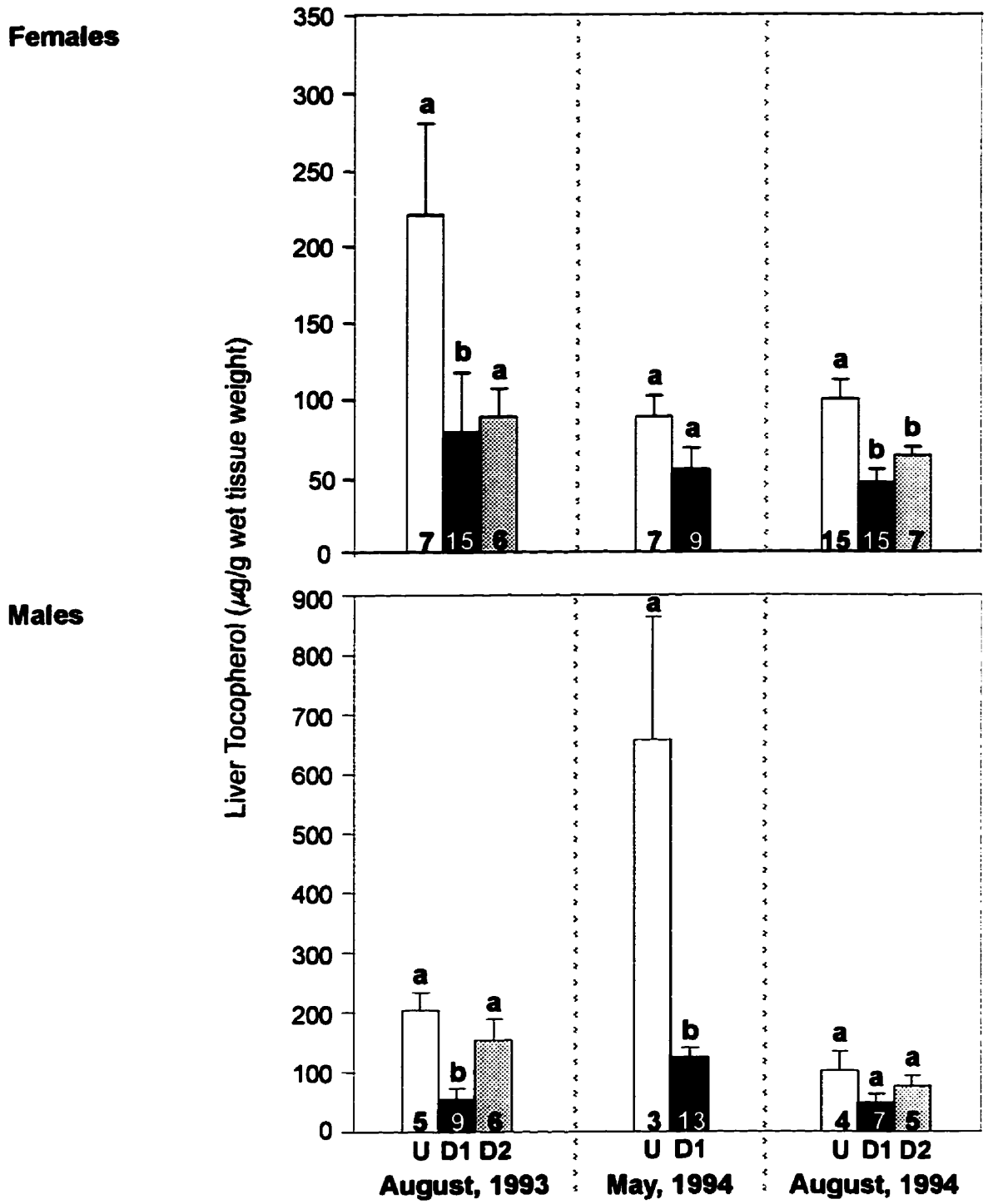


Figure 11: Mean hepatic tocopherol concentration of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars wth the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

and this may be why there were no significant differences between U and D2 in August, 1993. Tocopherol levels were never reduced in males from site D2, and were not significantly reduced at site D1 in August, 1994 indicating that tocopherol levels in males may be less sensitive. The lack of a significant site difference in spring for retinol, retinyl palmitate and tocopherol in female suckers suggests better nutrition of the downstream fish in the spring, but also appears to be due to a decline in the levels of these vitamins in fish from upstream. As mentioned in the sections on MFOs and hormones the reduction in significant vitamin differences in the spring may be due to the overwintering of the fish in the lake, the potential increased mobility of the fish in the spring or to the presence of non-resident fish at spawning time.

Deficiencies in vitamins A and E may lead to a decrease in growth, decreased appetite and immunity, muscular weakness, nephritis, liver necrosis, nervous system disorders, and reproductive and visual impairments (Combs, 1992). Studies have demonstrated a reduced immune response in fish deficient in tocopherol (Blazer and Wolke, 1984). Watanabe and Takashima (1977) found that a tocopherol deficiency in carp (*Cyprinus carpio*) inhibited ovarian development and hypothesized that tocopherol was essential for healthy gonad development. Vitamin A and/or some of its precursor carotenoid pigments have also been hypothesized to play a role in fish ovarian development (Steven, 1949; Woodhead and Plack, 1968; Harris, 1984 and Luquet and Watanbe, 1986). Unfortunately, these studies did not quantify the degree of vitamin deficiency and the species used were not white sucker; so we cannot conclude that the reduction in vitamins noted downstream of this mill were sufficient to effect other biochemical, physiological and morphological parameters.

There is little information on vitamins in fish downstream of pulp mill effluents, however, Brown and Vandenbyllaardt (1996) reported a decrease in retinyl palmitate in longnose sucker (*Catostomus catostomus*) downstream of a chlorinating pulp mill effluent and Brown and Munkittrick (unpublished data) found a similar decrease in retinoids in white suckers downstream from a bleached kraft mill effluent. White suckers sampled from a river contaminated with moderate to high levels of PCBs, PAHs and heavy metals had hepatic vitamin A stores that were only 9.3% (females) and 30% (males) of those of fish from a reference location, indicating that contaminants can alter vitamin levels in white suckers (Branchaud et al., 1995). The actual amount of retinyl palmitate in the contaminant exposed white suckers described by Branchaud et al. (1995) was much lower than the lowest levels in fish from site D1, although their retinol levels were similar.

Vitamin levels have been shown to be reduced in organisms exposed to a wide variety of environmental contaminants (Zile, 1992) and contaminants in the mill effluent may be responsible for the reductions in vitamins A and E noted in these fish. Another explanation for these vitamin differences could be the diets of the fish in the area. Because there were few weight differences among the sites (see "Size and Age Comparisons" below) the caloric intake of the fish at the different sites could not have been substantially different. It appears unlikely that the vitamin depletion was due to a lack of food, however, while the food organisms may have been abundant (Wong et al., 1996) they may have been less nutritious, possibly because of the wood fibre contamination (Wong et al., 1996). There was no vitamin data collected on chironomids, oligochaetes or mayflies, but it seems worth determining whether the depletion of vitamins in the fish could be induced by the change in diet from the

benthic community found upstream to that found downstream. It is possible that the white suckers downstream from this mill had less vitamins available to them in their diet.

The higher retinyl palmitate levels in 1994 suggest that feeding conditions were better at this time. The reason for the better nutrition in 1994, as indicated by the retinyl palmitate stores is unknown at this time.

Morphological Parameters (GSI, LSI and CFAC) (Figures 12 and 13)

FEMALES Gonadosomatic indices were never significantly different among the sites (Table A2).

Liver somatic indices were significantly higher at site D1 at all sampling times but were never higher at site D2 (Figure 12).

Condition factor was significantly reduced at site D1 in August, 1993, but there were no site differences found in May or August, 1994 (Figure 13).

MALES Male gonadosomatic indices could only be calculated for May and August, 1994, as male gonads were not collected in August, 1993. There was no difference in GSI between the sites at either sampling time (Table A2).

LSIs at site D1 were greater in August, 1993, and May, 1994, but were not significantly elevated in August, 1994 and were never elevated at site D2 (Figure 12).

Condition factors were not significantly different among the sampling sites at any sampling time (Figure 13).

The lack of significant effects on GSI suggest that the lower fecundity in the spring and the reductions in egg size in August, 1994, were not sufficient to cause a decrease in the

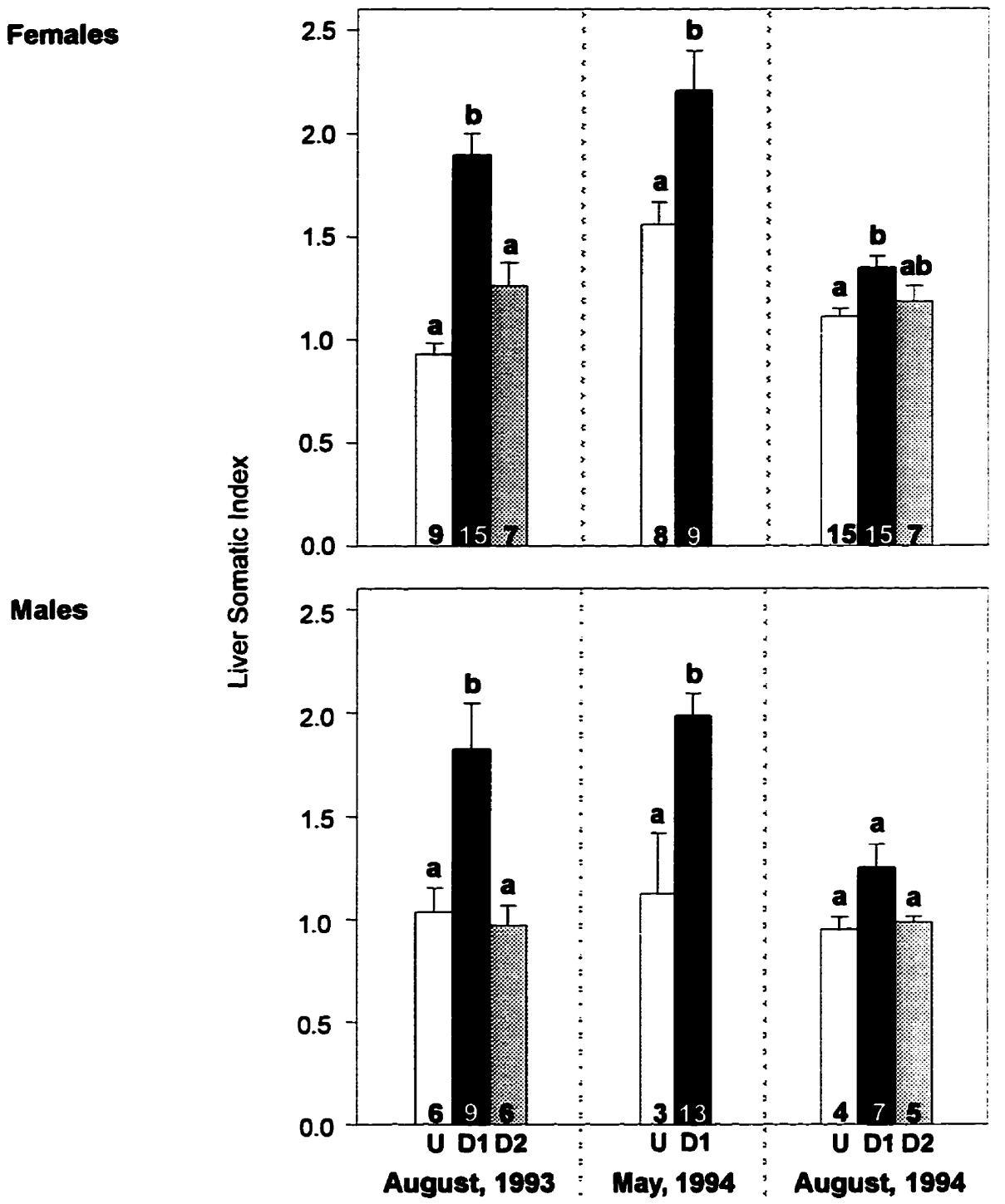


Figure 12: Mean liver somatic index ((body weight - liver weight) / body weight) * 100) of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

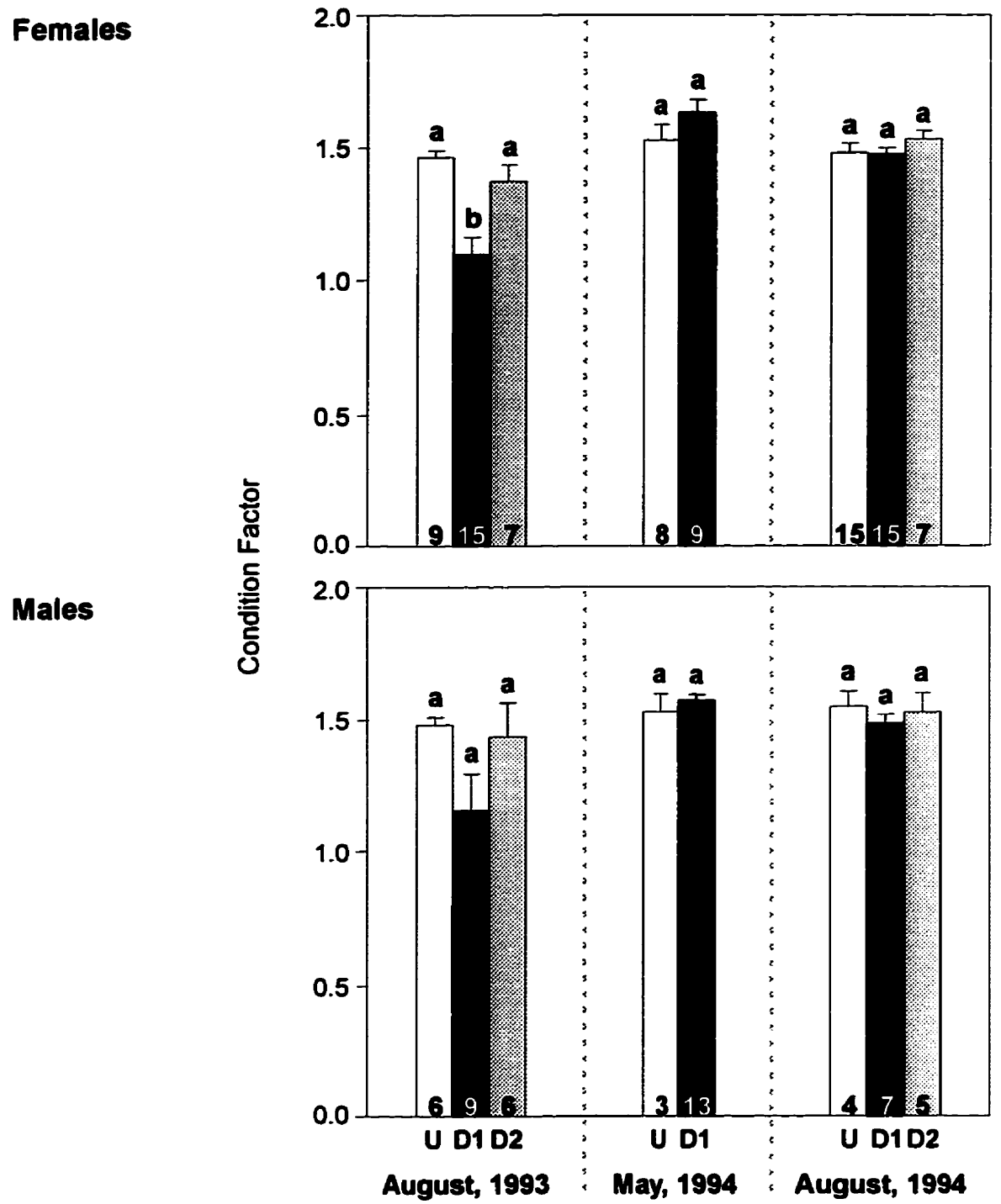


Figure 13: Mean condition factor ((body weight (g) / length^3(cm)) * 100) of female (top) and male (bottom) white suckers from the Winnipeg River. Fish were obtained from three sampling sites (U, upstream; D1, near downstream and D2, far downstream) in August, 1993 and August, 1994 and from two sampling sites in May, 1994 (U and D1). Lines above the bars represent the S.E.M. and bars with the same letter are not significantly different ($p < 0.05$). The number at the base of each bar indicates the sample size.

overall amount of gonad tissue. Female white sucker captured downstream from seven out of eight pulp mills in Ontario had reduced GSIs regardless of the presence of secondary treatment or absence of chlorine bleaching (Munkittrick et al., 1994). This leaves only one of the eight mills with no impact on gonad size. Similar to our results, Gagnon et al. (1995) reported no significant difference in GSI immediately downstream from a secondary-treated bleached kraft mill effluent in Quebec.

Liver somatic indices may be influenced by feeding conditions and/or contaminant exposure. Contaminant exposure can increase liver size by causing metabolic disturbances and/or by increasing the amount of protein produced by the liver (as occurs with the increase in biotransformation enzymes, such as the mixed-function oxygenases; Andersson et al., 1988). Liver somatic indices were higher at the immediate downstream site at all sampling times (Figure 12). This response has frequently been reported in fish downstream from other pulp mill effluent discharges (Kloepper-Sams et al., 1994), including those from other non-chlorinating mills (Larsson et al., 1988; Munkittrick et al., 1994). It could not be determined whether the increase in LSI was due to differences in feeding conditions between the sites or to contaminant exposure.

Condition factor generally reflects the nutritional status of the fish and may be higher due to better feeding conditions, (Busacker et al., 1990), but, may also be affected by contaminants. The decrease in condition factor at site D1 in August, 1993, indicates that the mill had some negative impact. It is uncertain whether the decreased condition factor is attributable to the chemical nature of the pulp mill effluent or to the diet of the fish downstream of the mill since the benthic invertebrate populations were different (Wong et al.,

1996). A reduced or similar condition factor downstream from a pulp mill is not new to the pulp mill literature, as increases, no effects and decreases have all been noted downstream of other pulp and paper mills (Munkittrick et al., 1994, Hodson et al., 1992 and Barker et al., 1994). The inconsistencies in the results for condition factor, accompanied by the nearness of the dam, town and lake, do not allow for a definitive conclusion to be drawn.

Size and Age Comparisons

FEMALES In August 1993, female fish from site D1 were older than those sampled from sites U or D2, but there were no differences in the lengths or weights of these fish at any sampling time. Females from site D1 were older in August 1993, relative to fish caught at this site in August, 1994, but were heavier in August, 1994.

MALES In August, 1993 male suckers from D1 were older than those from U or D2 and fish from site D2 were longer than those from site U. In August 1994, males from both downstream sites were longer and heavier than those from upstream. Male suckers were older at site D1 in August, 1993 when compared to those caught in August, 1994, but there were no weight differences.

It is not known why fish from site D1 were older than those from the other sites in August, 1993; this difference in age was not noted at the other sampling times. One might expect that if fish from site D1 were significantly older that they should also be significantly larger, but this was not the case. Fish from D1 were older but were not longer or heavier than those from the reference site in August, 1993. This is not unusual, because all of the fish had reached sexual maturity (i.e. were able to spawn) and growth tends to slow down in mature

white suckers (Dr. K. Mills, personal communication). The trend of increasing lengths and weights in male suckers was not noted in the female suckers, but indicates that the condition of the fish was not adversely affected.

The reason for the decrease in fish age at site D1 in 1994 relative to 1993 is unknown, but the increase in weight of the females in 1994, accompanied by their younger age, indicates that conditions for growth at this site were better in August 1994, than they were in August 1993. Evidence from the males does not contradict or support this hypothesis, as they were younger and of similar weight at site D1 in August, 1994 relative to August, 1993.

White suckers downstream from a primary treated bleached kraft mill in Jackfish Bay were older and shorter than those from a reference site (McMaster et al., 1991), while white suckers below the Pine Falls mill tended to be longer (males) and of similar age (except at D1 in August, 1993). Gagnon et al. (1995) sampled white suckers from a bleached kraft mill-impacted river below a dam and a reference river below a dam, and found that fish downstream from dams and small towns exhibited an increased rate of growth and were longer than those caught upstream, regardless of their exposure to bleached kraft mill effluent.

Due to the relatively small sample sizes and inconsistencies in the differences for age (only noted in 1993), length and weight (sporadically significant), together with the close proximity of the Powerview Dam, town of Pine Falls and Lake Winnipeg it cannot be concluded whether these parameters were or were not affected by effluent from the Pine Falls mill.

Correlations

A summary of correlations obtained from the white suckers caught in August, 1993 and 1994 and May, 1994 is provided in Table 3. Variables which can be considered "autocorrelative" (i.e. length and weight, gonad weight and egg size etc.) have been omitted. Generally, EROD correlated positively with AHH, liver weight, LSI and relative fecundity and negatively with hepatic vitamins, testosterone, egg diameter, egg weight and condition factor. Vitamins were positively correlated with each other and were also positively correlated with condition factor, testosterone, estradiol, egg diameter, egg weight and relative fecundity.

EROD and AHH activities were very highly correlated (R^2 .961, $p < 0.001$) which suggests that only one of these parameters actually requires measurement. The fact that they do correlate so strongly, however, does serve as a check to help assure that the readings are correct. The correlations indicate that fish with elevated EROD activities and LSIs tend to have lower levels of hormones, vitamins and egg sizes and are generally in poorer condition.

Retinol and retinyl palmitate negatively correlated with EROD to a greater degree than tocopherol where no significant correlation was noted, a finding previously reported by Palace et al. (1996). Palace et al. (1997) attributed a decrease in retinol to the possibility of direct metabolism of retinol by MFO and phase II conjugating enzymes in lake trout exposed to PCB 126. The decreases in hepatic retinol (reduced by up to 82%) and retinyl palmitate (reduced by up to 77%) in the white suckers in this study were accompanied by EROD induction of less than 10-fold; induction levels produced by Palace et al. (1997) were well over 100-fold. The hypothesis that the vitamin depletion may be entirely due to increased

Table 3: Correlations between condition factor (CFAC), liver weight (Livwt.), gonadosomatic index (GSI), liver somatic index (LSI), plasma testosterone concentration (Test.), plasma estradiol concentration (Estra.), absolute fecundity (Absfec.), relative fecundity (Relfec.), egg diameter (Eggdiam.), egg weight (Eggwt.), liver retinol concentration (Livret.), liver retinyl palmitate concentration (Livretp.), liver tocopherol concentration (Livtoc.), 7-ethoxyresorufin O-deethylase enzyme activity (EROD) and aryl hydrocarbon hydroxylase enzyme activity (AHH). N is the number of samples used in the correlation, p is the significance level of the correlation and Corr. is the correlation coefficient. Dashes indicate that no significant correlation exists between the variables in question.

Correlation	August 93 + 94 Males			August 93 + 94 Females			May, 1994 Males			May, 1994 Females		
Variables	N	p	Corr.	N	p	Corr.	N	p	Corr.	N	p	Corr.
CFAC. vs. GSI	-	-	-	68	<0.001	0.522	16	0.025	0.577	-	-	-
CFAC. vs. LSI	37	0.001	-0.517	68	<0.001	-0.558	-	-	-	-	-	-
CFAC. vs. Relfec.	-	-	-	68	<0.001	-0.534	-	-	-	-	-	-
CFAC. vs. Livret.	-	-	-	65	0.036	0.260	-	-	-	-	-	-
CFAC. vs. Livretp.	-	-	-	65	<0.001	0.422	-	-	-	-	-	-
CFAC. vs. EROD	-	-	-	68	<0.001	-0.419	-	-	-	-	-	-
CFAC. vs. AHH	-	-	-	68	0.002	-0.361	-	-	-	-	-	-
Livwt. vs. GSI	15	0.039	-0.537	-	-	-	16	0.047	0.503	-	-	-
Livwt. vs. Test.	-	-	-	46	0.012	-0.368	-	-	-	-	-	-
Livwt vs. Absfec.	-	-	-	68	<0.001	0.541	-	-	-	16	0.021	0.569
Livwt. vs. Livret.	-	-	-	65	0.011	-0.315	-	-	-	-	-	-
Livwt. vs. Livretp.	-	-	-	65	0.010	-0.317	-	-	-	-	-	-
Livwt. vs. Livtoc.	-	-	-	-	-	-	16	0.028	-0.549	-	-	-
Livwt. vs. EROD	-	-	-	68	0.002	0.365	-	-	-	-	-	-
Livwt vs. AHH	36	0.001	0.511	68	0.016	0.292	-	-	-	-	-	-
GSI vs. LSI	-	-	-	68	0.003	0.357	-	-	-	-	-	-
LSI vs. Test.	29	0.004	-0.517	46	0.018	-0.348	-	-	-	-	-	-
LSI vs. Relfec.	-	-	-	68	0.013	0.298	-	-	-	-	-	-
LSI vs. Livret.	-	-	-	65	<0.001	-0.508	16	0.007	-0.643	-	-	-
LSI vs. Livretp.	36	0.026	-0.370	65	<0.001	-0.515	16	0.025	-0.556	-	-	-
LSI vs. Livtoc.	-	-	-	65	0.045	-0.249	16	<0.001	-0.836	-	-	-

Correlation Variables	August 93 + 94 Males			August 93 + 94 Females			May, 1994 Males			May, 1994 Females		
	N	p	Corr.	N	p	Corr.	N	p	Corr.	N	p	Corr.
LSI vs. EROD	-	-	-	68	<0.001	0.692	-	-	-	-	-	-
LSI vs. AHH	-	-	-	68	<0.001	0.650	-	-	-	-	-	-
Livret. vs. Test.	28	0.033	0.395	43	<0.001	0.519	-	-	-	-	-	-
Livret. vs. Estra	-	-	-	65	0.009	0.321	-	-	-	14	0.018	0.620
Livret. vs. Livretp.	36	0.009	0.426	65	<0.001	0.749	16	<0.001	0.853	-	-	-
Livret. vs. Livtoc.	-	-	-	65	<0.001	0.455	16	0.002	0.720	16	0.039	0.520
Livret vs. EROD	-	-	-	65	<0.001	-0.469	-	-	-	-	-	-
Livret. vs. AHH	36	0.031	-0.360	65	<0.001	-0.449	-	-	-	-	-	-
Livretp. vs. Test.	28	0.033	0.403	43	0.001	0.492	-	-	-	-	-	-
Livretp. vs. Estra.	-	-	-	65	0.035	0.261	-	-	-	-	-	-
Livretp. vs. Relfec.	-	-	-	65	0.028	0.273	-	-	-	-	-	-
Livretp. vs. Eggdiam.	-	-	-	65	0.018	0.292	-	-	-	-	-	-
Livretp. vs. Eggwt.	-	-	-	65	0.001	0.391	-	-	-	-	-	-
Livretp. vs. Livtoc.	-	-	-	65	0.025	0.278	16	0.005	0.660	-	-	-
Livretp. vs. EROD	-	-	-	65	<0.001	-0.491	-	-	-	-	-	-
Livretp. vs. AHH	36	0.034	-0.354	65	<0.001	-0.501	-	-	-	-	-	-
EROD vs. Test.	-	-	-	46	0.002	-0.454	-	-	-	15	0.027	0.568
EROD vs. Relfec.	-	-	-	68	0.015	0.295	-	-	-	-	-	-
EROD vs. Eggdiam.	-	-	-	68	0.049	-0.239	-	-	-	-	-	-
EROD vs. Eggwt.	-	-	-	68	0.003	-0.355	-	-	-	-	-	-
EROD vs. AHH	36	<0.001	0.838	68	<0.001	0.961	15	0.002	0.730	16	<0.001	0.813
AHH vs. Test.	-	-	-	68	0.001	-0.457	-	-	-	-	-	-
AHH vs. Relfec.	-	-	-	68	0.035	0.256	-	-	-	-	-	-
AHH vs. Eggdiam.	-	-	-	68	0.019	-0.284	-	-	-	-	-	-
AHH vs. Eggwt.	-	-	-	68	0.002	-0.368	-	-	-	-	-	-
Estra. vs. Test.	-	-	-	46	0.002	0.450	-	-	-	-	-	-
Estra vs. Eggdiam.	-	-	-	68	0.048	0.240	-	-	-	14	0.011	0.657
Eggwt. vs. Relfec.	-	-	-	68	<0.001	-0.437	-	-	-	-	-	-

metabolism by MFOs seems unlikely because retinol and tocopherol were reduced in the spring even though there was no increase in MFO activity then, and there was little or no MFO induction at site D2, yet fish from this site had reduced retinol and tocopherol in August, 1994. The negative correlation between liver vitamins (especially retinol and retinyl palmitate) and EROD suggest a number of possibilities: these vitamins may have been utilized as antioxidants (because an increase in EROD results in an increase in oxidative stress which in turn increases the demand for antioxidant molecules such as vitamins A and E, Palace et al., 1996); vitamin metabolism may have been altered by MFOs; MFO induction is correlated with some unknown factor responsible for preventing vitamin absorption and/or increasing vitamin excretion; fish with increased EROD activities live in areas where their food is low in vitamins.

The positive correlation between LSI and vitamin levels supports data presented by Taveekijakarn et al. (1994) who reported an increase in LSI in cherry salmon (*Oncorhynchus masou*) that were depleted in vitamin A. Perhaps a deficiency in vitamin A could account for the increased liver somatic indices.

The positive correlations between vitamin stores and reproductive parameters may indicate that poorer nutrition may relate to at least some of the reproductive effects, although direct chemical effects likely also occur (Van Der Kraak et al., 1992; McMaster et al., 1996). Watanabe and Takashima (1977) found that a tocopherol deficiency in carp affected the pituitary-ovarian system, decreased the production of certain fatty acids, and inhibited ovarian development. Mammals deficient in vitamin A or E have been shown to have reduced levels of testosterone (Kutsky, 1973). There is no work in fish which directly links such vitamin

depletions with depletions in hormones, however, there is some evidence that nutrition does affect gonad and offspring development. Woodhead and Plack (1967) noted that vitamin A levels in female tomcod (*Microgadus tomcod*) were correlated with gonad development and Hubbs and Stavenhagen (1958) found that greenthroat darters (*Etheostoma lepidum*) fed a carotenoid and vitamin A deficient diet produced eggs which had a lower survival rate than those on a vitamin A sufficient diet. These effects were temporary, however, as offspring survival increased once carotenoid/vitamin A levels were increased. As previously mentioned, tocopherol also appears to play an important role in the proper functioning of the pituitary-ovarian system (Watanabe and Takashima, 1977). There are indications that cells which secrete gonadotropin are reduced in fish which have been depleted in tocopherol (Watanabe and Takashima, 1977). Gonadotropin is the hormone responsible for stimulating the gonads to produce the reproductive hormones, such as testosterone and estradiol. The findings of Watanabe and Takashima (1977), together with a similar response in mammals, indicates that deficiencies of vitamin E could possibly result in reduced testosterone and estradiol production. Van Der Kraak et al. (1992) concluded that bleached kraft mill effluent affected reproduction in white suckers by acting at multiple sites within the pituitary-gonadal axis. One of these effects was a reduction in gonadotropin secretion from the pituitary; further supporting the hypothesis that a vitamin depletion may be partially responsible for the reproductive effects noted in the white suckers downstream from the Pine Falls pulp mill.

The reduced testosterone and fecundity levels noted in these fish may be linked to nutritional status and/or they may result directly from exposure to components in the effluent. Common carp *(Cyprinus carpio)* exposed to phenol or sulfide for one month had smaller

gonads than controls (Kumar and Mukherjee, 1988), and exposure to β-sitosterol (a plant sterol found in pulp mill effluent) has been shown to cause a dose-dependent decrease in plasma hormone levels (MacLatchy and Van Der Kraak, 1995). The impacts of β-sitosterol appear to be confined to the gonad, as the pituitary was functioning normally in these fish (although the exposure was run for less than one week). These results indicate that β-sitosterol may be responsible for some of the reproductive effects, but also indicates that there are likely other effluent components or reasons for these effects because there was no impact on gonadotropin production in the β-sitosterol exposed fish and gonadotropin production has been affected in feral white sucker exposed to BKME, (Van Der Kraak et al., 1992). It is possible that both contaminant and dietary factors operate simultaneously to cause reproductive changes. At the present time there are no clear indications of whether these vitamin depletions are due to a decrease in available vitamins or to altered vitamin metabolism.

Chapter 2: Toxicity and MFO-inducing properties of the untreated Pine Falls pulp mill effluent in rainbow trout.

INTRODUCTION

There are hundreds of individual compounds in pulp mill effluents and site specific differences in mill processes, effluent treatments and receiving ecosystems complicate the assessment of effluent impacts, making it difficult to relate specific environmental effects to particular effluent contaminants (Owens, 1991; Hodson et al., 1992; Hodson et al., 1996). To complicate the issue further, many pulping effluents, aside from discharging toxic chemicals, have also discharged large amounts of organic contaminants, nutrients and wood fibres (Owens, 1991; Wong et al., 1996). Historical habitat degradation from these types of compounds further complicates the examination of effluent impacts.

There have been numerous process and treatment upgrades at pulp mills within the last decade and assessments at many mills have been made prior to and following these upgrades in an attempt to assess whether the prior impacts have been alleviated (Munkittrick et al., 1992b; Sandström, 1994; Nener et al., 1995; van den Heuvel et al., 1996). If the impacts have been caused by compounds that persist within the sediments or from general habitat degradation, it may not be possible to observe rapid improvements in feral fish populations as impacts noted in feral fish may not be attributable to the effluent that is currently being discharged (Owens, 1991; Servos et al., 1994). To determine whether the effluent itself is responsible for effects noted in feral fish, laboratory studies which examine similar responses to those reported in feral fish are proposed. This provides confirmation that

the present effluent is responsible for impacts noted in the feral fish. In the laboratory we can control the effluent concentration, exposure period, and the species used, thus allowing for a more detailed assessment of effluent impacts and more direct comparisons of different effluents and methods of effluent treatment (Jokinen et al., 1995).

The acute toxicity of pulp mill effluents is largely due to resin and fatty acids and biological treatment removes a majority of these compounds (Leach and Thakore, 1977; Owens, 1991). While largely eliminating effluent lethality, secondary treatment and mill process changes have not been successful in eliminating a variety of sub-lethal effluent impacts (Munkittrick et al., 1992b; Gagne and Blaise, 1993; Martel et al., 1994; Priha, 1996; Williams et al., 1996). The most sensitive and commonly reported of these impacts being the induction of the mixed function oxygenase system (Hodson, 1996). The effluent components responsible for MFO induction are not known and the toxicological and ecological relevance of the response cannot be determined without first identifying the inducing compound(s) (Williams et al., 1996; Hodson, 1996). MFO induction is largely controlled by the molecular size and shape of the inducer and information as to inducer strength and stability can be obtained by examining the nature of the induction; i.e. how long it takes for induction to occur after contaminant exposure, how strong the induction is and how long it persists once the exposed organisms are moved to clean water (Bucheli and Fent, 1995; Hodson, 1996). This information helps to determine the basic chemical structure of the inducer and can indicate whether there are different inducers in different types of effluents and whether the inducing compound(s) are likely to be persistent and/or bioaccumulative.

A number of biochemical parameters were altered in white suckers downstream from

the Pine Falls pulp mill including the induction of MFOs and reductions in plasma hormone levels and hepatic vitamin stores. Rainbow trout were exposed to the untreated effluent from the Pine Falls pulp mill to examine the nature of the effluent's toxicity and MFO inducing properties. These experiments were done to determine whether the effluent could in and of itself be responsible for the MFO effects noted in the feral fish and to document the nature of the MFO response. Effects on hormones and vitamins were not done in the laboratory because the fish used in the laboratory were not sexually mature and would not be suitable for hormone studies and the livers of the fish were too small to conduct both MFO and vitamin analyses. Rainbow trout were chosen because they are easy to culture in the laboratory, much is known about their biology, their MFO system is known to be inducible, they have previously been used in toxicity tests of the Pine Falls effluent and they are used in virtually all previous laboratory studies conducted with other pulp mill effluents, thus allowing for a more direct comparison between laboratory studies using different effluents (Gagne and Blaise, 1993; Martel et al., 1994; Goksøyr and Förlin, 1992; Priha, 1996; Williams et al., 1996).

MATERIALS AND METHODS

Effluent Collection and Storage

Twenty-four hour composite effluent samples (20 - 80 L) were collected by a chain-and-bucket sampler from the mill sewer prior to release to the river. The effluent was stored in plastic containers (20 - 40 L) and kept in the dark at 10°C.

Fish Care

All rainbow trout (*Oncorhynchus mykiss*) used in the laboratory experiments were juveniles of the Mount Lassen strain and were obtained as swim up fry (1 month old, average weight 0.12 g) from the Rockwood Aquaculture Research Centre in the summer of 1993. The fish were held in the laboratory in large tanks with an ample flow-through of 10°C Winnipeg dechlorinated tap water, which was aerated to facilitate tank circulation. The fish were maintained on an a diet of Martin Mills Trout Chow and were fed three times a week until feeding activity diminished. The daily light cycle was 12 hours of light and 12 hours of dark and was maintained in all experiments. Fish age and size are provided in the detailed methods for each experiment. Mortalities in the test fish prior to an experiment were always negligible.

Preliminary Toxicity Experiments

Experimental Conditions and Fish Sampling

A series of experiments was run to determine the toxicity of the effluent over time; whether the toxicity was primarily in the dissolved or particulate fraction; and whether aeration of effluent and/or tanks altered effluent toxicity. These tests were also run to determine the appropriate effluent concentrations to be used in the experiments examining the EROD response. All of these preliminary experiments were conducted on unfed (feeding ceased 24 hours prior to experimental exposure) juvenile rainbow trout under semi-static conditions (i.e. 50% of the tank volumes of appropriate solutions were changed daily) and all treatments were run in duplicate in 6-L tanks with five fish per tank. The tanks were arranged

in a random fashion in a 10°C controlled environment chamber and the fish were added to the tanks 1 at a time until each tank had 5 fish. After 96 hours all remaining fish were anaesthetized in tricaine methanesulphonate (MS 222) prior to sampling, and the weight in grams and fork length in mm was recorded for each fish. Tank conditions such as temperature, pH and dissolved oxygen concentrations were recorded daily.

1.) Effect of Effluent Storage on Toxicity.

One standard 96-hour LC50 test was run after effluent samples were stored for either 2 , 14 or 330 days. Each of these experiments was run with identical duplicate concentrations of 0 (control), 0.5, 1, 5, 10 and 50% effluent to observe if changes in effluent toxicity occurred with storage time. Table 4 lists the average ages, weights and lengths of the fish used in these experiments.

Table 4: Average age, weight and length of rainbow trout used in the 96-hour LC50 experiments with Pine Falls effluent stored for varying amounts of time. (mean ± S.E.M.)

Effluent Storage Time	n	Fish Age (months)	Fish Weight (g)	Fish Length (mm)
2 Days	59	2.0	0.19±0.006	29.6±0.28
14 Days	60	2.5	0.29±0.009	32.1±0.27
330 Days	57	14.0	3.20±0.156	65.0±1.05

The same effluent sample was used in the 2 day and 14 day trials, but a different effluent sample was used in the 330 day trial. The effluent used in the 330 day trial was received within a few weeks of the sample used in the 2 and 14 day trials.

2.) Toxicity of Solid and Liquid Effluent Fractions.

One 96-hour LC50 experiment was conducted to determine if the toxicity was primarily in the liquid or solid fraction of the effluent. The effluent (less than one week old) was centrifuged at 17 000 rpm for a half hour in a flow-through centrifuge and was then decanted at a rate of 45 mL per minute. Dilutions were made with the resulting supernatant, otherwise referred to as the liquid fraction of the effluent and with the left over fibres or solid fraction of the effluent. Duplicate controls and liquid effluent concentrations of 1.25, 2.5, 4, 5, and 10% were prepared, as well as 2 tanks with dilutions of fibres of 10 and 50%. Fibre dilutions were prepared by adding the fibres obtained by centrifuging an appropriate amount of whole effluent, e.g. to prepare a 50% fibre concentration in a 6-L tank, fibres from 3 L of centrifuged effluent were added to the tank and the tank was topped up with water to the 6 L mark. Because the amount of fibres was limited, the water in these tanks could not be changed during the experiment (i.e. 50% tank replacement daily). Daily aeration for a short period was substituted to maintain oxygen levels. The fish used in this experiment were 2 months old with an average weight and length of 0.57 ± 0.024 g and 37.8 ± 0.43 mm respectively (mean ● S.E.M.).

3.) Effect of Effluent and/or Tank Aeration on Effluent Toxicity.

To determine whether the lethal contaminants were highly volatile and whether tank aeration would affect the toxicity of the effluent, a 1 day experiment was set up using 15% effluent. Four litres of effluent were placed in an open glass jar and aerated vigorously for 66 hours prior to the experiment and an identical 4-L glass jar was filled with effluent and

capped for the 66 hour period. Four tanks were prepared with either the aerated or non-aerated effluents and two tanks within each treatment were aerated during the experiment. This resulted in four treatments; 1. effluent not aerated and tank not aerated, 2. effluent not aerated and tank aerated, 3. effluent aerated and tank not aerated and 4. effluent and tank aerated. The age of the fish used in this experiment was 14 months with an average weight and length of 5.6 ● 0.35 g and 74 ± 1.5 mm respectively (mean ● S.E.M.).

EROD Laboratory Experiments

Experimental Conditions and Fish Sampling

Following the preliminary experiments one flow-through effluent dose-EROD response experiment was conducted over a seven day period and another flow-through experiment was run for 28 days to monitor the time-course of EROD induction and decline. In each experiment, effluent concentrations and controls were run in duplicate. Prior to being used in these experiments the effluent was filtered through a 2 to 3 mm plastic mesh to remove the large particulates and clumps of cellulose fibres. This was necessary to avoid clogging of the continuous flow apparatus tubing. The effluent was slowly stirred with a magnetic stirrer during the flow-through experiments to ensure a uniform suspension.

In addition to monitoring temperature, pH and dissolved oxygen, the actual concentration of the effluent in each tank was monitored using fluorometry. Samples of effluent, control water and tank samples were filtered to remove particulates. An appropriate range of effluent dilutions in control water was prepared and all standards and tank samples were then read on a Perkin-Elmer LS50 luminescence spectrofluorometer with excitation and

emission wavelengths of 355 and 398 nm respectively, with slit widths of 5 nm. The concentration of effluent in the tanks was determined by using the regression of the standard dilution curve (sample fluorometric emission vs. known dilution, Figure 14).

When fish were found dead or when the experiment ended, the weights and lengths of the fish were recorded and their livers (minus gallbladders) were removed and placed into prechilled 2.5 mL glass homogenization tubes. The homogenization tubes containing the livers were kept on ice until post-mitochondrial supernatant could be prepared (approximately 1 hour).

Determination of MFO Activity

Post-Mitochondrial Supernatant Preparation

Post-mitochondrial supernatant was prepared as described in chapter 1; methods for microsome preparation, with the following exceptions: Tris buffer was used for homogenization instead of KCl-HEPES buffer; whole livers or less than 0.4 g of liver were used; the amount of buffer used per sample was as follows:

Amount of Liver (g)	Volume of Tris Buffer (μL)
<0.1	500
0.1 - 0.2	1000
0.2 - 0.3	1500
0.3 - 0.4	2000

Homogenate was transferred to microcentrifuge tubes and centrifuged in an Eppendorf microcentrifuge at 15 600 x g for 20 minutes; the resultant post-mitochondrial supernatant was transferred to cryovials for storage in liquid nitrogen; and protein dilutions were prepared by adding 20 μL of post-mitochondrial supernatant to each of three tubes containing 2 mL

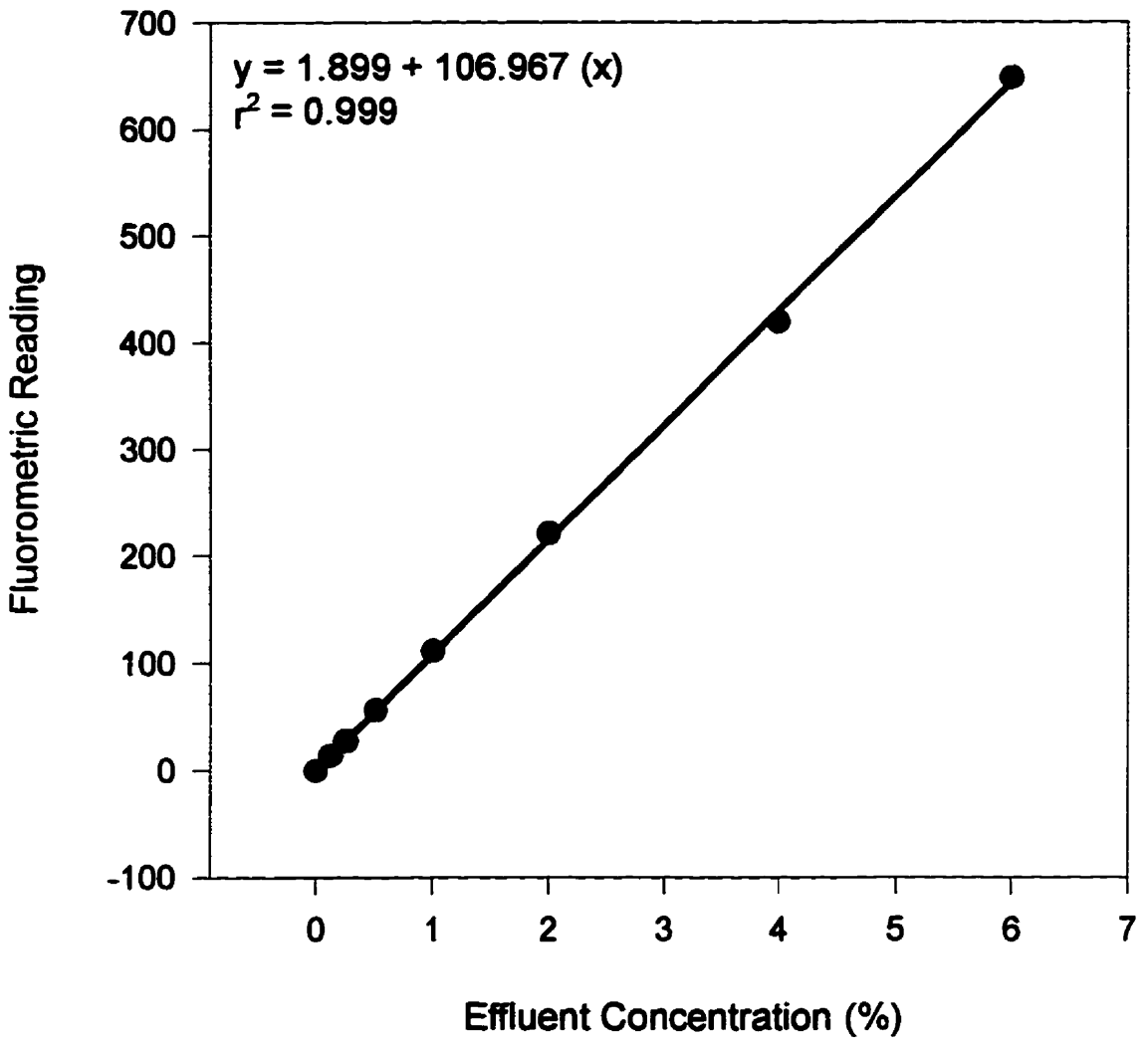


Figure 14: Regression of effluent concentration versus fluorometric readings taken with excitation and emission wavelengths of 355 and 398 nm respectively, with slit widths of 5 nm. This standard dilution curve was prepared on January 24, 1995, and is typical of the standard effluent dilution curves that were obtained every time tank effluent concentrations were determined.

of redistilled water.

Protein Analysis

Protein analysis was done as described in chapter 1, except that the post-mitochondrial supernatant was diluted 1 to 101 with redistilled water.

Determination of Ethoxyresorufin *O*-Deethylase (EROD) Activity

EROD activity was determined as described in chapter 1, except that a 10 minute incubation was used and results were calculated as pmol/mg protein/minute.

1.) Effluent Dose-EROD Response Experiment.

Replicate concentrations of 0.0 (controls) 0.25, 0.50, 1.0, 2.0 and 4.0% effluent were used for this effluent dose-EROD response experiment. Five juvenile rainbow trout were exposed to these concentrations via a flow-through apparatus (modified proportional diluter, Mount and Brungs, 1967) in 30-L tanks. The 12 tanks were randomly arranged in a 10°C controlled environment chamber and the fish were added to the tanks one at a time until each tank contained 5 fish. The age of the fish used in this experiment was 21 months and their average weight and length was 16.2 ± 0.85 g and 110.2 ± 2.2 mm respectively (mean ± S.E.M.). The average flow rate provided 2.33 L of solution per gram of fish per day. The experiment was run for seven days during which the fish were fed every second day at a rate of 1.2% body weight.

2.) EROD Time-Course Experiment.

Following the dose-response experiment an effluent concentration of 1% was chosen for the EROD time-course experiment. This was also set up as a flow-through experiment except that the fish were kept in the lab in 160-L tanks with 60 fish per tank (at the start) and as only 1 effluent concentration was required a different dosing apparatus was used. The 1% effluent concentration was achieved and maintained by pumping an appropriate amount of water and effluent into a bucket which was constantly mixed with a magnetic stirrer. The bucket was equipped with a swing arm on the outside connected to an opening in the bucket near the bottom. The swing arm level and water volumes were set so there was a constant overflow from the swing arm to each of the 1% effluent tanks. The age of the fish used in this experiment was 22 months and their average weight and length was 25.7 ± 0.67 g and 130.9 ± 1.32 mm respectively (mean ± S.E.M.). The minimum flow rate during the experiment was 1.45 L per gram of fish per day, this increased as fish were removed from the tanks. Fish were exposed to a control or 1% effluent concentration for a period of 8 days and were sampled for EROD activity after 1, 2, 4 and 8 days. On day 8 the fish exposed to 1% effluent were moved to clean tanks with control water, and EROD activity was monitored after 1, 2, 4, 8, and 18 days in the control water. The control fish were not moved to different tanks, but the stress of a move was simulated by stirring their tank water with a dip net and catching some of them in the net. The effluent concentration in the 1% tanks was monitored using fluorometry (as described above) to ensure that the they were receiving the appropriate amount of effluent. At each sampling time 5 fish were taken from each tank for a total of 20 fish per sampling time (5 from both of the control tanks = 10 and 5 from both of the

"treatment" tanks = 10).

Statistical Analyses

Statistics were done with a nested ANOVA model using concentration and tank replicate within concentration as independent variables, following the general linear model in Systat 5.0 (Wilkinson et al., 1992). Weight was used as a covariate when comparing similar concentrations between different trials, because different trials were run with fish of different ages/sizes. The F ratios were calculated manually by dividing the mean square error for concentration by the mean square error for tank replicate within concentration. These F values were compared to those obtained from F tables using the degrees of freedom (D.F.) for concentration and the D.F. for tank replicate within concentration, at a p value of 0.05. Pairwise comparisons were done using Fisher's least significant differences (LSD) test with mean square error and degrees of freedom entered as those for the variable tank replicate within concentration and not that of the entire model (to avoid problems of pseudo-replication). EROD data were log transformed and time to death data were not transformed prior to statistical analysis. A probability level of <0.05 was considered significant. To simplify presentation, arithmetic means with standard errors have been used in the figures.

RESULTS AND DISCUSSION

Preliminary Laboratory Experiments

1.) Effect of Effluent Storage on Toxicity. (Figure 15)

The effluent caused mortality in rainbow trout (within 96 hours) at concentrations of 5% or greater when stored for periods of 2 or 14 days. There was no mortality at concentrations of 10% or less when tests were run with effluent that had been stored for 330 days. Effluent stored for 14 days was slightly more toxic at concentrations of 5 and 10% than effluent stored for only 2 days, however, effluent toxicities at 1 and 50% were similar in both of these trials. Mean time to death was less than 35 hours at 5% and less than 10 hours in 10% effluent in both experiments using samples stored for 2 or 14 days, but there was no mortality at either of these concentrations with effluent stored for 330 days. The effluent stored for 330 days did retain some toxicity since all fish were killed in the 50% dilution within 70 hours.

No partial mortalities occurred at any concentration within the 96 hours. In this situation the LC50 may be estimated by averaging the highest concentration with no mortality (1%) and the lowest concentration with 100% mortality (5%) (Parrish, 1985). The LC50 was estimated as 3%, regardless of whether the effluent was stored for a period of 2 or 14 days. The LC50 increased to 30% after 330 days of effluent storage.

The results of these experiments revealed that effluent can be stored (in the dark at 10°C) for up to 2 weeks without losing toxicity, that a narrow concentration range needs to be used for an accurate 96-hour LC50 determination, and that the 96-hour LC50 is approximately 3%. This corresponds well with the 3 to 4% reported by the mill in 1993 (T.

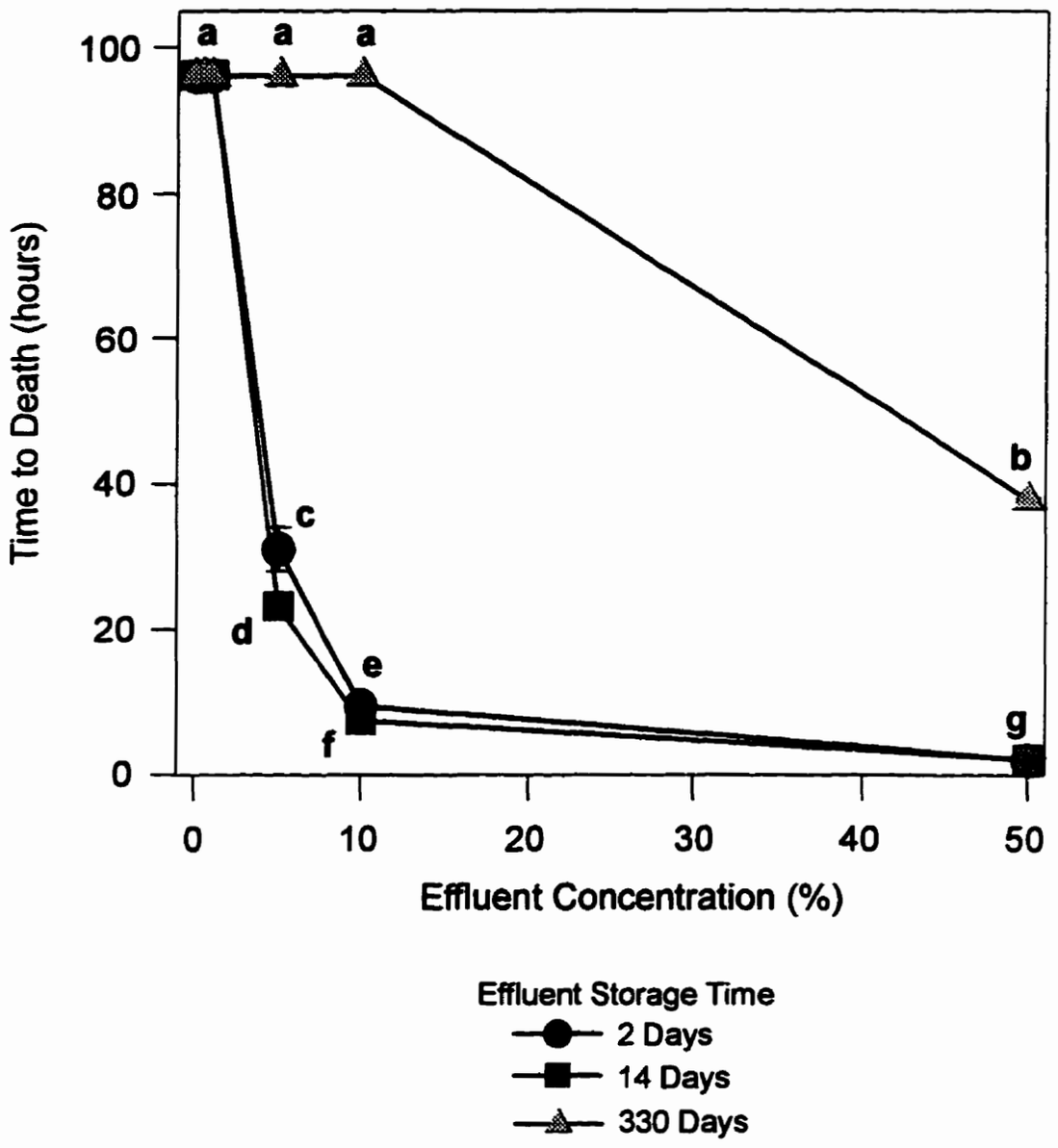


Figure 15: Change in effluent toxicity against juvenile rainbow trout with increasing effluent storage time. Each point represents the arithmetic mean of 2 tanks, with 5 fish per tank. Lines indicate ± S.E.M. A nested ANOVA (fish in tank within concentration) was used to determine significant differences between the different effluent concentrations within the same trial and weight was used as a covariate when comparing the same effluent concentrations of different trials. Points with the same letter are not significantly different ($p < 0.05$). Note: Effluent used in the 330 day trial was not the same sample used in the 2 and 14 day trials.

Youmans, Health Canada, personal communication). In comparison with other pulp mill effluents, the effluent from the Pine Falls mill was highly toxic. Gagne and Blaise (1993) tested 13 pulp mill effluent samples from a variety of pulping processes and effluent treatment types and LC50s ranged from 4.2 - 100%. The toxicity of the Pine Falls effluent should be greatly reduced if not completely eliminated now that the mill has installed secondary treatment facilities. Secondary-treated pulp mill effluents are much less toxic than effluents with only primary treatment, with secondary-treated effluents often resulting in no acute toxicity to fish even at concentrations as high as 100% (Gagne and Blaise, 1993; Priha, 1996; Williams et al., 1996). The components of the untreated effluent most likely responsible for a majority of the toxic effects are resin acids, unsaturated fatty acids, diterpene alcohols and juvabiones, which are all compounds naturally present in the wood used in the pulping process (Leach and Thakore, 1977).

2.) Toxicity of Solid and Liquid Effluent Fractions. (Figure 16)

The effluent was a suspension which did not clear readily on standing and the separation of solid and liquid effluent fractions was not complete by the centrifugation procedure. A small amount of liquid was left in the solid fraction and small particulate matter remained in the liquid effluent fraction. There was no significant toxicity in the liquid effluent fraction at concentrations of 4% or less, or in the particulate fraction at 10%. In general, toxicity appeared to be somewhat lower in the liquid fraction than the whole pulp mill effluent, although there was no significant difference between these two at the concentrations tested. The liquid fraction was more toxic than the isolated particulate fraction, as the

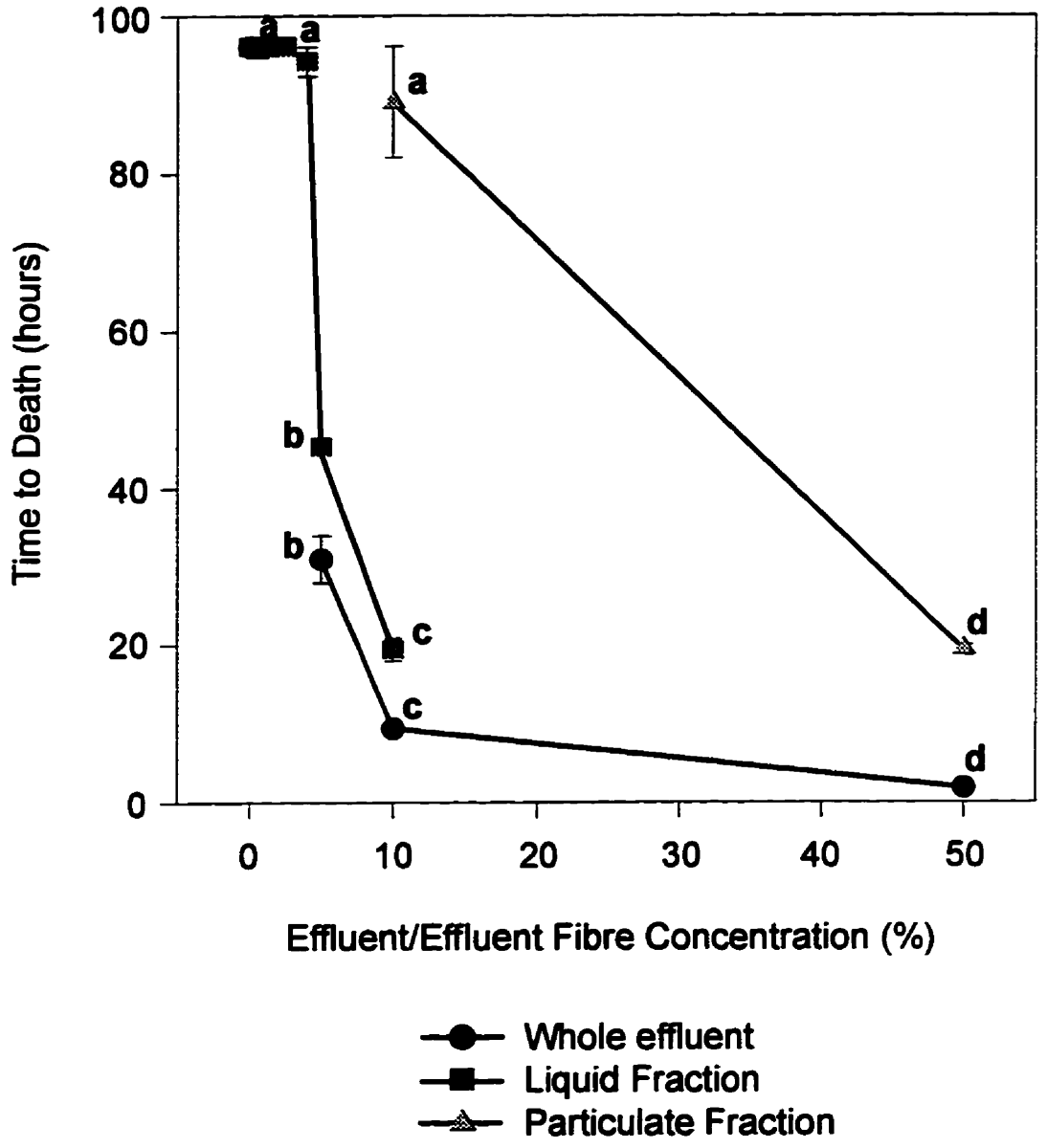


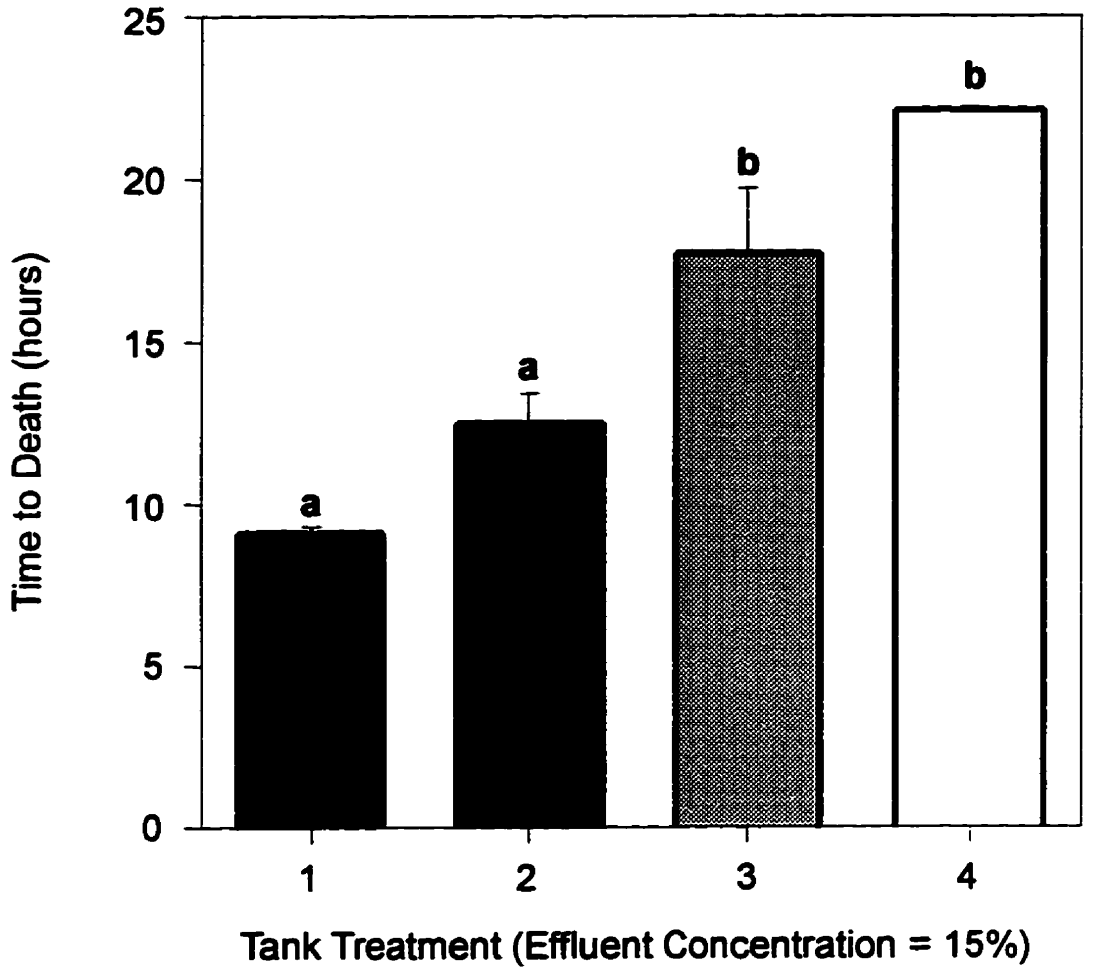
Figure 16: Toxicity of pulp mill effluent fractions to juvenile rainbow trout compared to that of whole pulp mill effluent. Effluent was centrifuged for 30 minutes at 17000 rpm and then decanted at a rate of 45 mL/minute. Each point represents the arithmetic mean of 2 tanks with 5 fish per tank. Lines indicate ± S.E.M.. A nested ANOVA (fish in tank within concentration) was used to determine significant differences ($p < 0.05$) between the different effluent concentrations of the same trial and weight was used as a covariate when comparing the same effluent concentrations of the different trials. Note: the toxicity of the whole effluent was determined in a different trial with a different effluent sample.

average time to death for fish in tanks with 10% fibres was 89 hours while that for fish in 10% liquid effluent was 19.4 hours. There was some toxicity in the fibre fraction because all fish in the 50% fibre tanks were dead within 24 hours. This mortality was slower than that of whole effluent where all fish in a 50% concentration were dead within 2 hours, although these times to morality were not significantly different when fish weight was used as a covariate.

Most effluent toxicity was associated with the liquid/small particulate fraction indicating that the removal of the larger components of the effluent would not diminish its toxicity greatly. The fibre toxicity may have been due to the physical clogging of the gills with particulate matter (particulate was noted in fish gills), to the presence of some effluent liquid (the separation of the liquid and solid fractions was not complete), and/or to the toxicity of particle ingestion or compounds leaching from the particles. Rainbow trout fed food contaminated with the solid fraction of a bleached kraft mill effluent (10%) grew more slowly and had increased hepatic lipids and MFO activities indicating that the solid fraction of other pulp mill effluents also have toxic properties (Lehtinen et al., 1991).

3.) Effect of Effluent and/or Tank Aeration on Effluent Toxicity. (Figure 17)

Prior effluent aeration did not significantly reduce effluent toxicity, although fish in treatments 2 and 4 (previously aerated) did take somewhat longer to die than those in treatments 1 and 3 (not previously aerated), respectively. Aeration of the tanks during the toxicity tests did significantly decrease effluent toxicity, regardless of prior effluent aeration (comparison of treatments 3 and 4 with treatments 1 and 2 respectively) as the fish took longer to die when the tanks were aerated than when they were not. The cause of death was



Treatment #1 = Effluent not aerated and tank not aerated
Treatment #2 = Effluent aerated and tank not aerated
Treatment #3 = Effluent not aerated and tank aerated
Treatment #4 = Effluent aerated and tank aerated

Figure 17: Effect of effluent and/or tank aeration on effluent toxicity against juvenile rainbow trout. Effluent was vigorously aerated in an open jar for 66 hours prior to the experiment or was not aerated at all; and during the experiment the tanks did or did not receive aeration. Bars represent the arithmetic mean of 2 tanks with 5 fish per tank and the lines above the bars represent the S.E.M.. A nested ANOVA (fish in tank within concentration) was used to determine differences between the treatments. Treatments with the same letter are not significantly different ($p < 0.05$).

not due to oxygen depletion because oxygen levels did not drop below 5.9 mg/L in the most oxygen depleted tanks by the end of the test. The water in the tank with the least oxygen was still more than 50% oxygen saturated and levels of 40% saturation are permissible in static bioassays (Parrish, 1985) Oxygen levels in the tanks averaged 6.4 mg/L (effluent and tank not aerated), 7.8 mg/L (effluent aerated, tank not aerated), 10.8 mg/L (effluent not aerated and tank aerated) and 11.2 mg/L (both effluent and tank aerated).

The toxic component(s) in the effluent were not highly volatile, as effluent aeration did not significantly diminish effluent toxicity (treatment 1 versus treatment 2, Fig. 16). Tank aeration during effluent toxicity experiments is not recommended as this would not provide an accurate toxicity assessment (LC50 values would be inflated, making the effluent appear less toxic than it actually is).

Although acute toxicity tests allow for the comparison of effluent toxicities at different times and between different types of effluents, it is important to note that using death as an end point may not be environmentally relevant. Kovacs et al. (1995) conducted acute toxicity, sub-chronic toxicity and life cycle tests with fathead minnows (*Pimephales promelas*) and found that the most sensitive endpoint was fish reproduction, which was significantly affected at an effluent concentration of less than 10 percent. This same effluent was found to be non-toxic to adults and did not affect their growth after 7 days at a concentration of 100%. Effluent exposure also had no affect on egg fertilization, hatching, larval survival or growth of the young when exposed to concentrations ranging from 1.25 to 20%. However, when the exposed fish matured their reproductive capacity was greatly reduced, with effects noted at an effluent concentration as low as 2.5% (no eggs were produced in fish exposed to

a concentration of 20% effluent). Effects on *Ceriodaphnia* reproduction as assessed in a 7 day bioassay were also incapable of predicting the effects on minnow reproduction. The results of the short-term tests could not predict the effects of chronic exposure to lower effluent concentrations. Robinson et al. (1994) reported similar findings; that short-term lab toxicity tests using fathead minnow growth or *Ceriodaphnia* survival as end points, were not predictive of the physiological responses noted in wild fish exposed to pulp mill effluents.

EROD Laboratory Experiments

During the course of the EROD induction experiments some of the fish became infected with a disease which caused patches of skin discolouration and loss of equilibrium. The cause of the condition is uncertain, but fungal infection is probable. Fish visibly affected by the disease were omitted from analyses, leaving 35 of the 40 living fish from the effluent dose-EROD response experiment and 155 out of 160 living fish from the EROD time-course experiment.

1.) Dose-Response Experiment. (Figure 18)

Only three of the five effluent concentrations tested were included in this analysis because the fish in the two highest concentrations (2 and 4%) were killed and MFO activity degrades rapidly after death. A dose-response relationship was evident (i.e. EROD activity increased with each increase in dose), however, due to the pattern noted in the residuals of the regression analysis a nested ANOVA statistic was used instead of regression. Enzyme induction occurred at all concentrations, thus defining the threshold for EROD induction as

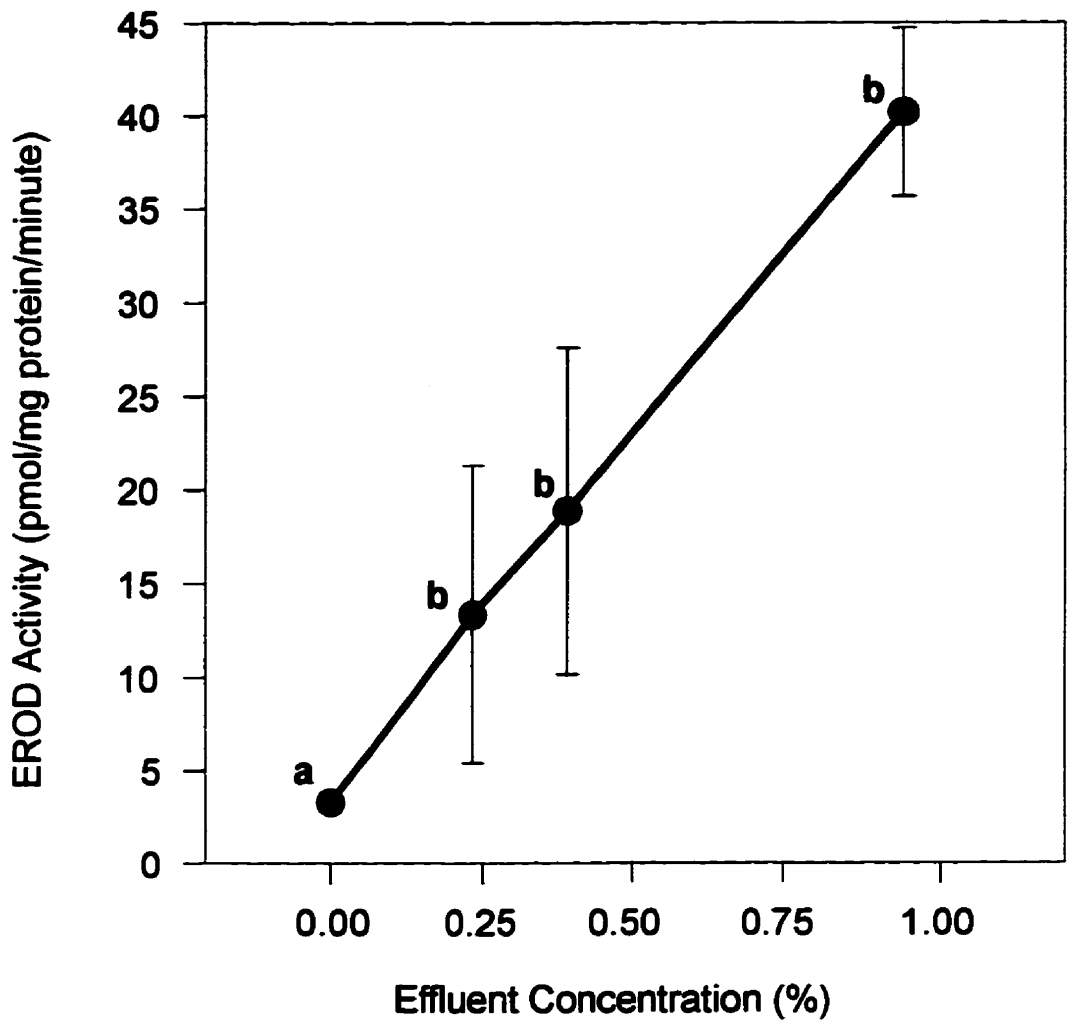


Figure 18: 7-ethoxyresorufin O-deethylase (EROD) enzyme activity in juvenile rainbow trout exposed to concentrations of whole pulp mill effluent under continous flow conditions for 7 days. Each point represents the mean of 2 tanks with 5 fish each and the lines indicate the S.E.M.. Differences between treatments were determined using a nested ANOVA (fish in tank within concentration). Points with the same letter are not significantly different ($p < 0.05$).

falling at or below 0.23% in laboratory rainbow trout. Average EROD induction was 4.5, 5.3 and 10.9-fold in 0.23, 0.39 and 0.94% effluent respectively. There was high variability in the EROD response of the fish; this has been reported by others working on the EROD-inducing properties of pulp mill effluents with rainbow trout (Martel et al., 1994; Gagne and Blaise, 1993).

While a precise threshold value could not be determined, (due to the non-random distribution of the residuals in the regression analysis) the MFO inducing properties of this effluent were quite strong, as induction occurred at only 0.23%. This level is lower than threshold values reported by Williams et al. (1996) in 5 kraft mill effluents which ranged from 0.57 to 9.1% effluent. Martel et al. (1994) tested 31 secondary-treated effluent samples from 8 different mills and found that a majority of samples from thermomechanical and chemi-thermomechanical mills did not cause MFO induction, while most samples from bleaching kraft pulp mills did cause MFO induction. Unfortunately, Martel et al. (1994) only examined one effluent concentration (10%), and since induction may occur at lower effluent concentrations, but be inhibited at higher concentrations (Pesonen and Andersson, 1992; Gagne and Blaise, 1993), the effluent concentration they chose may have been too high for some of the effluents to show induction. Lehtinen (1990) reported up to 6-fold induction in rainbow trout exposed for 7 weeks to 0.25% and greater than 2-fold induction at 0.05% effluent, from a bleaching kraft mill in Sweden with no effluent treatment. Gagne and Blaise (1993) tested three sublethal concentrations of 12 pulp mill effluent samples for MFO inducing properties in rainbow trout, including 9 effluents that were not from the bleached kraft pulping process, and found MFO induction after 4 days in a majority of these effluents,

although induction levels were usually low. The highest level of MFO induction noted by Gagne and Blaise (1993) was 9.4-fold, which occurred in 5.6% sulphite/groundwood effluent with secondary treatment. This level of induction corresponds well with the induction found here, and suggests that the new secondary treatment facility at the Pine Falls mill may not alleviate the MFO response of the fish. Munkittrick et al. (1992b) also observed that secondary treatment of a bleaching kraft mill effluent was not sufficient to remove the MFO response in white suckers from Jackfish Bay (Lake Superior) and Martel et al. (1994) found that secondary treatment at kraft mills did not eliminate the MFO response in laboratory fish.

This laboratory data can serve as background information which may be used to assess the effectiveness of the de-inking and secondary treatment systems which began operation in the winter of 1995. If enzyme induction is not completely reduced, a comparison of this threshold value with a threshold value determined for the treated effluent would provide an estimate of the effectiveness of the treatment in decreasing the enzyme response. Gagne and Blaise (1993) found that MFO induction generally occurred at higher concentrations in secondary-treated effluents than in primary-treated effluents. The toxicity of the Pine Falls pulp mill effluent was approximately 3% and the MFO inducing threshold was below 0.23%, this means that sub-lethal effects of this effluent occurred at less than 7.7% the LC50 values. The results of this experiment support the hypothesis that it is in fact the effluent discharge responsible for MFO effects in the white sucker from the river.

2.) EROD Time-Course Experiment. (Figure 19)

EROD activities were greater in fish from the 1% effluent tanks than those from the control tanks after 2 days of exposure. The 1% effluent exposed fish retained this level of induction (5.8 to 8.5-fold) for the remainder of the exposure period. Upon moving the 1% effluent-exposed fish to clean water (day 8) EROD activities remained significantly elevated for 1 more day, but declined to control levels thereafter. Induction dropped from 8.9-fold after 1 day in clean water to 6.2 and 2.8 after 2 and 4 days in clean water respectively, although the induction on days 2 and 4 was not significant. By day 8, EROD activity was identical to that of the control fish. Induction occurred within 48 hours and was decreased within 48 hours, however, due to the large degree of variability between the tanks on the second day in clean water (day 10), this value must be used with caution. For this reason the half-life for EROD induction will be guesstimated as falling between 2 and 4 days.

The time-course experiment showed that the contaminant responsible for the enzyme induction was readily taken up and eliminated or metabolized by the fish, as induction reached a steady level within 2 days and decreased within this same amount of time after exposure ceased. This indicates that the inducer (as expected due to a lack of chlorine bleaching) was not a highly chlorinated, non-metabolizable compound. Our findings are similar to those reported by Munkittrick et al. (1992b) in white sucker exposed to a bleached kraft mill effluent, indicating that the inducer at this non-chlorine bleaching mill may be similar to that from the bleached kraft mill at Jackfish Bay.

It has been thought that chlorine-containing organic compounds, especially pentachlorodibenzodioxins (PCDD) and pentachlorodibenzofurans (PCDF) were probable

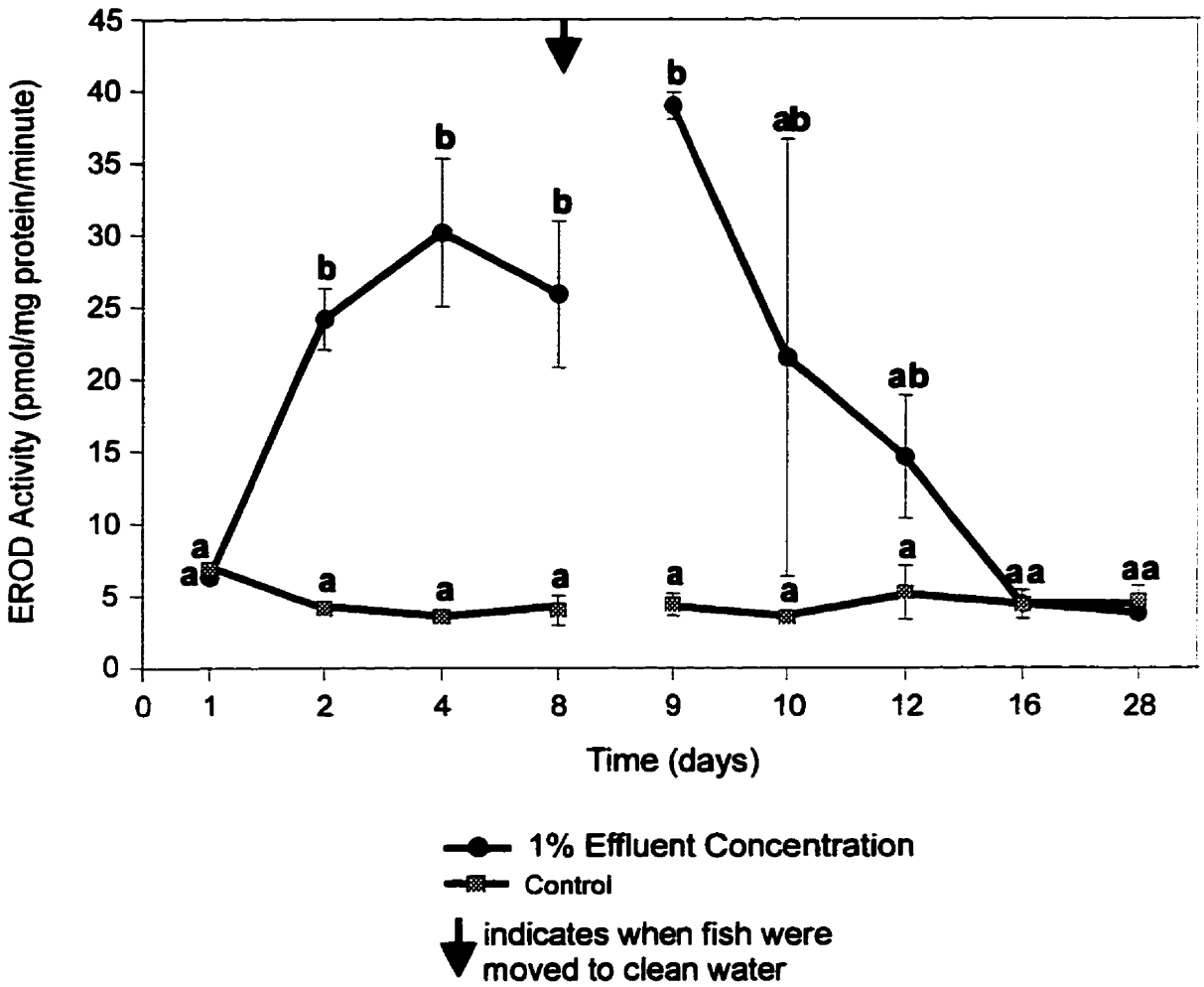


Figure 19: Time dependence of the 7-ethoxyresorufin O-deethylase (EROD) enzyme response of juvenile rainbow trout exposed to a control or 1% effluent concentration. The fish were maintained in tanks under flow-through conditions and were monitored for EROD activity during and following effluent exposure. Each point represents the arithmetic mean of 2 tanks with 5 fish per tank. Lines indicate ± S.E. M.. A nested ANOVA (fish in tank within concentration) was used to determine significant differences between the treatments. Points with the same letter are not significantly different ($p < 0.05$).

causes of the MFO induction noted downstream from bleaching kraft mills, although recent evidence indicates that this is not exclusively the case (Courtenay et al., 1993; Servos et al., 1994; Bankey et al., 1994; Munkittrick et al., 1994 van den Heuvel et al., 1995; Burnison et al., 1996; van den Heuvel et al., 1996;). The level and duration of induction caused by such substances tends to be much greater than that noted in this and many other pulp mill effluents. Muir et al. (1990) and Delorme (1995) reported EROD induction from a dietary or intraperitoneal injection of 2,3,4,7,8-PCDF that persisted for more than 180 and 300 days in juvenile and adult rainbow trout respectively. The level of EROD induction was also high, up to 84-fold in juvenile rainbow trout fed PCDF-spiked food for 31 days (Muir et al., 1990) and up to 340-fold in male rainbow trout exposed to an i.p. injection of 3 ng/g 10 months prior (Delorme, 1995). Parrott et al. (1995) orally dosed fish at time zero with varying concentrations of 5 PCDDs and 4 PCDFs and monitored induction after 2,4,8 or 16 days. Maximal EROD activity achieved at these sublethal concentrations was up to 250-fold for each contaminant and it was concluded that these compounds would not be rapidly metabolized. The above evidence indicates that if the inducer(s) in the effluent was one of these PCDDs or PCDFs that induction would have been greater and its decline to control levels would have taken longer than was observed (Figure 19).

There is experimental evidence to indicate that the MFO inducers in some bleached kraft mill effluents may be similar to those in the non-bleaching effluent from the Pine Falls' mill. Channel catfish (*Ictalurus punctatus*) exposed to 8% bleached kraft mill effluent for 263 days had 13-fold EROD induction which declined to the control levels when fish were exposed to clean water for 7 days (Bankey et al., 1994). Van den Heuvel et al. (1996) found

that white suckers caged in a bleached kraft mill effluent plume were readily induced within 2 days and remained at this induced level for the remainder of the 8 day exposure, with little or no measurable uptake of PCDDs or PCDFs. Munkittrick et al. (1992b) report a 40% decrease in MFO activity in bleached kraft mill effluent exposed white suckers after a 2 week mill shutdown. Munkittrick et al. (1995) later showed a rapid decline in EROD activity in white sucker, but only after the fish had been exposed to the effluent for a period of 14 days. Fish exposed for 4 days then placed in clean water did not show any reduction in EROD activity when sampled up to 8 days later, those exposed for 8 days then placed in clean water did not show any reduction in EROD activity until day 16, while those exposed for 14 days showed a decline in EROD activity beginning after only 2 days in clean water, with a decrease to control values within 8 days. Rainbow trout exposed for 2 or 4 days did not decline to reference levels after 16 or 8 days in clean water respectively (Munkittrick et al., 1995). The discrepancies in Munkittrick et al. (1995) may be due to the length of the exposure period, but may also be due to the presence of different types of inducers.

Further evidence for other types of inducers can be found in Courtenay et al., (1993). Courtenay et al. (1993) report a decrease in CYP1A mRNA induction in Atlantic tomcod (*Microgadus tomcod*) after 14 days of being caged in effluent these fish showed an 11-fold increase in CYP1A mRNA, after 1 day in clean water this increased to 14-fold, after 3 days it increased to 20-fold and after 10 days induction was still 4-fold (although this was no longer significant). Courtenay et al. (1993) concluded that the inducer(s) at this mill, while not behaving like a highly chlorinated compound(s), did also not behave like a readily metabolizable PAH. Similar results have been found by Muir et al. (1990) with low doses of

PCDF. Muir et al. (1990) reported a relatively low level of EROD induction (approximately 4-fold) after 31 days of feeding rainbow trout a low dose (0.82 ng/g) of PCDF and induction was not sustained up to 180 days as it was for the high dose group (9 ng/g). Muir et al. (1990) also found that EROD activity reached a maximal level 2 days after contaminant exposure ceased. Thus, the decrease in induction noted by Courtenay et al. (1993) is very similar to that noted for a low dose of a highly chlorinated compound. While research at many mills would seem to indicate that the inducer(s) are quite readily metabolizable, possibly indicative of PAH compounds (van den Heuvel et al., 1995) research at other mills indicates the presence of a more stable type of inducer (Courtenay et al., 1993; Kloepper-Sams and Benton, 1994). Whitefish (*Prosopium williamsoni*) with elevated EROD activities were found 200 km downstream and 70 km upstream from a secondary-treated bleached kraft mill effluent in northern Alberta and these same fish had elevated muscle tetrachlorodibenzodioxin (TCDD) and had not been exposed to the effluent for a number of days (Kloepper-Sams and Benton, 1994). The lack of recent exposure accompanied by EROD induction indicates that the inducer(s) at this Alberta mill is/are not readily eliminated/metabolized and this was further supported by a caging study with whitefish. Whitefish placed in reference water for 8 days showed no change in their relationship between EROD activity and TCDD concentration. The association between EROD activity and TCDD concentration, together with the lack of recovery when moved to clean water suggests that the inducer(s) in this Alberta mill's effluent may be TCDD or that the inducer was some other compound that was not readily metabolizable.

The above evidence indicates that different mills may release different types of

inducers and individual mills may have more than one inducer, as well as having effluent components which may increase and decrease the EROD response. Different fish species may also show different levels of responsiveness to the same types of inducers (Kloepper-Sams and Benton, 1994). These factors demonstrate the usefulness of characterizing pulp mill effluents in the lab, where effluent characteristics can be examined in the same species, at a similar temperature and at a range of known concentrations and durations. These types of studies provide information as to the type of inducer present and allow for a more direct comparison of results. Results from field data are influenced by the species used, the time of year (especially in sexually reproducing individuals), and the characteristics of the receiving environment, including: effluent dilution ratio, sediment composition, diet of the fish in the area, background water quality etc.. Furthermore, some potential impacts noted downstream of pulp mill effluents may be due to historical site degradation (Owens, 1991), which means that there may be effects in the fish population downstream that are not attributable to the existing effluent. Laboratory tests which examine similar characteristics to those in the field would also be valuable to separate these types of environmental effects from those caused directly from effluent exposure.

SUMMARY AND CONCLUSIONS

Summary of the Field Study

Feral white suckers captured downstream from the Pine Falls Pulp Mill effluent discharge exhibited a number of biochemical and morphological differences when compared with reference fish which were isolated from the effluent discharge by the Powerview Dam (Table 5). The parameters most impacted were plasma testosterone levels, hepatic retinoid and tocopherol stores, MFOs and liver somatic indices. Fecundity was also reduced, although this was only detectable in mature gonads from fish captured in the spring.

The decrease in site differences noted in the spring may be due to the spawning migration, which could result in the presence of fish from populations other than those that normally reside in this reach of the Winnipeg River. The possible migration of the downstream fish to the lake in the fall/winter and/or the potential increased mobility of the fish in the spring would also decrease the site differences, because it would mean that the fish would not be exposed to the effluent for as long a period of time prior to being captured compared to those caught in the summer.

Although cause/effect relationships cannot be rigorously proven from the fish taken from the river, there are a number of findings which would indicate that the effluent/mill operations are responsible for these effects. There was a trend towards increasing impacts in August, 1994, relative to August, 1993, because egg weights and diameters were not affected in August, 1993, but were reduced in August, 1994, and hepatic retinol levels (males), hepatic tocopherol levels (females) and EROD activities (females) were not affected at the further downstream site (D2) in August, 1993, but were affected in August, 1994. These increased

Table 5: Summary of differences noted between the upstream reference and two downstream sites. A dash indicates no significant difference, an up arrow indicates a significant increase above values at the reference site, and a down arrow indicates a significant decrease below values at the reference site. NA indicates that the analysis was not applicable. Differences were considered significant if $p < 0.05$. Units for all variables can be found in the Appendix (Table A2).

Variable	Females					Males				
	August, 1993		May, 1994	August, 1994		August, 1993		May, 1994	August, 1994	
	D1	D2	D1	D1	D2	D1	D2	D1	D1	D2
Length	-	-	-	-	-	-	↑	-	↑	↑
Weight	-	-	-	-	-	-	-	-	↑	↑
Age	↑	-	-	-	-	↑	-	-	-	-
Condition Factor	↓	-	-	-	-	-	-	-	-	-
Liver Somatic Index	↑	-	↑	↑	-	↑	-	↑	-	-
Gonadosomatic Index	-	-	-	-	-	-	-	-	-	-
Testosterone	↓	-	-	↓	NA	-	-	-	↓	NA
Estradiol	-	-	-	-	-	NA	NA	NA	NA	NA
Relative Fecundity	-	-	↓	-	-	NA	NA	NA	NA	NA
Absolute Fecundity	-	-	↓	-	-	NA	NA	NA	NA	NA
Egg Weight	-	-	-	↓	-	NA	NA	NA	NA	NA
Egg Diameter	-	-	-	↓	-	NA	NA	NA	NA	NA
Liver Retinol	↓	-	-	↓	-	-	-	↓	↓	↓
Liver Retinyl Palmitate	↓	-	-	↓	-	↓	-	↓	-	-
Liver Tocopherol	↓	-	-	↓	↓	↓	-	↓	-	-
EROD	↑	-	-	↑	↑	-	-	-	-	-
AHH	↑	-	-	↑	-	↑	-	-	-	-

impacts coincided with an increase in effluent concentration in the Winnipeg River, indicating that the effluent may be responsible for the effects. Most significant differences were noted between the upstream reference site and the site immediately below the effluent outfall and these same differences were not usually displayed between the upstream reference and further downstream sites, further signalling the presence of the mill as the source of the effects. Finally, one of the parameters quantified in the feral fish, the MFO response, was induced in laboratory fish exposed only to the effluent. Whether the responses in feral fish were due entirely to the (then) currently released effluent discharges or to the historical environmental degradation of the sediments/benthos in the area is uncertain at this time.

Conclusions from the Field Study

- Aspects of white sucker biochemistry and morphology were altered downstream of the Pine Falls pulp mill prior to the installation of the secondary treatment facility. These differences included increases in MFO activities and liver somatic indices, reductions in plasma testosterone, reductions in hepatic concentrations of retinol, retinyl palmitate and tocopherol and reductions in fecundity. Parameters that were somewhat less impacted, as evidenced by their reduced occurrence and/or severity were: an increase in length (males), weight (males) and age and a decrease in condition factor (females), egg weight and egg diameter.

- Many of these effects have also been reported in feral fish downstream from bleaching and non-bleaching pulp mills at other locations, including some with secondary effluent

treatment; indicating that the new secondary treatment facility at the Pine Falls pulp mill may not alleviate all of the responses observed in this study.

- Although fecundity was reduced, it was still high in comparison with white suckers from other locations as described in the literature.

- EROD correlated positively with LSI and negatively with hormones, vitamins and condition factor; vitamins were positively correlated with condition, hormones and other measures of reproductive fitness (egg diameter, egg size and fecundity).

- Vitamin levels may be depleted for a number of reasons, one may be accelerated metabolism (Palace et al., 1997) another may simply be a lack of vitamin availability downstream of the effluent discharge.

- Effects noted in feral fish may be due to historical site degradation which has altered the composition of the sediments/benthos downstream of the Pine Falls mill and/or the direct exposure of feral fish to the effluent that was being released at the time of this study.

Summary of the Laboratory Experiments

The preliminary laboratory experiments revealed that effluent toxicity did not degrade rapidly upon effluent storage, that the toxic components in the effluent were soluble and not highly volatile and that aeration of exposure tanks would not be desired for reliable LC50

estimates.

The MFO experiments confirmed that one of the impacts noted in the feral fish could be caused by effluent exposure alone, indicating that the current pulp mill effluent contains compound(s) with MFO inducing properties. The characteristics of the MFO induction resembled those caused by PAH type compounds and not PCDDs or PCDFs.

Conclusions from the Laboratory Experiments

Preliminary Experiments

- Effluent toxicity declined after an extended period of effluent storage (330 days), but was maintained after similar storage (in the dark at 10°C) for at least 14 days.

- A majority of the effluent's toxicity was within the liquid/small particulate fraction, indicating that the coarse filtration of the effluent, as required in the MFO experiments, would not diminish its toxicity.

- The toxic compounds in the effluent were not highly volatile because effluent toxicity was not significantly reduced after vigorous effluent aeration for 66 hours.

- Tank aeration significantly reduced effluent toxicity, suggesting that aeration of exposure tanks may give spuriously high LC50 values.

MFO Experiments

- Aqueous effluent exposure was capable of inducing EROD activities in rainbow trout.

- The threshold effluent concentration required for EROD induction in rainbow trout was below 0.23%.

- EROD induction occurred within 2 days of exposure of rainbow trout to a 1% effluent concentration and remained at a similar level for the remainder of the 8 day exposure period.

- EROD induction declined within 2 days after the fish were removed to clean water, with an estimated half life of less than 4 days; indicating that the contaminant responsible for the induction was apparently readily eliminated or metabolized by the fish.

Combined Field and Laboratory Discussion

Although the rainbow trout used in the lab are not the same species as the suckers sampled from the river, it is interesting to note that the MFO systems of both species were induced by similar concentrations of effluent (less than 1%). The range of MFO induction noted in the field was between 3.4 to 8.6-fold and that in the lab ranged from about 5 to 11-fold. The finding that the laboratory fish were induced after only 2 days of effluent exposure indicates that the fish sampled from the river may also be induced after exposure of a

relatively short duration, indicating that they do not have to be stationary for an extended period of time prior to the detection of effluent exposure using EROD induction.

Combined Field and Laboratory Conclusions

- Fish caught within 1 km of the mill were exposed to effluent concentrations of less than 1% and laboratory fish exposed to concentrations ranging from 0.23 to 1.0% showed similar MFO effects.

- The EROD induction in fish exposed to effluent in the lab offers strong support for the argument that the enzyme induction noted in the field was directly caused by the exposure of fish to the pulp mill effluent and not by some other variable.

- Maximum EROD induction of white sucker from the Winnipeg River was 8.6-fold in 1993 and 4.1-fold in 1994; a similar induction of 10.9-fold was found in rainbow trout in the lab.

- These field and laboratory studies provide background information for monitoring the effectiveness of the recently installed secondary treatment system.

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APPENDIX

Table A1: Description of maturity index categories for female and male white suckers taken from the Winnipeg River in August, 1993, May, 1994 and August, 1994; including values reported as occurring in the fish at the time of sampling as well as a description of fully mature fish (stage 11 for females and stage 7 for males).

Females

Stage 9: ovarian samples with a distinct vitellogenic clutch of developing oocytes plus a core of pre-vitellogenic resting oocytes

Stage 10: ovarian samples with a distinct vitellogenic clutch of mature oocytes plus a core of pre-vitellogenic resting oocytes.

Stage 11: fish have ovulated, ovarian samples comprised almost entirely of loose clutch oocytes, cannot be used for fecundity estimates as eggs may have been discharged from the body cavity

Males

Stage 3: the tunica is clearly defined; lobule formation is complete; many cysts containing spermatocytes; spermatids and spermatozoa are present; lobules are wider than in stage 2

Stage 4: within sperm cysts spermatocytes are mostly replaced by spermatids and spermatozoa

Stage 5: lobules are tightly packed with spermatozoa; no cysts, spermatocytes or spermatids present

Stage 6: testes are "ripe and running"; there is an absence of sperm from some lobules; lobule walls are thickened

Stage 7: fibrous connective tissue is thickened by contraction; tunica is thick and folded; lobules are distorted and collapsed; relic sperm and cell debris can be found in the lobules

Table A2 Summary statistics for length, weight, age, condition factor (CFAC.), liver weight (Livwt.), gonad weight (Gowt.), gonadosomatic index (GSI), liver somatic index (LSI), plasma testosterone (Test.), plasma estradiol (Estra.), absolute fecundity (Absfec.), relative fecundity (Relfec.), egg diameter (Eggdiam.), egg weight (Eggwt.), maturity index, liver retinol (Livret.), liver retinyl palmitate (Livretp.), liver tocopherol (Livtoc.), 7-ethoxyresorufin O-deethylase enzyme activity (EROD) and aryl hydrocarbon hydroxylase activity (AHH) in male and female white suckers from the Winnipeg River.

Variable	Time	Sex	Site	N	Min.	Max.	Mean	Variance	Standard Deviation	Standard Error
Length (cm)	93AUG	F	D1	15	41.8	49.2	45.94	4.90	2.213	0.571
Length (cm)	93AUG	F	D2	7	33.3	47.5	41.21	28.84	5.370	2.030
Length (cm)	93AUG	F	U	9	31.6	46.6	42.58	21.71	4.660	1.553
Length (cm)	93AUG	M	D1	9	40.0	47.8	45.11	5.43	2.329	0.776
Length (cm)	93AUG	M	D2	6	38.1	46.9	41.92	11.21	3.348	1.367
Length (cm)	93AUG	M	U	6	32.0	45.8	39.90	22.78	4.773	1.949
Length (cm)	94AUG	F	D1	15	38.6	50.2	44.33	14.26	3.776	0.975
Length (cm)	94AUG	F	D2	7	35.5	49.6	41.66	17.98	4.241	1.603
Length (cm)	94AUG	F	U	15	36.8	50.2	44.22	19.69	4.438	1.146
Length (cm)	94AUG	M	D1	7	33.8	45.7	40.39	18.38	4.287	1.620
Length (cm)	94AUG	M	D2	5	38.0	43.3	40.20	4.82	2.195	0.982
Length (cm)	94AUG	M	U	4	38.3	43.9	40.33	6.40	2.530	1.265
Length (cm)	94MAY	F	D1	9	34.4	42.5	38.66	8.78	2.962	0.987
Length (cm)	94MAY	F	U	8	33.8	49.7	40.50	27.68	5.261	1.860
Length (cm)	94MAY	M	D1	13	33.9	39.6	36.49	2.82	1.680	0.466
Length (cm)	94MAY	M	U	3	33.7	40.8	38.00	14.29	3.780	2.183
Weight (g)	93AUG	F	D1	15	678.5	1530.5	1060.60	70800	266.1	68.7
Weight (g)	93AUG	F	D2	7	540.5	1485.0	988.21	126209	355.3	134.3
Weight (g)	93AUG	F	U	9	449.0	1582.5	1167.28	116273	341.0	113.7
Weight (g)	93AUG	M	D1	9	428.0	1719.0	1070.39	172454	415.3	138.4
Weight (g)	93AUG	M	D2	6	846.0	1510.0	1052.17	62816	250.6	102.3
Weight (g)	93AUG	M	U	6	451.0	1477.5	983.33	126097	355.1	145.0
Weight (g)	94AUG	F	D1	15	866.0	1847.0	1296.53	82079	286.5	74.0
Weight (g)	94AUG	F	D2	7	721.0	1773.0	1129.71	111373	333.7	126.1
Weight (g)	94AUG	F	U	15	650.0	2082.0	1323.13	190057	436.0	112.6
Weight (g)	94AUG	M	D1	7	614.0	1345.0	988.71	61264	247.5	93.6
Weight (g)	94AUG	M	D2	5	868.0	1103.0	985.40	7353	85.8	38.3
Weight (g)	94AUG	M	U	4	852.0	1231.0	1015.75	25688	160.3	80.1
Weight (g)	94MAY	F	D1	9	707.0	1176.0	943.00	27268	165.1	55.0
Weight (g)	94MAY	F	U	8	529.0	1846.0	1061.13	186188	431.5	152.6
Weight (g)	94MAY	M	D1	13	611.0	1016.0	769.31	16519	128.5	35.6
Weight (g)	94MAY	M	U	3	601.0	1101.0	853.33	62516	250.0	144.4
Age (years)	93AUG	F	D1	15	5	17	8.60	9.257	3.043	0.786
Age (years)	93AUG	F	D2	7	3	6	4.14	1.143	1.069	0.404
Age (years)	93AUG	F	U	9	3	14	6.89	8.611	2.934	0.978
Age (years)	93AUG	M	D1	9	4	13	10.11	6.111	2.472	0.824
Age (years)	93AUG	M	D2	5	4	9	5.60	4.300	2.074	0.927
Age (years)	93AUG	M	U	6	4	12	7.50	7.900	2.811	1.147
Age (years)	94AUG	F	D1	15	4	8	6.13	2.124	1.457	0.376
Age (years)	94AUG	F	D2	7	3	7	4.86	1.476	1.215	0.459
Age (years)	94AUG	F	U	15	4	9	6.07	2.495	1.580	0.408
Age (years)	94AUG	M	D1	7	3	8	5.71	3.238	1.799	0.680
Age (years)	94AUG	M	D2	5	4	5	4.80	0.200	0.447	0.200
Age (years)	94AUG	M	U	4	6	8	7.00	1.333	1.155	0.577
Age (years)	94MAY	F	D1	9	4	7	4.67	1.000	1.000	0.333
Age (years)	94MAY	F	U	8	4	8	5.75	1.929	1.389	0.491
Age (years)	94MAY	M	D1	13	4	9	4.62	1.923	1.387	0.385
Age (years)	94MAY	M	U	3	4	7	6.00	3.000	1.732	1.000
CFAC.	93AUG	F	D1	15	0.75	1.55	1.09	0.065	0.255	0.066
CFAC.	93AUG	F	D2	7	1.06	1.51	1.37	0.027	0.164	0.062
CFAC.	93AUG	F	U	9	1.36	1.58	1.46	0.007	0.081	0.027
CFAC.	93AUG	M	D1	9	0.52	1.77	1.15	0.172	0.415	0.138
CFAC.	93AUG	M	D2	6	1.10	1.90	1.44	0.098	0.313	0.128
CFAC.	93AUG	M	U	6	1.38	1.56	1.48	0.005	0.068	0.028
CFAC.	94AUG	F	D1	15	1.26	1.61	1.47	0.009	0.094	0.024
CFAC.	94AUG	F	D2	7	1.39	1.62	1.53	0.007	0.086	0.032
CFAC.	94AUG	F	U	15	1.24	1.65	1.48	0.019	0.138	0.036
CFAC.	94AUG	M	D1	7	1.40	1.64	1.48	0.009	0.094	0.035
CFAC.	94AUG	M	D2	5	1.36	1.75	1.53	0.028	0.168	0.075
CFAC.	94AUG	M	U	4	1.46	1.70	1.55	0.013	0.114	0.057
CFAC.	94MAY	F	D1	9	1.46	1.95	1.63	0.021	0.145	0.048
CFAC.	94MAY	F	U	8	1.33	1.78	1.53	0.027	0.163	0.058
CFAC.	94MAY	M	D1	13	1.41	1.65	1.57	0.006	0.076	0.021
CFAC.	94MAY	M	U	3	1.39	1.62	1.53	0.015	0.121	0.070

Variable	Time	Sex	Site	N	Min.	Max.	Mean	Variance	Standard Deviation	Standard Error
Liwt. (g)	93AUG	F	D1	15	14.0	28.9	18.97	12.708	3.565	0.920
Liwt. (g)	93AUG	F	D2	7	6.1	16.0	11.97	14.859	3.855	1.457
Liwt. (g)	93AUG	F	U	9	5.0	15.8	10.66	13.248	3.640	1.213
Liwt. (g)	93AUG	M	D1	9	13.0	22.5	17.11	14.531	3.812	1.271
Liwt. (g)	93AUG	M	D2	6	6.6	15.3	10.12	10.614	3.528	1.330
Liwt. (g)	93AUG	M	U	6	3.4	17.8	10.38	28.442	5.333	2.177
Liwt. (g)	94AUG	F	D1	15	9.7	26.5	17.27	23.591	4.857	1.254
Liwt. (g)	94AUG	F	D2	7	6.9	17.9	13.19	16.875	4.108	1.553
Liwt. (g)	94AUG	F	U	15	7.5	22.0	14.37	22.071	4.698	1.213
Liwt. (g)	94AUG	M	D1	7	5.3	18.7	12.60	27.457	5.240	1.981
Liwt. (g)	94AUG	M	D2	5	8.3	10.8	9.62	1.397	1.182	0.529
Liwt. (g)	94AUG	M	U	4	7.2	11.0	9.58	2.909	1.706	0.853
Liwt. (g)	94MAY	F	D1	9	14.1	28.8	20.10	33.990	5.830	1.943
Liwt. (g)	94MAY	F	U	8	9.5	28.2	15.91	44.833	6.696	2.367
Liwt. (g)	94MAY	M	D1	13	10.2	20.3	14.81	9.616	3.101	0.860
Liwt. (g)	94MAY	M	U	3	5.8	18.2	10.00	50.440	7.102	4.100
Gowt. (g)	93AUG	F	D1	15	23.6	55.1	40.90	82.203	9.067	2.341
Gowt. (g)	93AUG	F	D2	7	10.5	76.6	39.54	442.793	21.043	7.953
Gowt. (g)	93AUG	F	U	9	7.4	51.1	35.08	172.269	13.125	4.375
Gowt. (g)	93AUG	M	D1	0	-	-	-	-	-	-
Gowt. (g)	93AUG	M	D2	0	-	-	-	-	-	-
Gowt. (g)	93AUG	M	U	0	-	-	-	-	-	-
Gowt. (g)	94AUG	F	D1	15	20.1	52.9	37.57	115.039	10.726	2.769
Gowt. (g)	94AUG	F	D2	7	6.2	56.7	36.47	262.239	16.194	6.121
Gowt. (g)	94AUG	F	U	15	17.3	75.8	45.28	280.242	16.740	4.322
Gowt. (g)	94AUG	M	D1	6	22.9	56.4	43.95	204.175	14.289	5.833
Gowt. (g)	94AUG	M	D2	5	31.8	64.4	50.38	149.912	12.244	5.476
Gowt. (g)	94AUG	M	U	4	24.3	71.6	56.88	490.296	22.143	11.071
Gowt. (g)	94MAY	F	D1	9	60.5	123.3	88.57	376.855	19.413	6.471
Gowt. (g)	94MAY	F	U	8	15.6	232.0	107.99	5238.827	72.380	25.590
Gowt. (g)	94MAY	M	D1	13	22.4	58.0	38.80	104.580	10.226	2.836
Gowt. (g)	94MAY	M	U	3	19.4	73.6	38.57	923.223	30.385	17.543
GSI	93AUG	F	D1	15	2.65	6.87	4.18	1.393	1.180	0.305
GSI	93AUG	F	D2	7	1.98	6.18	4.03	2.035	1.427	0.539
GSI	93AUG	F	U	9	1.66	6.10	3.11	1.563	1.250	0.417
GSI	93AUG	M	D1	0	-	-	-	-	-	-
GSI	93AUG	M	D2	0	-	-	-	-	-	-
GSI	93AUG	M	U	0	-	-	-	-	-	-
GSI	94AUG	F	D1	15	1.93	3.61	2.98	0.283	0.532	0.137
GSI	94AUG	F	D2	7	0.87	4.03	3.21	1.149	1.072	0.405
GSI	94AUG	F	U	15	2.57	4.87	3.56	0.649	0.806	0.208
GSI	94AUG	M	D1	6	2.23	8.37	5.37	7.421	2.724	1.112
GSI	94AUG	M	D2	5	3.22	6.39	5.42	1.740	1.319	0.590
GSI	94AUG	M	U	4	2.94	7.91	5.91	5.249	2.291	1.145
GSI	94MAY	F	D1	9	7.87	12.35	10.38	2.058	1.435	0.478
GSI	94MAY	F	U	8	1.26	19.69	11.45	25.983	5.097	1.802
GSI	94MAY	M	D1	13	3.39	7.08	5.31	1.127	1.061	0.294
GSI	94MAY	M	U	3	2.31	7.16	4.47	6.097	2.469	1.426
LSI	93AUG	F	D1	15	1.15	2.61	1.89	0.165	0.406	0.105
LSI	93AUG	F	D2	7	1.04	1.93	1.26	0.092	0.303	0.115
LSI	93AUG	F	U	9	0.66	1.13	0.93	0.025	0.158	0.053
LSI	93AUG	M	D1	9	0.88	3.24	1.82	0.443	0.665	0.222
LSI	93AUG	M	D2	6	0.76	1.40	0.97	0.055	0.234	0.096
LSI	93AUG	M	U	6	0.48	1.28	1.04	0.083	0.288	0.117
LSI	94AUG	F	D1	15	1.00	1.78	1.35	0.045	0.213	0.055
LSI	94AUG	F	D2	7	0.97	1.45	1.18	0.042	0.205	0.077
LSI	94AUG	F	U	15	0.88	1.42	1.11	0.022	0.149	0.038
LSI	94AUG	M	D1	7	0.87	1.68	1.25	0.092	0.304	0.115
LSI	94AUG	M	D2	5	0.89	1.06	0.98	0.004	0.062	0.028
LSI	94AUG	M	U	4	0.85	1.12	0.95	0.014	0.118	0.059
LSI	94MAY	F	D1	9	1.34	2.99	2.20	0.334	0.578	0.193
LSI	94MAY	F	U	8	1.10	1.91	1.56	0.093	0.304	0.108
LSI	94MAY	M	D1	13	1.36	2.87	1.98	0.153	0.392	0.109
LSI	94MAY	M	U	3	0.68	1.68	1.12	0.260	0.510	0.294
Test. (nmol/L of plasma)	93AUG	F	D1	15	0.003	0.319	0.072	0.008	0.088	0.023
Test. (nmol/L of plasma)	93AUG	F	D2	7	0.128	0.662	0.360	0.048	0.220	0.083
Test. (nmol/L of plasma)	93AUG	F	U	9	0.049	1.331	0.335	0.156	0.395	0.132
Test. (nmol/L of plasma)	93AUG	M	D1	9	0.021	0.215	0.100	0.005	0.069	0.023

Variable	Time	Sex	Site	N	Min.	Max.	Mean	Variance	Standard Deviation	Standard Error
Test. (nmol/L of plasma)	93AUG	M	D2	6	0.257	1.182	0.667	0.116	0.341	0.139
Test. (nmol/L of plasma)	93AUG	M	U	6	0.003	0.430	0.337	0.028	0.166	0.068
Test. (nmol/L of plasma)	94AUG	F	D1	11	0.003	0.218	0.084	0.005	0.072	0.022
Test. (nmol/L of plasma)	94AUG	F	D2	0	-	-	-	-	-	-
Test. (nmol/L of plasma)	94AUG	F	U	4	0.111	0.551	0.398	0.039	0.197	0.098
Test. (nmol/L of plasma)	94AUG	M	D1	5	0.045	0.454	0.157	0.029	0.170	0.076
Test. (nmol/L of plasma)	94AUG	M	D2	0	-	-	-	-	-	-
Test. (nmol/L of plasma)	94AUG	M	U	3	0.260	0.981	0.506	0.169	0.411	0.238
Test. (nmol/L of plasma)	94MAY	F	D1	8	0.191	1.540	0.787	0.222	0.472	0.167
Test. (nmol/L of plasma)	94MAY	F	U	7	0.156	1.793	0.983	0.436	0.661	0.250
Test. (nmol/L of plasma)	94MAY	M	D1	13	0.170	0.992	0.360	0.051	0.227	0.063
Test. (nmol/L of plasma)	94MAY	M	U	3	0.347	0.641	0.495	0.022	0.147	0.085
Estra. (nmol/L of plasma)	93AUG	F	D1	15	0.040	0.470	0.153	0.011	0.107	0.028
Estra. (nmol/L of plasma)	93AUG	F	D2	7	0.158	0.951	0.418	0.090	0.301	0.114
Estra. (nmol/L of plasma)	93AUG	F	U	9	0.040	0.988	0.282	0.079	0.282	0.094
Estra. (nmol/L of plasma)	94AUG	F	D1	15	0.004	0.400	0.221	0.015	0.121	0.031
Estra. (nmol/L of plasma)	94AUG	F	D2	7	0.059	0.749	0.271	0.053	0.231	0.087
Estra. (nmol/L of plasma)	94AUG	F	U	15	0.018	0.723	0.266	0.033	0.180	0.047
Estra. (nmol/L of plasma)	94MAY	F	D1	8	0.037	1.435	0.335	0.221	0.470	0.166
Estra. (nmol/L of plasma)	94MAY	F	U	6	0.022	1.112	0.423	0.145	0.381	0.155
Absfec. (eggs/fish)	93AUG	F	D1	15	42772	95034	60301	236648000	15383	3972
Absfec. (eggs/fish)	93AUG	F	D2	7	24917	87639	52468	479519000	21898	8277
Absfec. (eggs/fish)	93AUG	F	U	9	18992	73577	55238	303661000	17426	5809
Absfec. (eggs/fish)	94AUG	F	D1	15	35443	80431	52951	201926000	14210	3669
Absfec. (eggs/fish)	94AUG	F	D2	7	28182	66549	41754	155607000	12474	4715
Absfec. (eggs/fish)	94AUG	F	U	15	21257	79455	47800	254308000	15947	4118
Absfec. (eggs/fish)	94MAY	F	D1	9	18114	30856	23639	268929000	5186	1729
Absfec. (eggs/fish)	94MAY	F	U	8	16968	54104	30942	226285000	15043	5686
Relfec. (eggs/g of fish)	93AUG	F	D1	15	32.9	93.1	59.75	353.421	18.800	4.854
Relfec. (eggs/g of fish)	93AUG	F	D2	7	35.2	75.5	53.51	183.491	13.546	5.120
Relfec. (eggs/g of fish)	93AUG	F	U	9	33.6	74.3	48.10	160.855	12.683	5.228
Relfec. (eggs/g of fish)	94AUG	F	D1	15	28.2	91.3	43.30	212.784	14.587	3.766
Relfec. (eggs/g of fish)	94AUG	F	D2	7	35.7	39.6	38.19	1.791	1.338	0.506
Relfec. (eggs/g of fish)	94AUG	F	U	15	27.2	51.1	37.87	40.848	6.391	1.650
Relfec. (eggs/g of fish)	94MAY	F	D1	9	18.9	33.8	27.86	20.970	4.579	1.526
Relfec. (eggs/g of fish)	94MAY	F	U	7	29.1	45.9	33.74	32.896	5.736	2.168
Eggdiam. (mm)	93AUG	F	D1	15	0.844	1.035	0.921	0.003	0.056	0.014
Eggdiam. (mm)	93AUG	F	D2	7	0.813	1.094	0.971	0.011	0.104	0.039
Eggdiam. (mm)	93AUG	F	U	9	0.784	0.932	0.887	0.003	0.052	0.017
Eggdiam. (mm)	94AUG	F	D1	15	0.760	1.004	0.884	0.004	0.066	0.017
Eggdiam. (mm)	94AUG	F	D2	7	0.588	1.045	0.949	0.026	0.161	0.061
Eggdiam. (mm)	94AUG	F	U	15	0.891	1.089	0.990	0.004	0.066	0.017
Eggdiam. (mm)	94MAY	F	D1	9	1.807	2.116	1.931	0.009	0.096	0.032
Eggdiam. (mm)	94MAY	F	U	7	1.671	2.013	1.904	0.015	0.122	0.046
Eggwt. (mg)	93AUG	F	D1	15	0.412	0.757	0.588	0.013	0.113	0.029
Eggwt. (mg)	93AUG	F	D2	7	0.395	0.907	0.667	0.043	0.207	0.078
Eggwt. (mg)	93AUG	F	U	9	0.294	0.688	0.539	0.020	0.143	0.048
Eggwt. (mg)	94AUG	F	D1	15	0.311	0.891	0.637	0.022	0.148	0.038
Eggwt. (mg)	94AUG	F	D2	7	0.142	0.990	0.762	0.083	0.288	0.109
Eggwt. (mg)	94AUG	F	U	15	0.539	1.144	0.865	0.018	0.135	0.035
Eggwt. (mg)	94MAY	F	D1	9	2.685	4.822	3.788	0.361	0.601	0.200
Eggwt. (mg)	94MAY	F	U	7	3.047	4.510	3.812	0.313	0.559	0.211
Maturity Index	93AUG	F	D1	15	9	9	9.00	0.000	0.000	0.000
Maturity Index	93AUG	F	D2	7	9	9	9.00	0.000	0.000	0.000
Maturity Index	93AUG	F	U	9	9	9	9.00	0.000	0.000	0.000
Maturity Index	93AUG	M	D1	0	-	-	-	-	-	-
Maturity Index	93AUG	M	D2	0	-	-	-	-	-	-
Maturity Index	93AUG	M	U	0	-	-	-	-	-	-
Maturity Index	94AUG	F	D1	15	9	9	9.00	0.000	0.000	0.000
Maturity Index	94AUG	F	D2	7	9	9	9.00	0.000	0.000	0.000
Maturity Index	94AUG	F	U	15	9	9	9.00	0.000	0.000	0.000
Maturity Index	94AUG	M	D1	7	3	4	3.67	0.267	0.516	0.211
Maturity Index	94AUG	M	D2	5	4	4	4.00	0.000	0.000	0.000
Maturity Index	94AUG	M	U	4	4	4	4.00	0.000	0.000	0.000
Maturity Index	94MAY	F	D1	9	10	10	10.00	0.000	0.000	0.000
Maturity Index	94MAY	F	U	7	10	10	10.00	0.000	0.000	0.000

Variable	Time	Sex	Site	N	Min.	Max.	Mean	Variance	Standard Deviation	Standard Error
Maturity Index	94MAY	M	D1	13	5	5	5.00	0.000	0.000	0.000
Maturity Index	94MAY	M	U	3	5	5	5.00	0.000	0.000	0.000
Livret. (µg/g wet tissue)	93AUG	F	D1	15	0.020	1.528	0.338	0.195	0.441	0.114
Livret. (µg/g wet tissue)	93AUG	F	D2	6	0.518	2.584	1.064	0.639	0.799	0.326
Livret. (µg/g wet tissue)	93AUG	F	U	7	0.138	7.677	2.600	9.264	3.044	1.150
Livret. (µg/g wet tissue)	93AUG	M	D1	9	0.020	1.750	0.674	0.292	0.540	0.180
Livret. (µg/g wet tissue)	93AUG	M	D2	6	0.752	2.009	1.272	0.301	0.548	0.224
Livret. (µg/g wet tissue)	93AUG	M	U	5	0.384	2.163	1.270	0.557	0.746	0.334
Livret. (µg/g wet tissue)	94AUG	F	D1	15	0.020	1.402	0.502	0.190	0.436	0.113
Livret. (µg/g wet tissue)	94AUG	F	D2	7	0.418	1.228	0.767	0.087	0.295	0.112
Livret. (µg/g wet tissue)	94AUG	F	U	15	0.279	8.022	1.869	4.149	2.037	0.526
Livret. (µg/g wet tissue)	94AUG	M	D1	7	0.306	1.880	0.760	0.386	0.621	0.235
Livret. (µg/g wet tissue)	94AUG	M	D2	5	0.020	0.843	0.446	0.109	0.331	0.148
Livret. (µg/g wet tissue)	94AUG	M	U	4	1.024	3.728	1.926	1.529	1.237	0.618
Livret. (µg/g wet tissue)	94MAY	F	D1	9	0.129	3.359	0.688	1.094	1.046	0.349
Livret. (µg/g wet tissue)	94MAY	F	U	7	0.074	3.336	1.632	1.579	1.257	0.475
Livret. (µg/g wet tissue)	94MAY	M	D1	13	0.146	0.747	0.375	0.033	0.183	0.051
Livret. (µg/g wet tissue)	94MAY	M	U	3	0.497	2.270	1.226	0.860	0.927	0.535
Livretp. (µg/g wet tissue)	93AUG	F	D1	15	0.120	177.530	31.254	2961	54.41	14.05
Livretp. (µg/g wet tissue)	93AUG	F	D2	6	51.300	269.430	127.105	6896	83.04	33.90
Livretp. (µg/g wet tissue)	93AUG	F	U	7	0.300	413.900	178.610	19779	140.64	53.16
Livretp. (µg/g wet tissue)	93AUG	M	D1	9	0.120	119.870	60.574	2379	48.78	16.26
Livretp. (µg/g wet tissue)	93AUG	M	D2	6	98.620	410.630	238.963	16455	128.28	52.37
Livretp. (µg/g wet tissue)	93AUG	M	U	5	54.260	320.470	189.464	11689	108.12	48.35
Livretp. (µg/g wet tissue)	94AUG	F	D1	15	0.120	554.470	107.026	22459	149.86	38.70
Livretp. (µg/g wet tissue)	94AUG	F	D2	7	239.100	744.260	477.083	35314	187.92	71.03
Livretp. (µg/g wet tissue)	94AUG	F	U	15	12.550	1072.320	458.657	85372	292.19	75.44
Livretp. (µg/g wet tissue)	94AUG	M	D1	7	70.340	684.800	315.861	51242	226.37	85.56
Livretp. (µg/g wet tissue)	94AUG	M	D2	5	0.120	438.100	251.910	33497	183.02	81.85
Livretp. (µg/g wet tissue)	94AUG	M	U	4	349.000	522.600	441.030	8189	90.50	45.25
Livretp. (µg/g wet tissue)	94MAY	F	D1	9	17.960	112.350	79.429	746	27.32	9.11
Livretp. (µg/g wet tissue)	94MAY	F	U	7	48.690	212.360	100.493	2817	53.08	20.06
Livretp. (µg/g wet tissue)	94MAY	M	D1	13	40.590	182.300	100.428	1634	40.43	11.21
Livretp. (µg/g wet tissue)	94MAY	M	U	3	53.520	738.050	358.627	121288	348.26	201.07
Livtoc. (µg/g wet tissue)	93AUG	F	D1	15	9.42	623.79	78.32	23281	152.58	39.40
Livtoc. (µg/g wet tissue)	93AUG	F	D2	6	47.49	168.91	88.62	2012	44.85	18.31
Livtoc. (µg/g wet tissue)	93AUG	F	U	7	35.01	467.99	220.53	24997	158.10	59.76
Livtoc. (µg/g wet tissue)	93AUG	M	D1	9	10.80	193.02	52.92	3233	56.86	18.95
Livtoc. (µg/g wet tissue)	93AUG	M	D2	6	62.64	307.93	153.27	7521	86.72	35.40
Livtoc. (µg/g wet tissue)	93AUG	M	U	5	127.14	272.22	203.83	4218	64.94	29.04
Livtoc. (µg/g wet tissue)	94AUG	F	D1	15	9.55	130.58	44.90	1230	35.07	9.06
Livtoc. (µg/g wet tissue)	94AUG	F	D2	7	39.73	78.20	63.05	215	14.65	5.54
Livtoc. (µg/g wet tissue)	94AUG	F	U	15	38.73	227.35	100.10	2346	48.43	12.51
Livtoc. (µg/g wet tissue)	94AUG	M	D1	7	14.23	131.45	46.64	1594	39.92	15.09
Livtoc. (µg/g wet tissue)	94AUG	M	D2	5	26.77	124.69	75.21	1682	41.02	18.34
Livtoc. (µg/g wet tissue)	94AUG	M	U	4	46.69	193.34	101.18	4117	64.16	32.08
Livtoc. (µg/g wet tissue)	94MAY	F	D1	9	14.68	159.50	53.62	1869	43.23	14.41
Livtoc. (µg/g wet tissue)	94MAY	F	U	7	48.72	145.01	88.65	1298	36.03	13.62
Livtoc. (µg/g wet tissue)	94MAY	M	D1	13	53.62	252.30	123.72	3438	58.64	16.26
Livtoc. (µg/g wet tissue)	94MAY	M	U	3	373.83	1057.42	656.95	127151	356.58	205.87
EROD (nmol/mg protein/minute)	93AUG	F	D1	15	0.011	0.375	0.146	0.009	0.095	0.024
EROD (nmol/mg protein/minute)	93AUG	F	D2	7	0.010	0.030	0.022	0.000	0.006	0.002
EROD (nmol/mg protein/minute)	93AUG	F	U	9	0.003	0.048	0.017	0.000	0.013	0.004
EROD (nmol/mg protein/minute)	93AUG	M	D1	9	0.003	0.300	0.153	0.010	0.098	0.033
EROD (nmol/mg protein/minute)	93AUG	M	D2	6	0.017	0.105	0.057	0.001	0.035	0.014
EROD (nmol/mg protein/minute)	93AUG	M	U	6	0.003	0.061	0.034	0.001	0.023	0.010
EROD (nmol/mg protein/minute)	94AUG	F	D1	15	0.010	0.073	0.037	0.000	0.020	0.005
EROD (nmol/mg protein/minute)	94AUG	F	D2	7	0.005	0.052	0.023	0.000	0.017	0.006
EROD (nmol/mg protein/minute)	94AUG	F	U	15	0.003	0.016	0.009	0.000	0.004	0.001
EROD (nmol/mg protein/minute)	94AUG	M	D1	7	0.031	0.216	0.091	0.005	0.068	0.026
EROD (nmol/mg protein/minute)	94AUG	M	D2	5	0.002	0.079	0.041	0.001	0.034	0.015
EROD (nmol/mg protein/minute)	94AUG	M	U	4	0.011	0.040	0.027	0.000	0.016	0.008
EROD (nmol/mg protein/minute)	94MAY	F	D1	9	0.006	0.026	0.018	0.000	0.006	0.002
EROD (nmol/mg protein/minute)	94MAY	F	U	8	0.008	0.041	0.022	0.000	0.011	0.004
EROD (nmol/mg protein/minute)	94MAY	M	D1	13	0.042	0.173	0.084	0.001	0.031	0.009
EROD (nmol/mg protein/minute)	94MAY	M	U	3	0.061	0.094	0.073	0.000	0.019	0.011

Variable	Time	Sex	Site	N	Min.	Max.	Mean	Variance	Standard Deviation	Standard Error
AHH (nmol/mg protein/minute)	93AUG	F	D1	15	0.052	0.450	0.249	0.011	0.105	0.027
AHH (nmol/mg protein/minute)	93AUG	F	D2	7	0.032	0.151	0.077	0.001	0.036	0.014
AHH (nmol/mg protein/minute)	93AUG	F	U	9	0.017	0.100	0.056	0.001	0.026	0.009
AHH (nmol/mg protein/minute)	93AUG	M	D1	9	0.036	0.438	0.265	0.018	0.134	0.045
AHH (nmol/mg protein/minute)	93AUG	M	D2	6	0.065	0.217	0.120	0.003	0.059	0.024
AHH (nmol/mg protein/minute)	93AUG	M	U	6	0.018	0.142	0.093	0.003	0.051	0.023
AHH (nmol/mg protein/minute)	94AUG	F	D1	15	0.035	0.234	0.112	0.004	0.061	0.016
AHH (nmol/mg protein/minute)	94AUG	F	D2	7	0.017	0.163	0.071	0.002	0.047	0.018
AHH (nmol/mg protein/minute)	94AUG	F	U	15	0.020	0.064	0.041	0.000	0.016	0.004
AHH (nmol/mg protein/minute)	94AUG	M	D1	7	0.114	0.403	0.191	0.011	0.106	0.040
AHH (nmol/mg protein/minute)	94AUG	M	D2	5	0.018	0.200	0.111	0.005	0.072	0.032
AHH (nmol/mg protein/minute)	94AUG	M	U	4	0.059	0.103	0.085	0.000	0.022	0.011
AHH (nmol/mg protein/minute)	94MAY	F	D1	8	0.010	0.058	0.036	0.000	0.017	0.006
AHH (nmol/mg protein/minute)	94MAY	F	U	8	0.017	0.128	0.060	0.001	0.035	0.012
AHH (nmol/mg protein/minute)	94MAY	M	D1	12	0.071	0.235	0.132	0.002	0.043	0.012
AHH (nmol/mg protein/minute)	94MAY	M	U	3	0.107	0.169	0.137	0.001	0.031	0.018