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Detailed Endocrine Assessments of Wild Fish in the Northern River Basins, Alberta, in Comparison to EEM Monitored Endpoints

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During the Northern River Basins Study (NRBS), several issues were highlighted regarding the health of resident fish populations. Based on a basin-wide study of gonad morphology and circulating sex steroid levels, there was preliminary evidence of potential endocrine disruption in fish within the basins. The main concern with this was the potential loss of reproductive capability and possible declining populations. The Northern Rivers Ecosystem Initiative was established in response to the NRBS and an endocrine study was conducted to evaluate in more detail reproductive endocrine function of fish within the identified areas. A suite of endocrine endpoints was used and compared to overall fish health assessments using the Environmental Effects Monitoring (EEM) Programs protocol in areas exposed to sewage and pulp and paper mill effluents. As these northern rivers are nutrient-limited, fish responded to effluent addition (increased nutrients) by showing signs of increased growth and development relative to the upstream reference fish. Some signs of altered reproductive function were observed downstream of the one mill site, however additional responses were observed downstream of a municipal sewage treatment plant discharge from the neighbouring community. The reproductive responses were minimal compared to earlier studies at other older pulp and paper facilities. Follow-up studies at the Grande Prairie site documented changes in the fish responses to the effluents in terms of enrichment. The sewage treatment plant had installed tertiary treatment prior to the latest studies and reductions in the release of nutrients may be the cause. It is not known at the present time whether the pronounced eutrophication response that is still present in these fish may mask other endocrine alterations, as these effluents have endocrine-active compounds in them. The results also emphasize the need for EEM-like monitoring downstream of municipal sewage discharges.

Key words: **endocrine disruption, environmental effects monitoring, municipal sewage effluents, pulp and paper mill effluents, fish**

Introduction

Impairment of reproductive fitness in fish exposed to pulp and paper mill effluents is one of the most well documented cases of wildlife endocrine disruption in Canada (McMaster 2001). Effects documented since the late 1980s at sites where in-depth reproductive studies have been conducted include reductions in gonadal size, reduced fecundity, increased age to maturity, diminished secondary sex characteristics and decreased circulating levels of reproductive sex steroids (McMaster et al. 1991, 1992a, 1996; Munkittrick et al. 1991, 1992, 1998; Van Der Kraak et al. 1992, 1998). Researchers then traced the reproductive effects back to the mechanisms responsible for the reproductive alterations through the development of additional endocrine tools such as in vitro steroid productive capacity (McMaster et al. 1995a; Van Der Kraak et al. 1992) measurement of apoptotic ovarian cells (Janz et al. 2001) and quantification of hepatic and ovarian oxidative stress (Oakes and Van Der Kraak 2003; Oakes et al. 2003). Although studies documenting reproductive effects existed, other studies failed to identify reproductive problems downstream of some more modern pulp and paper facilities (Kloepper-Sams and Benton 1994; Kloepper-Sams et al. 1994; Servos et al. 1992; Swanson et al. 1993, 1994). There has also been some recovery at the original sites of study following the installation of secondary treatment and a number of process changes made in response to the new federal *Pulp and Paper Effluent Regulations* (PPER) (Munkittrick et al. 1997). Under the new PPER, mills are also required to conduct Environmental Effects

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Monitoring (EEM) Programs which include an evaluation of adult fish populations downstream of their discharges. Following the second and third cycles of the program (1992–2004), national assessments of the results indicated an overall pattern of a metabolic disruption type of response, with fish downstream of mill discharges generally having increased growth and development but reduced input of energy into gonadal development (Lowell et al. 2003, 2005).

During the Northern River Basins Study (NRBS) several issues were highlighted regarding the health of resident fish populations in northern Alberta. Based on a basin-wide analysis of gonad morphology and circulating sex steroid levels, there was preliminary evidence of sex hormone depressions in specific reaches of the basin (NRBS 1992). These data are the first evidence of potential endocrine disruption in fish within these basins. It was recommended at this time by the governments of Canada, Alberta and the Northwest Territories that a study specifically designed to investigate the endocrine disruption issue in the Northern River Basins was warranted based on potential implications for fish populations and the integrity of aquatic systems (Recommendation #15.1, Report to the Ministers; Canada, Alberta and Northwest Territories response to the NRBS). The mills identified in this study represented "modern" mills, indicating that they were all relatively new, had secondary treatment for the life of the mill and met the new PPER.

The objective of this study was to compare reproductive endocrine function in wild fish with other measures of overall fish performance such as growth, energy storage and age structure as measured in the Pulp and Paper EEM Program. An assortment of endocrine-related endpoints was selected from previous studies examining reproductive function and the mechanisms involved. These included circulating steroid hormone levels, gonadal steroid productive capacity, circulating vitellogenin levels, gonadal histological staging, intersex, gonadal and hepatic oxidative stress (Oakes et al. 2003), gonadal apoptosis and circulating levels of sex steroid binding protein globulins (Pryce-Hobby et al. 2003). As municipal sewage discharges were also present in the two areas identified in the earlier studies, we also examined endocrine function in fish downstream of these discharges.

The wild fish field studies focused on two reaches of the Northern River Basins: (1) the mainstream of the Athabasca River in the vicinity of Whitecourt, Alberta; and (2) the Wapiti-Smoky river systems near Grande Prairie, Alberta. Both areas were identified during the NRBS as "hot spots" showing evidence of steroid depressions, tissue abnormalities (increased numbers of immature fish and external alterations) and increased contaminant levels in resident fishes. This paper describes two years of studies conducted on the Wapiti-Smoky river system. Results from one year of detailed studies on the Athabasca River are presented in McMaster et al. (2004) along with forage fish results from the Wapiti River. Longnose sucker (*Catostomus catostomus*) was selected as a sentinel species due to its widespread distribution within and between the study areas. This species has previously been identified as having depressed steroid levels within the study areas (NRBS 1992). In addition, pulp mills within each study area use longnose sucker as a sentinel species for their EEM programs (Stantec and Golder Associates Ltd. 2000; Golder Associates Ltd. 2000).

Materials and Methods

Study Site and Fish Collection

Fish were sampled from 4 sites in the Wapiti River watershed (Fig. 1) during the fall of 1999. Longnose sucker (*Catostomus catostomus*) were selected as the sentinel species for all sites and site selection depended heavily on site descriptions from previous studies in the area (SENTAR 1993). The fish collections were conducted from September 20 to 29, at two reference locations, one on the Little Smoky River at Wasaahigan and one on the upper Wapiti River at the confluence of Pipestone Creek, upstream of the two effluent discharges (Fig. 1). The two exposed sites included downstream of the municipal sewage discharge from the City of Grande Prairie and approximately 10 km further downstream below the discharge of a bleached kraft mill. During the fall of 2001, follow-up studies were conducted on the Wapiti River (September 10–22) with fish collections occurring at 3 sites—the upstream reference site at Pipestone Creek, the sewage exposed site and downstream of the bleached kraft mill (Fig. 1). Boat electrofishing (Smith-Root SR-20 electrofishing workboat, provided by Alberta Environment) was used to collect the longnose sucker at all sites. Removal of stunned fish was accomplished using dip nets (approx. 0.5-cm mesh size) followed by transportation to the on-site laboratory.

Sampling Protocol

Longnose sucker were immobilized in a foam block and blood samples were taken from the caudal vessels using a syringe and heparinized vaccutainer immediately prior to sampling. Blood was held on ice prior to separation of the plasma by centrifugation; plasma was immediately frozen in liquid nitrogen. Circulating steroid levels of testosterone (both sexes), 17β-estradiol (females) and 11-ketotestosterone (males) from the plasma samples were quantified by radioimmunoassay (RIA) procedures (McMaster et al. 1992b). Each fish was rendered unconscious by concussion, and was measured for fork length (±1 cm) and body weight (±0.01 g). The internal organs were removed and the gonads (±0.01 g) and liver (±0.01 g) were weighed.

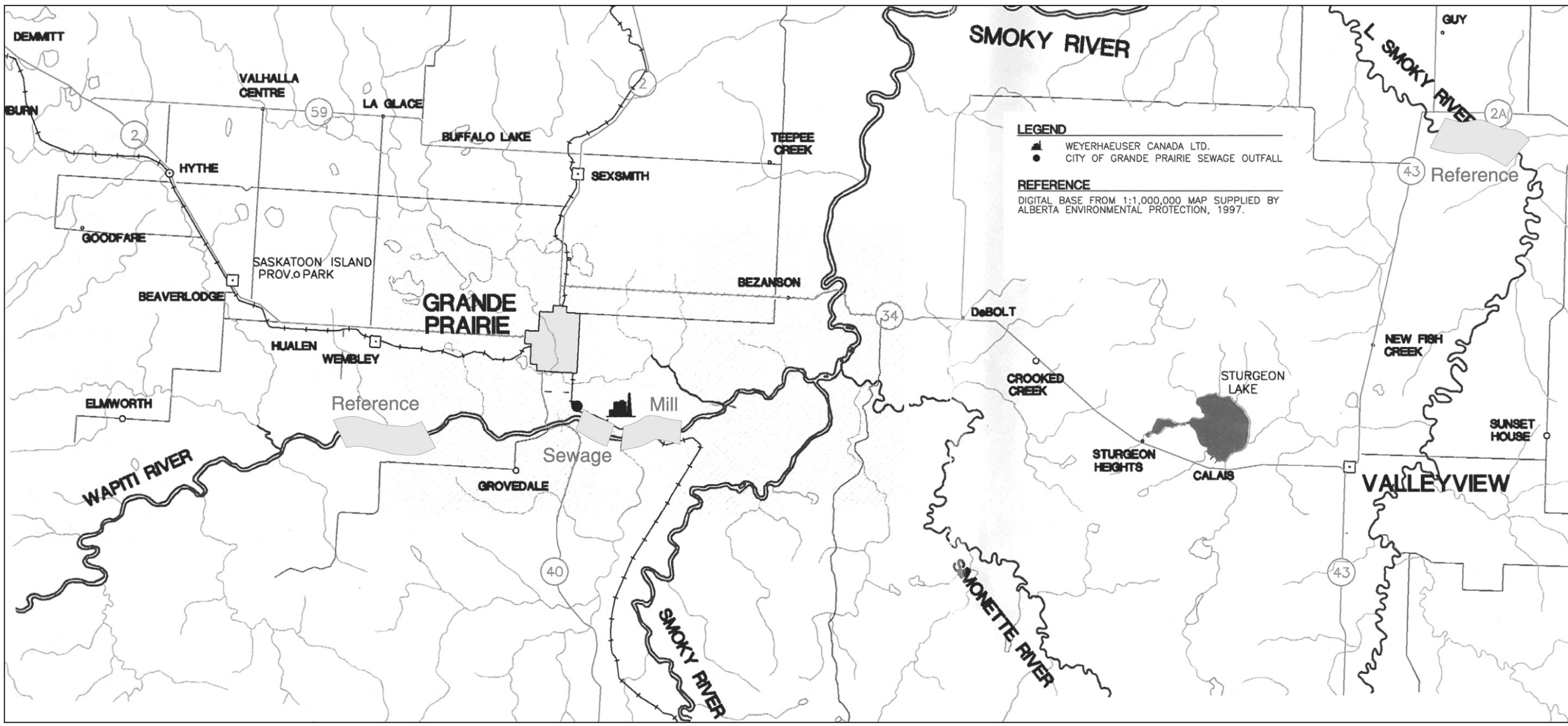

Fig. 1. Map of Grande Prairie sampling locations including the reference site on the Little Smoky River at Wasaahigan, a reference site on the Wapiti River at Pipestone, an exposed site downstream of the Grande Prairie municipal sewage effluent discharge and an exposed site downstream of the bleached kraft mill effluent discharge.

Male fish were rated with respect to the number and distribution of nuptial tubercle expression according to a subjective scale which ranged from 0 (no tubercles) to 6 (tubercles over entire body) (McMaster et al. 1991). Both sexes of fish were also rated with respect to their visceral lipid stores using a subjective scale ranging from 1 to 5 also adapted from McMaster et al. (1991) (with 1 representing very little visceral lipid and 5 representing large amounts).

A portion of liver tissue was placed in cryovials and frozen in liquid nitrogen for transportation back to the Canada Centre for Inland Waters for ethoxy-resorufin-o-dethylase (EROD) analysis (Parrott et al. 1999). EROD induction has been used as an indicator of exposure to a number of xenobiotics in aquatic organisms (Hodson et al. 1991). A subsample of liver tissue was also frozen for measurement of oxidative stress as quantified by increases in 2-thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LPO) according to Oakes and Van Der Kraak (2003). During the sampling, a subsample of gonadal tissue was taken from 12 female longnose sucker, and placed in incubation media for in vitro production of steroid hormones (McMaster et al. 1995b). A subsample of ovarian tissue was weighed and the number of follicles were manually counted and then multiplied by the gonadal weight to estimate total fecundity (total number of eggs/fish). An additional sample of both male and female gonadal tissue was also fixed in 10% buffered formalin for histological evaluation. Aging structures (opercula) were obtained from all fish for age analysis that was conducted at the National Water Research Institute in Burlington, Ontario.

Steroid Production

Incubations of gonadal tissues were used to determine the steroid hormone productive capacity of wild fish collected in the field (McMaster et al. 1995b). From female longnose sucker, subsamples of follicles were immediately placed directly into Media 199 (M199, containing Hank's salts without bicarbonate; GIBCO, Burlington, Ontario), which was supplemented with 25 mM Hepes, 4.0 mM sodium bicarbonate, 0.01% streptomycin sulfate and 0.1% bovine serum albumin (pH 7.2). Intact follicles were then placed in groups of 10 into polystyrene tissue culture plates (Corning Cell Well 25820 Corning Costar Corporation, Oneonta, New York) in nutrient media at 16 to 18°C for a period of 18 h. Replicates were either unstimulated (basal—media alone) or were stimulated with 1.0 IU/mL of human chorionic gonadotropin (hCG). The level of stimulated steroid production provides further information about the integrity and maximal capacity of the tissue to produce steroid hormones. Following incubation the media were drawn from the wells and frozen in liquid nitrogen for transportation to the laboratory. Concentrations of testosterone, and 17-estradiol released into the media during the incubation period were quantified by RIA procedures (McMaster et al. 1992b).

Vitellogenin

Circulating longnose sucker vitellogenin levels were measured using a direct ELISA with the white sucker 2C11 antibody (Nancy Denslow, University of Florida MCC/Biomarkers/ICBR/UF) using purified longnose sucker vitellogenin as the standard. Measurements were made in both male and female fish from all sites with a detection limit of 5 µg/mL.

Gonadal Histology

Gonadal sections were examined for differences in gonad development at sites downstream of the various discharges. Oocytes were categorized as previtellogenic, endogenous vitellogenic, vitellogenic or atretic according to their stage of maturation. Five cell types mark the stages of spermatogenesis: stem cells, spermatogonia, spermatocytes, spermatids or spermatozoa. The mean size and relative abundance of each cell type were used to compare gonad development at the various sites. Gonadal tissue was stained with haemotoxylin and eosin and fixed onto slides prior to examination with a Zeiss compound light microscope. To ensure an unbiased representation of each slide, standard stage coordinates were used to select 20 sections for sampling. An image of the field-of-view (FOV) at each section was recorded using AxioVision. For ovaries, all recognizable cell types within the FOV were tallied; however, cell size was measured only for cells with a visible nucleus (Soto et al. 1992; Nichols et al. 1999). In the case where a cell with a visible nucleus was not completely contained in the FOV, a second image was taken to facilitate measuring. The cell tally was pooled across images for each fish, and the proportion of cells at each stage of oocyte maturation was calculated by dividing by the total number of cells counted in a fish. The relative abundance of cells in the various stages of spermatogenesis was calculated as the area covered by each cell type divided by the total area of the FOV. Data were pooled across images within a fish. The sum area covered by a cell type was divided by the sum of the total area measured to calculate the relative abundance for a fish. The average proportion was then taken across all fish in a site.

Statistical Analysis

Analysis of fish data collected was conducted with sexes separate. Examination of the potential site differences in fish length and carcass weight were evaluated using analysis of variance. Condition factor (length versus carcass weight), gonadosomatic index (GSI) (ratio of gonad weight to carcass weight) and liver-somatic index (LSI) (ratio of liver weight to carcass weight) were evaluated using analysis of covariance (ANCOVA). Data were checked for normality and evaluated for homogeneity using the Levine's test prior to analysis. Logarithmic transformations were used if data did not meet these assumptions. Nonparametric Kruskal-Wallis tests were used to compare circulating steroid, in vitro steroid production, vitellogenin, EROD and age data between sites. A nonparametric one-way ANOVA (Kruskal-Wallis) was used to test for site effects on the proportion of each cell type for both males and females. A nested ANOVA was conducted to test for a significant difference between the size of the various ovarian cell types (area, μm^2), with SITES as the fixed effect and individual FISH as random effects nested within SITES. Tukey multiple comparisons tests were used to identify the site differences if one was found. All data analyses were conducted using SYSTAT 9.0 statistical software (Wilkinson 1990).

Results

Longnose Sucker Fish Collections

In 1999, male longnose sucker collected from the Little Smoky reference site were significantly shorter than males from the other three locations ($p < 0.001$), and no other significant site differences were found (Table 1). In terms of fish weight, Little Smoky fish were lighter than fish from the other three sites ($p < 0.001$), and Pipestone males were also lighter than the sewage and the pulp mill exposed males ($p < 0.001$). Examination of the condition factor of these fish (how heavy they are at a given length or how fat they are) indicated significant site differences ($p < 0.001$). Males collected downstream of both the sewage discharge and the pulp mill discharge had increased condition factor relative to both reference locations ($p < 0.001$) (Table 1) (Fig. 2A). Internal fat stores help support these findings, as male longnose sucker downstream of both discharges had increased fat stores relative to the reference fish ($p < 0.001$) (Fig. 3). However, males downstream of the sewage outfall also had significantly more internal fat stores than males downstream of the pulp mill discharge ($p = 0.008$).

Female longnose sucker collected from the Little Smoky were lighter than females from all three other sites ($p < 0.001$), and females from the Pipestone reference site were lighter than the downstream sewage and pulp mill exposed females ($p < 0.006$) (Table 1). The Little Smoky females were also shorter than fish from the other three sites ($p < 0.002$) although females from the Pipestone Creek reference site were similar to the two downstream locations. Condition factor of female longnose sucker indicated a significant interaction between sites. Examination of the data, however, indicated that fish collected downstream of both the sewage and pulp mill discharge were significantly fatter than fish from either reference location (Table 1). Internal fat stores correspond well, as female longnose sucker downstream of the sewage outfall had significantly more internal fat stores than that of females from the other three sites ($p < 0.001$). Females downstream of the pulp mill discharge had increased fat stores relative to both reference sites ($p \leq 0.008$), but reduced amounts relative to the sewage site (data not shown).

Little Smoky males were also younger than males from the other three sites ($p < 0.002$) (Table 1). Examination of male longnose sucker growth identified site differences in the relationships between fish length and age ($p < 0.001$) and weight and age ($p = 0.017$). Examination of site differences indicated that Little Smoky males were shorter and lighter at any given age relative to males from the other three sites ($p < 0.031$). Pipestone males were also lighter at each age relative to the sewage and the pulp mill exposed fish.

Little Smoky females were also younger than fish collected from the Wapiti River ($p < 0.05$) (Table 1). Examination of female longnose sucker growth demonstrated significant site differences in the relationship between fish length and age ($p < 0.001$). Evaluation of site differences indicated that both exposed sites (sewage and pulp mill) were significantly longer at any given age relative to the two reference sites ($p < 0.05$). Fish weight at age indicated the same response with exposed fish being heavier at any given age relative to both reference sites ($p < 0.001$).

Examination of male liver weights relative to the body weight of the fish indicated significantly larger livers in male fish collected downstream of both the sewage and pulp mill discharges relative to the two reference locations ($p < 0.001$) (Table 1). Examination of female liver weights indicated significant interactions between fish weight and site ($p = 0.044$). Examination of the data suggested that, in general, female fish downstream of both of the discharge sites had increased liver weight relative to the two reference locations (Table 1).

Hepatic mixed function oxygenase activity was examined in fish collected from all four locations. Reference male longnose sucker had low relative levels of EROD activity (Fig. 4A). EROD activity in males collected downstream of the sewage site was similar to both reference locations ($p > 0.386$). Activity in male fish collected downstream of the pulp mill discharge was significantly induced compared to fish from the other three locations ($p < 0.018$). Reference female longnose sucker demonstrated low relative levels of EROD activity, which increased in fish downstream of the sewage discharge ($p < 0.010$) (Fig. 4B). Female longnose sucker collected downstream of the pulp mill discharge, however, had significantly higher levels of hepatic EROD activity

TABLE 1. Length, weight, condition factor (k), liver somatic index (LSI), gonad somatic index (GSI), age and sample size (n) of longnose sucker collected at two reference sites, downstream of a sewage discharge and downstream of a bleached kraft mill on the Wapiti River in 1999 and 2001[a]

Sex	*Parameter*	*Year*	*Little Smoky*		*Pipestone Creek*		*Downstream sewage*		*Downstream mill*	
		1999								
Male	Length, cm		35.05 ± 0.31	a	37.74 ± 0.34	b	38.84 ± 0.43	b	38.72 ± 0.52	b
	Corrected body weight, g		456.82 ± 12.80	a	551.37 ± 17.31	b	728.50 ± 20.88	c	725.88 ± 26.05	c
	K		1.06 ± 0.02	a	1.03 ± 0.03	a	1.24 ± 0.02	b	1.25 ± 0.02	b
	LSI		1.37 ± 0.04	a	1.51 ± 0.05	a	2.70 ± 0.11	b	2.40 ± 0.07	b
	GSI		4.36 ± 0.12	a	4.82 ± 0.37	a	5.37 ± 0.19	ab	6.07 ± 0.19	b
	Age, y		6.20 ± 0.41	a	9.29 ± 0.47	b	9.10 ± 0.57	b	9.35 ± 0.56	b
	n		21		22		20		20	
Female	Length, cm		37.50 ± 0.47	a	41.03 ± 0.50	b	41.55 ± 0.62	b	41.67 ± 0.84	b
	Corrected body weight, g		571.45 ± 22.60	a	701.94 ± 38.67	b	894.53 ± 35.36	c	942.90 ± 50.38	c
	K*		1.07 ± 0.02	a	1.01 ± 0.04	a	1.24 ± 0.02	b	1.32 ± 0.08	b
	LSI		1.55 ± 0.04	a	2.02 ± 0.15	a	2.45 ± 0.06	b	2.13 ± 0.16	b
	GSI		5.78 ± 0.28	a	5.90 ± 0.41	a	7.07 ± 0.44	a	6.88 ± 0.62	a
	Fecundity, no. eggs		16719.84 ± 1271.82	a	21213.46 ± 1426.72	b	29,456.25 ± 2300.44	c	30,889.74 ± 1726.18	c
	Age, y		7.38 ± 0.36	a	11.00 ± 0.76	b	9.65 ± 0.66	b	10.45 ± 0.56	b
	n		25		16		20		20	
		2001								
Male	Length, cm		NS		36.97 ± 0.45	a	38.21 ± 0.36	ab	39.45 ± 0.31	b
	Corrected body weight, g		NS		531.23 ± 18.29	a	651.57 ± 17.26	b	726.09 ± 19.53	c
	K		NS		1.05 ± 0.01	a	1.16 ± 0.01	b	1.18 ± 0.01	b
	LSI		NS		0.90 ± 0.06	a	1.37 ± 0.04	b	1.83 ± 0.07	b
	GSI		NS		6.0 ± 0.24	a	5.65 ± 0.25	b	6.22 ± 0.24	ab
	Age, y		NS		7.5 ± 0.48	a	7.91 ± 0.42	a	8.46 ± 0.43	a
	n		NS		20		21		23	
Female	Length, cm		NS		41.53 ± 0.47	a	42.11 ± 0.35	a	42.76 ± 0.45	a
	Corrected body weight, g		NS		742.02 ± 25.77	a	836.45 ± 21.25	b	897.80 ± 33.86	b
	K		NS		1.03 ± 0.02	a	1.12 ± 0.01	b	1.14 ± 0.02	b
	LSI		NS		1.26 ± 0.04	a	1.56 ± 0.05	b	1.97 ± 0.06	b
	GSI		NS		5.88 ± 0.27	a	6.29 ± 0.22	a	8.08 ± 1.17	a
	Fecundity, no. eggs		NS		24,511.95 ± 1402.58	a	27,177.09 ± 948.02	ab	32,644.99 ± 2082.07	b
	Age, y		NS		9.6 ± 0.53	a	9.05 ± 0.42	a	8.14 ± 0.44	a
	n		NS		20		20		20	

[a]Values represent the mean ± S.E. and different lower case letters identify significant differences within a row. NS indicates not sampled.

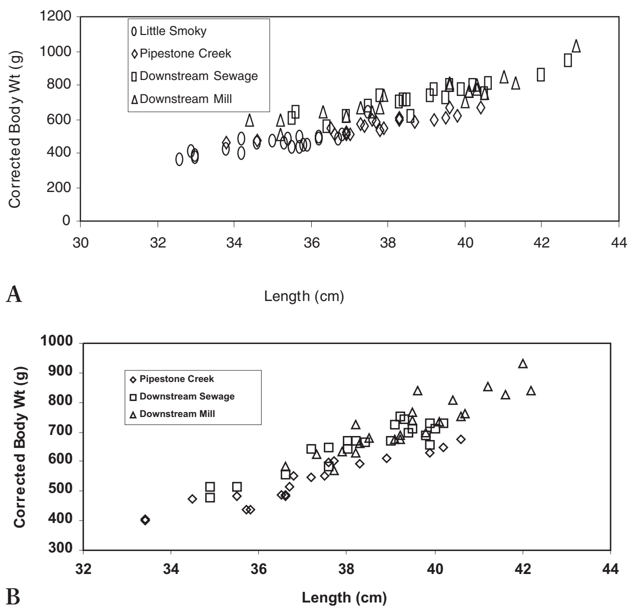

Fig. 2. Fork length (cm) versus corrected body weight (g) (body weight - gonad and liver weights) for male longnose sucker (*Catostomus catostomus*) collected downstream of the Grande Prairie sewage treatment plant, a bleached kraft pulp mill and two reference sites in the Smoky River basin during the fall of 1999 (A) and 2001 (B).

relative to the two reference locations (p < 0.001) as well as higher than females from the sewage site (p = 0.039) (i.e., more of a graded response).

Longnose Sucker Reproductive Indices

Examination of gonadal development in male longnose sucker indicated that relative to fish length and weight, males downstream of both discharges have increased gonadal development (p < 0.001) (Table 1). Relative to fish weight, the males downstream of the pulp mill discharge had increased testicular development relative to both reference sites (p < 0.001). Males downstream of the sewage site had intermediate gonads not different than reference or the pulp mill exposed males. Examination of external male secondary sexual characteristics indicated no significant differences in tubercle expression in males at any site (p = 0.188) (data not shown).

Examination of gonadal development in female longnose sucker indicated that relative to fish length, females collected downstream of the pulp mill discharge had increased gonad size compared to females from the reference locations (p < 0.001) (Table 1). Females collected downstream of the sewage site also had increased gonadal development relative to the Pipestone Creek reference females (p < 0.001). Relative to fish weight, there were no significant differences between sites in terms of female gonadal development (p = 0.06) although fish downstream of both discharges appeared to have larger gonads (Table 1). Female longnose sucker from the Little Smoky reference site had fewer eggs than females from the other three locations (p < 0.038) (Table 1). Females from Pipestone were intermediate with fewer eggs than females collected downstream of both the sewage discharge and the pulp mill effluent discharge (p < 0.042).

Male testicular tissue was preserved and prepared for histological evaluation for the presence of eggs in the testicular tissue, a condition referred to as intersex. No evidence of such a condition was found in any of the male longnose sucker collected from the four locations within the watershed (data not shown). Female longnose sucker gonadal tissue was also evaluated histologically

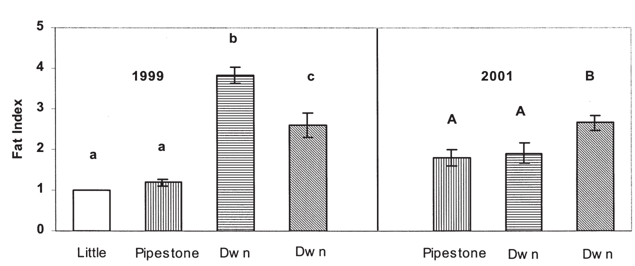

Fig. 3. A subjective evaluation of internal fat stores for male longnose sucker (*Catostomus catostomus*) collected downstream of the Grande Prairie sewage treatment plant and a bleached kraft pulp mill and two reference sites on the Waipti River in the fall of (A) 1999 and (B) 2001. Values represent the mean ± S.E. and different lower case letters identify significant differences. Sample size is 20 per site.

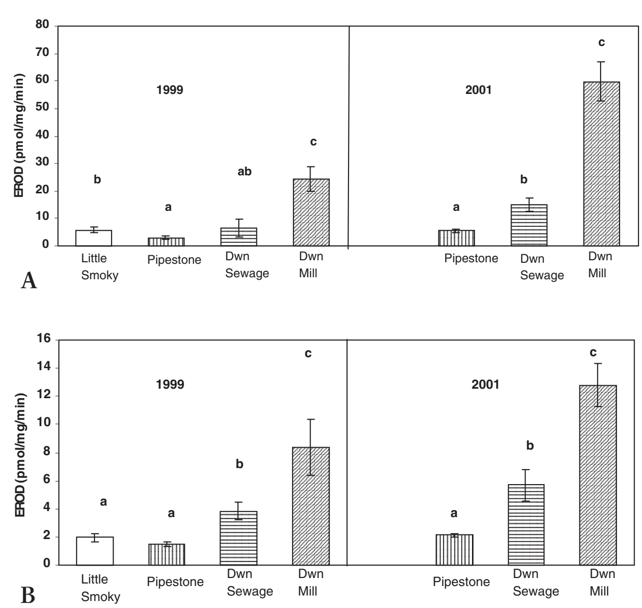

Fig. 4. Ethoxyresorufin-o-dethylase (EROD) activity in male (A) and female (B) longnose sucker (*Catostomus catostomus*) collected downstream of the Grande Prairie sewage treatment plant and a bleached kraft pulp mill and two reference sites on the Wapiti River. Values represent the mean ± S.E. and different lower case letters identify significant differences. Sample size is 20 per site.

TABLE 2. Circulating plasma levels of testosterone (both sexes), 11-ketotestosterone (males), 17β-estradiol (females) and vitellogenin in longnose sucker collected at two reference sites, downstream of a sewage discharge and downstream of a bleached kraft mill on the Wapiti River in 1999 and 2001[a]

Sex	Parameter	Year	Little Smoky		Pipestone Creek		Downstream sewage		Downstream mill	
		1999								
Male	T		1373.95 ± 110.18	a	877.09 ± 94.15	a	2130.04 ± 219.88	b	1288.31 ± 80.58	a
	11-KT		3937.97 ± 350.82	a	2336.72 ± 183.22	b	4608.83 ± 344.35	a	2733.08 ± 171.65	b
Female	T		1327.02 ± 152.06	a	1172.59 ± 200.78	a	2550.96 ± 323.19	b	1221.20 ± 250.20	a
	E2		1184.46 ± 148.42	a	934.32 ± 97.99	a	534.22 ± 81.7	b	1001.81 ± 164.97	ab
	Vtg (mg/mL)		1.21 ± 0.20	a	1.63 ± 0.10	a	14.59 ± 1.35	b	3.79 ± 0.74	c
		2001								
Male	T		NS		1716.18 ± 118.35	a	1936.21 ± 97.85	a	2971.63 ± 229.63	b
	11-KT		NS		9163.93 ± 542.60	a	9677.61 ± 565.45	a	10,458.19 ± 563.49	a
	Vtg (mg/mL) (ng/mL)		NS		0.006 ± 0.003	a	0.04 ± 0.01	a	0.12 ± 0.004	a
Female	T		NS		1656.36 ± 253.13	a	1766.02 ± 120.87	a	1928.01 ± 111.83	a
	E2		NS		2975.55 ± 630.86	a	2544.93 ± 253.13	a	2198.95 ± 196.46	a
	Vtg (mg/mL)		NS		6.41 ± 0.53	a	11.82 ± 1.28	b	23.42 ± 2.61	c

[a]Values represent the mean ± S.E. and different lower case letters identify significant differences within a row. NS indicates not sampled and sample size equals 15.

for differences in the stages of ovarian development. There were no significant differences in the mean size of the three cell types in terms of cell diameter. The frequency of each cell type found in the histological sections was also evaluated. Although some slight differences were found between the four sites, the general trends were the same indicating similar levels of gonadal development between sites (data not shown).

Female circulating 17β-estradiol levels were significantly lower in fish collected downstream of the sewage discharge relative to both reference locations ($p < 0.05$) (Table 2). Levels in females collected downstream of the pulp mill discharge were intermediate and not different than any of the three other sites ($p > 0.141$). Female longnose sucker circulating testosterone levels were significantly higher in fish collected downstream of the sewage effluent discharge relative to both the reference locations and the fish collected downstream of the pulp mill discharge ($p < 0.05$) (Table 2).

Male longnose sucker circulating levels of testosterone demonstrated identical patterns to those of females as males collected downstream of the sewage discharge had higher circulating levels than fish collected from the other three locations (Table 2). Circulating levels of 11-ketotestosterone showed altered responses as fish from the Little Smoky reference site had similar levels to the sewage site, and fish from the Pipestone Creek reference site had similar levels to the fish collected downstream of the pulp mill discharge (Table 2).

Production of 17β-estradiol by follicles under basal incubation conditions was reduced downstream of the sewage discharge relative to both of the reference locations (Table 3). Levels produced by the follicles from females collected downstream of the pulp mill discharge were similar to the Pipestone reference site but reduced relative to the Little Smoky reference location. Following stimulation with hCG, follicles from fish collected downstream of both discharges produced similar levels of 17β-estradiol, compared to follicles from fish at the reference locations ($p \geq 0.117$) (Table 3). Production of testosterone by follicles from females collected from the four locations showed no differences under basal incubation conditions ($p > 0.05$). Following stimulation with hCG, follicles collected from fish downstream of the pulp mill discharge demonstrated increased production of testosterone (Table 3).

Circulating levels of vitellogenin in females were relatively low at the two reference sites ranging from 1 to 2 mg/mL of plasma (Table 2). These levels rise significantly ($p < 0.001$) downstream of the Grande Prairie sewage discharge to approximately 15 mg/mL. Vitellogenin levels then decrease significantly ($p < 0.001$) further downstream on the Wapiti River downstream of the pulp mill discharge to levels ranging around 4 mg/mL. These levels downstream of the pulp mill discharge are, however, still higher than those in female fish collected

TABLE 3. In vitro production of testosterone and 17β-estradiol under basal and hCG induced conditions by ovarian follicles from female longnose sucker collected at two reference sites, downstream of a sewage discharge and downstream of a bleached kraft mill on the Wapiti River in 1999 and 2001[a]

Sex	*Year*	*Little Smoky*		*Pipestone Creek*		*Downstream sewage*		*Downstream mill*	
	1999								
T - basal		18.24 ± 1.29	a	21.33 ± 2.04	a	16.89 ± 1.27	a	18.27 ± 1.36	a
T - hCG		85.70 ± 12.40	a	117.98 ± 12.78	a	105.04 ± 14.25	a	235.39 ± 29.64	b
E2 - basal		105.22 ± 4.94	a	78.28 ± 4.81	b	59.10 ± 4.59	c	70.58 ± 5.38	bc
E2 - hCG		354.79 ± 29.43	ab	353.48 ± 21.27	ab	276.94 ± 27.31	a	398.06 ± 30.81	b
	2001								
T - basal		NS		66.89 ± 19.88	a	179.13 ± 28.13	b	140.46 ± 30.10	b
T - hCG		NS		813.54 ± 182.68	a	3036.55 ± 560.94	b	1015.43 ± 224.19	a
E2 - basal		NS		166.15 ± 25.15	a	340.85 ± 100.90	b	125.19 ± 13.89	a
E2 - hCG		NS		914.19 ± 86.17	a	1195.33 ± 100.77	a	825.39 ± 85.74	a

[a]Values represent the mean ± S.E. for 10 fish with incubations conducted in triplicate and different lower case letters identify significant differences within a row. NS indicates not sampled.

at the two reference sites ($p < 0.004$) (Table 2). Male longnose sucker at all sites had circulating levels of vitellogenin that were either below or very close to the level of detection of the assay.

2001 Longnose Sucker Confirmation Studies

Male longnose sucker collected downstream of the pulp mill discharge in the fall of 2001 were longer than males from the Pipestone Creek reference site ($p < 0.001$) with males from the sewage exposed site intermediate in length, not different from either location ($p > 0.05$) (Table 1). Longnose sucker weights demonstrated a gradient type of response with males from the sewage location heavier than the Pipestone reference males and the pulp mill effluent exposed males heavier than both reference ($p < 0.001$) and sewage exposed fish ($p = 0.031$) (Table 1). Males exposed to both the sewage and the pulp mill effluent, demonstrated increased condition factors relative to the Pipestone reference males ($p < 0.001$) (Fig. 2B). Males downstream of the pulp mill discharge had increased fat stores relative to the two other sites ($p = 0.006$) with the sewage exposed fish similar to the Pipestone reference males (Fig. 3). Examination of male longnose sucker growth identified site differences as fish collected downstream of both the sewage and the pulp mill discharges were longer ($p \leq 0.007$) and heavier ($p \leq 0.001$) at any given age when compared to the Pipestone Creek reference fish. Male suckers collected downstream of the mill effluent discharge were also heavier at any given age when compared to fish collected immediately downstream of the sewage outfall ($p = 0.026$).

Male longnose sucker collected downstream of both effluent discharges had larger livers relative to the male fish collected at the Pipestone reference site ($p < 0.001$) (Table 1). Hepatic EROD levels demonstrated a gradient type of response as males collected downstream of the sewage discharge had elevated EROD relative to the reference males and males collected downstream of the pulp mill effluent had even higher induction, greater than both the reference and sewage exposed males ($p \leq 0.001$) (Fig. 4A).

Female longnose sucker collected from all three locations were similar in length, but those collected downstream of both the sewage and the pulp mill effluent discharges were heavier than the Pipestone reference females ($p < 0.006$) (Table 1). There were no significant site differences in age of female longnose suckers between the three sites ($p = 0.086$) (Table 1). Examination of the condition factor of female longnose sucker found that fish collected downstream of both the sewage and pulp mill discharges were significantly fatter than fish from the upstream reference site ($p \leq 0.001$). Again, internal fat stores do not correspond completely, as female longnose sucker showed a gradient type response with fish getting fatter downstream ($p \leq 0.008$). Examination of female longnose sucker growth demonstrated significant site differences in the relationship between fish length and age ($p = 0.034$). Evaluation of site differences indicated that fish from the mill exposed site were significantly longer at any given age relative to the reference site ($p = 0.027$), but there was no difference between the sewage exposed site and the reference site ($p = 0.543$). Fish weight at age indicated that fish from both exposed sites (sewage and pulp mill) were heavier at any given age relative to the reference site ($p \leq 0.013$).

Examination of female longnose sucker liver size indicated significantly larger livers in fish collected downstream of both the sewage and pulp mill discharges relative to the reference location ($p < 0.001$) (Table 1). Hepatic EROD measurements indicated a gradient response similar to that found in male fish as reference fish were relatively low, sewage exposed fish induced somewhat and pulp mill exposed females higher than both other locations ($p \leq 0.001$) (Fig. 4B).

2001 Longnose Sucker Reproductive Indices

Examination of gonadal development in male longnose sucker downstream of the two effluent discharges indicated that relative to fish length, no site differences were present compared to males from the Pipestone reference site ($p = 0.644$). With respect to fish weight, males from the sewage site had reduced testicular development relative to the upstream Pipestone creek males ($p = 0.011$) and no other site differences were present (Table 1). Examination of external male secondary sexual characteristics indicated no significant differences in tubercle expression in males from all sites in 2001 ($p > 0.254$) (data not shown).

Examination of gonadal development in female longnose sucker in 2001 indicated that relative to fish length, females collected downstream of the pulp mill discharge had increased gonadal size compared to females from the reference location ($p < 0.001$). Relative to fish weight, there were no significant differences between sites in terms of female gonadal development ($p = 0.06$) (Table 1), although it appears that there are trends to increased development downstream of the pulp mill discharge. Female longnose sucker downstream of the pulp mill discharge had increased total number of eggs relative to the upstream reference Pipestone females ($p = 0.001$) (Table 1).

Multiple comparison tests indicated that the proportion of atretic oocytes was significantly higher in Pipestone reference females, compared to fish downstream of the sewage outflow, although this was still relatively low at about 1%. Endogenous vitellogenic oocytes were significantly larger in fish collected downstream of the mill discharge compared to those at Pipestone Creek. A Tukey multiple comparisons test indicated that the proportion of spermatocytes was significantly higher in fish collected downstream of the pulp mill location compared to the Pipestone reference site males and no evidence of intersex was found (data not shown).

Female circulating 17β-estradiol and testosterone levels did not differ significantly in fish collected downstream of the sewage or pulp mill discharges relative to the Pipestone reference location ($p = 0.529$ and 0.798, respectively) (Table 2). Male longnose sucker circulating levels of testosterone demonstrated a different pattern to those of females as males collected downstream of the pulp mill discharge had higher circulating levels than fish collected from the other two locations ($p < 0.005$). Circulating levels of 11-ketotestosterone showed fish collected downstream of the discharges had similar levels to those from the Pipestone Creek reference site.

Production of 17β-estradiol by follicles under basal incubation conditions was elevated in fish collected downstream of the sewage discharge relative to the reference location ($p = 0.002$) (Table 3). Levels produced by the follicles from females collected downstream of the pulp mill discharge were similar to the Pipestone reference site ($p = 0.45$). Following stimulation with hCG, follicles from fish collected downstream of both discharges produced similar levels of 17β-estradiol, compared to follicles from fish at the reference location ($p \geq 0.117$). Production of testosterone showed increased levels of basal steroid production downstream of both discharges when compared to the reference site ($p \leq 0.013$) (Table 3). Examination of site differences in this stimulated production demonstrated increased production of testosterone by follicles collected from fish downstream of the sewage discharge ($p < 0.001$).

Circulating levels of vitellogenin in females were relatively low at the Pipestone reference site and rise significantly ($p = 0.010$) downstream of the Grande Prairie sewage discharge (Table 2). Vitellogenin levels then increased again downstream of the pulp mill discharge to levels ranging around 25 mg/mL. Male longnose sucker at all sites had similar circulating levels of vitellogenin.

Discussion

In this Northern Rivers Ecosystem Initiative endocrine study, fish health was examined and compared to a number of reproductive endpoints developed to assess endocrine function. The research methodology that was used to assess fish health was identical to that developed for the Environment Effects Monitoring (EEM) Program for the pulp and paper sector which evaluates fish growth, reproduction, age structure and energy storage (Environment Canada 1998). The additional endocrine methodology was a result of research studies conducted over the last ten years examining endocrine function in wild fish at Jackfish Bay and the Moose River basin (Munkittrick et al. 1998; Janz et al. 1997; Oakes and Van Der Kraak 2003). As recent studies had also demonstrated reproductive effects in fish downstream of municipal sewage treatment plant outfalls (Jobling et al. 1998; Purdom et al. 1994), additional sites were added downstream of the sewage outfalls from the two towns around the pulp mills identified.

In 1999, it was quite evident during sampling of the longnose sucker that site differences existed as fish collected downstream of the sewage effluent outfall (Wapiti Sewage) and downstream of the pulp mill discharge (Wapiti Downstream) were larger and fatter than fish collected from the two reference sites (Little Smoky and Pipestone Creek). Responses such as these are a result of increased nutrients from both the municipal sewage discharge and the pulp mill discharge. The rivers in the Northern River Basins are known to be nutrient-limited and increased nutrient input results in increased food availability and a corresponding increase in fish growth and condition relative to the upstream reference fish. As with fish, the primary cumulative effects on benthic macroinvertebrates appeared to occur in the areas immediately downstream from pulp mill and municipal discharges. Measures of benthic macroinvertebrate community structure in the areas immediately above and below pulp mills indicated that while communities did not differ in terms of major taxa, there was a tendency for downstream communities to show higher diversity and density (Wrona et al. 1997). This was also a consequence of nutrient enrichment, an observation supported by studies of Chambers (1997) that indicated that pulp mills contributed approximately 13% of annual total nitrogen and phosphorus loads in the Athabasca and Wapiti-Smoky rivers.

Exposed fish also had increased liver weights relative to the two upstream reference sites. Increases in liver size are often seen in fish that reside downstream of pulp and paper mill effluent discharges. In a survey of 10 Canadian mills in the early 1990s, Munkittrick et al. (1994) found that in cases where fish were captured, fish at 6 of 7 sites had larger livers than those captured at reference locations. While increased liver size has been attributed by some authors to induced liver detoxification enzymes, increases in liver size have been seen at some pulp and paper mills without EROD induction (Munkittrick et al. 1994), and may be due to changes in lipid or glycogen storage associated with effluent exposure and food supply. In this study, although fish downstream of the Grande Prairie sewage discharge demonstrated some induction of MFO activity, it was minor compared to that in fish collected downstream of the bleached kraft mill effluent discharge. Increased liver size in longnose sucker downstream of both effluent discharges is therefore more likely a direct result of the increased food supply than increased liver enzyme activity.

Both male and female longnose sucker collected downstream of the two effluent discharges also had increased gonad size relative to fish from the two reference locations. Increased investment in gonadal development has also been associated with increased nutrients or food supply (Munkittrick et al. 2002). Unlike some of the previous studies (McMaster et al. 1991; Munkittrick et al. 1994), fish collected from the Wapiti River appear to be able to convert the increased food supply into gonadal development. Fish collected from a number of earlier pulp mill studies demonstrated increased condition and liver size, but a reduction in gonad size relative to reference fish (McMaster et al. 1991; Munkittrick et al. 1991, 1998). This was associated with some form of metabolic disruption as fish appeared to have enough food but were unable to convert that food to gonadal development (Munkittrick et al. 2000, 2002).

Lake chub (*Couesius plumbeus*) were also collected from three sites on the Wapiti River in the fall of 1999, the same sites as the longnose sucker except for the second reference site on the Little Smoky River. These smaller forage fish are thought to be less mobile than the larger longnose sucker and may be useful in separating out confounding factors from multiple discharges (McMaster et al. 2002). Lake chub also demonstrated increased condition factors downstream of the two effluent outfalls compared to their upstream reference counterparts (McMaster et al. 2004). Liver size failed to show a corresponding increase as it did in longnose sucker, and both male and female lake chub collected downstream of the sewage outfall showed signs of reduced gonadal development. This reduction in gonadal development may be a result of endocrine-active compounds in sewage effluents altering normal reproductive development in these fish (McMaster et al. 2004). Although the opposite was found for longnose sucker, differences in species sensitivity could be responsible.

Over the last several years of study at a number of pulp and paper locations, a number of reproductive endocrine tools were developed during studies examining the mechanisms of action of pulp mill effluents on reproductive function (McMaster et al. 2003). The first of these was the measurement of circulating reproductive steroid hormone levels (McMaster et al. 1992b) as well as the measurement of the ability of gonadal tissue to produce these steroids in vitro (McMaster et al. 1995b). It was preliminary evidence of sex hormone depressions that flagged these areas in the Northern River Basins as areas of potential endocrine disruption during the original NRBS program (NRBS 1992). Examination of circulating steroid levels in longnose sucker collected from these sites also demonstrated significant site differences. However, most of the differences were found downstream of the Grande Prairie municipal sewage effluent discharge. Female longnose sucker appeared to have trouble converting testosterone into 17β-estradiol as females have higher levels of testosterone and reduced circulating levels of 17β-estradiol. The enzyme responsible for this conversion is aromatase and previous studies examining pulp and paper mill effluents have documented impacts on this enzyme (McMaster et al. 1995a). Male longnose sucker also demonstrated alterations in circulating steroid levels downstream of the sewage discharge. Males however had higher levels of both testosterone and 11-ketotestosterone compared to reference and pulp mill exposed fish. These altered levels, however, did not result in changes in the expression of male secondary sexual characteristics. Previous studies at some pulp mill locations have demonstrated impacts on male sex characteristics including both increasing and decreasing the expression (McMaster et al. 2003).

Examination of the ability of female ovarian tissue from longnose sucker to produce these biologically active steroid hormones corresponded well with circulating levels for 17β-estradiol as follicles collected from sewage exposed females produced reduced levels of this hormone. Higher levels of testosterone production were not found, however, numerous control mechanisms are present for circulating levels of steroid hormones, only one of which is the production of these steroids. Previous studies at some pulp mill locations have demonstrated a strong correlation between reduced circulating levels of steroid hormones and the ability of the gonadal tissue to produce these steroids (McMaster et al. 1995a, 1996; Van Der Kraak et al. 1992).

In the mid-1980s in the United Kingdom, a research team led by John Sumpter sought to address possible causes underlying the observation of hermaphroditic fish caught by anglers from waters below sewage treatment plants. They hypothesized that one factor contributing to the phenomenon was the presence of estrogenic substances found in effluents (Purdom et al. 1994). Recent work by that group revealed that in many effluents the likely cause of the vitellogenin induction (female egg yolk protein) in male or immature fish was the presence of natural estrogens such as estradiol and estrone and the synthetic estrogen ethinylestradiol (used in birth control pills) (Brighty 1997; Giesy and Snyder 1998). Further studies demonstrated a clear relationship between concentration of sewage effluent and the percentage of intersex fish (male fish with eggs present in testicular tissue) (Jobling et al. 1998). As sewage discharges were associated with all three of the mills identified in the NRBS studies and as increased numbers of immature fish were found (NRBS 1992), we decided to add these estrogenic indicators to our list of endocrine tools. We were able to demonstrate significant site differences in the levels of vitellogenin in mature female fish. Females collected downstream of the sewage effluent discharge had significantly higher circulating levels of vitellogenin relative to the two reference locations. Although levels dropped significantly in females collected downstream of the pulp mill discharge, these levels were still higher than reference female levels. With this increased circulating vitellogenin level, one might expect differences in the stage of ovarian development in these fish. Examination of the frequency of the various ovarian developmental stages however showed no differences between sites and the cell size of the three stages of ovarian development were also similar between sites. Exposure of fish to ethinylestradiol has been shown to alter gonadal development using similar staging techniques (Palace et al. Accepted for publication). Male longnose sucker testicular sections were also examined for evidence of intersex and no signs of egg development were found. The increased levels of vitellogenin in female fish collected downstream of the sewage discharge are also difficult to interpret given the reduced circulating levels of 17β-estradiol in these female fish. Previous pulp and paper studies have demonstrated reduced circulating levels of vitellogenin corresponding to reduced circulating levels of 17β-estradiol (Nickel et al. 2003).

New techniques were also developed to examine levels of gonadal apoptosis or programmed cell death. Previous studies that had documented increased levels of gonadal apoptosis correlated well with reductions in steroid productive capacity and reduced gonadal development in fish exposed to pulp and paper mill effluents (Janz et al. 1997, 2001). However, examination of ovarian samples collected from female longnose sucker from the four sites showed no increased levels in fish exposed to either of the effluent sources (McMaster et al. 2004). It is thought that increased ovarian apoptosis may contribute to the reduced steroid biosynthetic capacity in some fish exposed to mill effluents (Janz et al. 2001). Another potential contributor to reduced steroid productivity and the corresponding reproductive alterations in fish exposed to some pulp and paper mill effluents is an increased level of hepatic and gonadal oxidative stress (Oakes and Van Der Kraak 2003). Samples collected over a number of years were recently evaluated for free radical damage and a strong correlation to reproductive effects was found at two locations (Oakes et al. 2003). Examination of hepatic tissue collected from longnose sucker at these sites demonstrated a graded response to the effluent sources. Levels in fish collected downstream of the sewage discharge showed intermediate levels of oxidative stress and fish collected downstream of the pulp mill discharge were even higher. Although these levels do not correlate with the reproductive changes, they do mirror the induction of hepatic MFO activity in these fish (Oakes et al. 2004). Oakes et al. (2003) also found a strong correlation between MFO induction and free radical damage and suggested that induced MFO activity may be one of the many sources of oxidative stress in these fish. Binding characteristics of sex steroid binding proteins were also examined in reference and exposed fish. There were only modest changes in binding capacity with a significant increase in longnose sucker exposed to mill effluent (Pryce-Hobby et al. 2003). As significant reproductive effects were not demonstrated downstream of the mill discharge, these changes in binding characteristics do not appear to represent significant impacts on reproductive fitness.

Overall, fish collected downstream of both the Grande Prairie municipal sewage effluent discharge and the discharge of the bleached kraft mill demonstrated signs of nutrient enrichment. This type of response (increased growth, condition, liver size and gonad size) was demonstrated in fish at a number of pulp and paper mill sites in the recent review of the Environmental Effects Monitoring Programs Cycle 2 and Cycle 3 data (Lowell et al. 2003, 2005). A similar nutrient enrichment response was also often found in the benthic invertebrate community data from freshwater effluent discharges in Canada (Lowell et al. 2003, 2005). No clear signs of endocrine disruption were demonstrated in fish collected downstream of the bleached kraft mill effluent discharge on the Wapiti River. Whether compounds capable of reproductive effects are present and are just masked by the large nutrient enrichment response, however, is not known. Effluent from this mill does contain compounds which bind to a number of endocrine receptors and wood furnish from this site also contains endocrine-active compounds (Hewitt et al. 2004; McMaster et al. 2002, 2004). Short-term exposures of immature rainbow trout to Grande Prairie effluent also failed to alter circulating steroid levels over a 21-day period (Oakes et al. 2005). Effluent from the municipal sewage discharge did, however, alter a number of endocrine endpoints relative to the reference sites. Circulating steroid levels as well as in vitro production of some steroids were altered in sewage exposed fish, and females contained higher levels of the female egg yolk protein. Although these changes did not appear to result in whole body reproductive alterations, with the increased growth of the city of Grande Prairie and higher municipal effluent flows, fish should continue to be monitored at this location.

Other important findings from this study are that a number of the additional endocrine tools, some additional fish endpoints (fat index) and some EEM endpoints (growth) were capable of distinguishing between longnose sucker collected at the sewage exposed site and fish collected downstream of the bleached kraft mill discharge. This is of importance as these sites are only 10 km apart and there are no barriers to fish movement or migration. This indicates that these fish do not mix during periods of gonadal development making them a suitable sentinel species for EEM studies. The issue of mobility has raised a number of questions about the use of larger bodied species in this program and the effects of confounding factors (Munkittrick et al. 2002). These results correspond well to tracking data for the white sucker on the Saint John River in New Brunswick (Doherty et al. 2005).

Very few differences in fish populations were demonstrated downstream of two mills on the Athabasca River in Whitecourt, Alberta, during field studies in 2000 (McMaster et al. 2002, 2004). As the majority of the changes in fish populations occurred downstream of the two effluent outfalls on the Wapiti River in 1999, follow-up confirmation studies were conducted in 2001. Both male and female longnose sucker again demonstrated signs of nutrient enrichment downstream of both the sewage and pulp mill discharges with increased growth, condition and liver size. Female longnose sucker collected downstream of the pulp mill discharge demonstrated signs of increased ovarian development relative to the reference site, although females downstream of the sewage source showed no differences. Unlike the 1999 sampling period, male longnose sucker downstream of both discharges did not show increased gonadal growth; in fact males collected downstream of the sewage discharge demonstrated reduced testicular development relative to the upstream reference fish. Internal fat stores also demonstrated different responses to those seen in 1999. Fish downstream of the sewage discharge in 1999 had the highest levels of internal fat, even higher than those collected downstream of the bleached kraft mill. In 2001 however, internal fat stores in fish collected downstream of the sewage site were similar to those of the reference fish. Internal fat stores in fish downstream of the pulp mill were higher than both other sites, roughly the same level as they were in 1999. The Grande Prairie municipal sewage treatment plant added tertiary treatment prior to the 2001 collections, which may explain the changes in available nutrients.

In 1999, sewage exposed fish also demonstrated alterations in circulating steroid hormone levels relative to the other locations. In 2001, however, no indications of impacts on circulating steroid hormone levels were evident. Circulating vitellogenin levels also changed significantly between the two years of collection. In 1999, females collected downstream of the sewage outfall had very high levels, which decreased significantly in fish downstream of the pulp mill discharge. In 2001, levels in sewage females were intermediate, higher than reference, but lower than pulp mill exposed fish. Whether changes in treatment processes at the sewage treatment plant also changed the levels of the chemicals responsible for the reproductive effects is unknown. Absolute levels of vitellogenin in the blood of females collected downstream of the pulp mill discharge also increased between 1999 and 2001. Whether additional estrogen-active compounds were being released is not known, however, males downstream of the pulp mill did not demonstrate vitellogenin induction suggesting that exogenous estrogenic substances were not responsible.

Although some signs of altered endocrine function were demonstrated in fish collected from the Northern Rivers Basins, the main response was one of nutrient enrichment or eutrophication. During recent reviews of Cycle 2 and Cycle 3 data from the Environmental Effects Monitoring Program, a number of large-scale national response patterns were identified downstream of Canada's pulp and paper mills. For fish, the average national response pattern was one of an increase in weight at age, condition and liver weight, and average decrease in gonad weight in exposed fish. This pattern of decreased gonad size, combined with increased energy use and storage, has been interpreted as a form of metabolic disruption or endocrine disruption, associated with difficulties in producing sufficient sex steroid hormones (Lowell et al. 2003). Examination of the national data in more detail indicated that there was also a large number of mills that demonstrated more of an overall nutrient enrichment response pattern with increases in all of these endpoints. In a multivariate statistical evaluation of the fish and benthic data from the Cycle 2 studies it became clear that the eutrophication response in fish was correlated with a pronounced eutrophication response in the benthic invertebrate data. The metabolic or endocrine disruption type of response in fish was found under conditions of less pronounced eutrophication in benthic invertebrate data. It was thought by the authors that it may be possible that metabolic disruption is masked at high levels of eutrophication (Lowell et al. 2003). If this is the case, the lack of endocrine alterations could be a result of the pronounced eutrophication responses demonstrated in these nutrient-poor Northern Rivers. It is recommended that fish performance continue to be monitored in these systems, especially as our second year of sampling on the Wapiti River demonstrated changes in the fish responses downstream of the municipal sewage discharge following the installation of tertiary treatment at this location. The results also emphasize the need for EEM-like monitoring programs downstream of municipal sewage discharges in Canada.

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