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Effects of Xenoestrogens on Male Reproduction

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By

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ABSTRACT

While previous studies have identified the presence of estrogenic substances in St. Lawrence river, along the Island of Montreal there is no information on the physiological and population consequences associated with exposure to low levels of estrogenic compounds in fish in these sites. Therefore, the objective of my first study was to assess the effects of such contaminants on male reproductive function in the spottail shiner (Notropis hudsonius). Disturbances in stages of spermatogenesis were seen in fish captured at sites having high levels of estrogenic contamination. Sperm concentration and various motility parameters were significantly lower in shiners from Ilet Vert as compared with those from Iles de la Paix (reference site). Histological analyses of testes revealed that more than one-third of the fish captured at sites with the highest estrogenic contamination displayed intersex, a condition in which ovarian follicles were developing within the testis. These data indicate that there is significant estrogenic contamination in the St. Lawrence River that is associated with impaired reproductive function in male fish.

After finding that the fish from the St. Lawrence River are exposed to xenoestrogens causing male reproductive dysfunction, in the second part of my study we wanted to determine if lactational exposure to contaminated fish could alter the development of the male reproductive system in rats. Three experimental groups were used: rats (dams) gavaged with (a) distilled water (control), or (b) homogenized fish from a reference site (Iles de la Paix) or (c) homogenized fish from a xenoestrogen-contaminated site (Ilet Vert). Pups were exposed via lactation and sampled on either day 21 (weaning) or day 91 (adults). Adult sperm concentrations and sperm motility parameters were all significantly decreased in the xenoestrogen group as compared to the reference and control groups. Furthermore, the distribution of stages of spermatogenesis was altered in the xenoestrogen group, indicating an

effect on the kinetics of spermatogenesis. Immunoreactivity of connexin43 (Cx43), a gap-junctional protein, was markedly decreased in the seminiferous epithelium of the xenoestrogen group, suggesting that the intercellular coordination of testicular function may be affected. These data indicate that contaminants from xenoestrogen environments may pass through the food chain and exert effects on male reproductive functions.

The mechanism responsible for alteration in gap junctions in Sertoli cells after exposure to xenoestrogens is unknown. The objectives of the third part of my study were to determine the effects of nonylphenol on GJIC and connexin 43 (Cx43) in a murine Sertoli cell line, TM4. A significant concentration-dependent reduction in GJIC was observed at nonylphenol concentrations between 1 and 50 microM. Cx43 immunofluorescent staining was reduced at both 10 and 50 microM doses of nonylphenol. Cx43 phosphorylation, as determined by Western blot analysis, was reduced at both 10 and 50 microM concentrations. A dose-dependent decrease in p38-MAPK activity was observed in nonylphenol-exposed Sertoli cells. Protein kinase C activity was also measured and was not influenced by nonylphenol. In contrast, no effect on GJIC or Cx43 protein was observed in cells exposed to 17beta-estradiol at these concentrations. These results suggest that nonylphenol inhibits GJIC between Sertoli cells and that this is modulated via nonestrogenic pathways.

In summary we have shown that xenoestrogens in the aquatic system can effect spermatogenesis in fish and this effect can be passed up the food chain. Also xenoestrogens like nonylphenol can have effects that are not mediated by the classical estrogen pathway.

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LIST OF COMMON ABBREVIATIONS

4-t-OP: 4-tert-octylphenol

4-n-NP : 4-n-nonylphenol

ANOVA: Analysis of Variance

ATF-2: Activating Transcription Factor-2

BCF: Beat Cross Frequency

BCF: Bioconcentration factor

Cx: Connexin

DDE: dichlorodiphenyldichloroethylene

DDT: 1,1,1-trichloro-2 (p-chlorophenyl) 2-(o-chlorophenyl) ethane

DEHP: di-2-ethylhexyl phthalate

DOP: Dioctylphthalates

EDC: Endocrine Disrupting Compound

EE2:17- α -ethinylestradiol

GJIC: Gap Junctional Intercellular Communication

LIN: Linearity

MAPK: Mitogen Activated Protein Kinase

MUC: Montreal Urban Community

NP: Nonylphenol

NP(1-16)EO: nonylphenol polyethoxylates

NP(1)EC and NP(2)EC: nonylphenol-mono and di-ethoxycarboxylic acids

OP(1)EC and OP(2)EC: octylphenol-mono and di-ethoxycarboxylic acids

PCB: Polychlorinated Biphenyls

PCDF: polychlorinated dibenzofurans

PCDD: polychlorinated dibenzo-p-dioxins

PVC: Polyvinyl Chloride

PKC: Protein Kinase C

SEM: Standard error of mean

STR: Straightness

TPA: tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate

VAP: Path Velocity

VCL: Curvilinear Velocity

VSL: Straight line Velocity

VTG: Vitellogenin

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INTRODUCTION

The first part of this thesis comprises a literature review, which focusses on endocrine disrupting compunds in the aquatic system and what is known about its effects on male reproductive system. This is followed by three chapters which details the experiments done at the laboratory, to build up on the information that is already known. A 'Discussion and Conclusion' section identifies the significance of this research project towards greater understanding of the significance of endocrine disrupting compunds on male reproductive system and more importantly elucidating the mechanisms by which such effects occur. Furthermore, a final section prepared in French discusses all the key topics of this research project.

The Montreal Urban Community (MUC) sewage treatment facility discharges all of its effluent at a single site near the eastern tip of the Island of Montreal, where it enters the St. Lawrence River. Thus, the sewage created by a population of approximately 1.8 million people enters the St. Lawrence at a single discharge point. Building up on previous studies, which reported the presence of alkylphenol ethoxylates at many sites in the sediments of the St. Lawrence River around the Island of Montreal and found levels to be low at Île de la Paix, high at Île Dorval, and much higher at Îlet Vert and Île Beauregard (Sabik et al.,2003), we wanted to determine the physiological and population consequences in fishes associated with exposure to low levels of estrogenic compounds under field conditions. Therefore my first part of the study was to determine the level of estrogenic contamination in fishes at various sites in the St. Lawrence river using biomarkers (Vitellogenin) and to further look at the effects on the male reproductive system in fishes.

Several reports suggest a deterioration in the quality of male human reproductive function over the last 50 years (Carlsen E et al.,1992; Aitken RJ et al.,2004). Male reproductive parameters that have been reported as altered include a decrease in sperm count, increased incidences of congenital malformations of the male reproductive tract, and testicular cancer among young men (Toppari J et al.,1996; Sharpe RM et al.,2001). While it is not clear what factors or changes in lifestyle may be responsible for these changes, it has been suggested that exposure to certain environmental contaminants may be contributing to these effects (Sharpe RM et al.,1993). When we sampled fish to study the presence and effects of endocrine-disrupting chemicals, we noted the presence of an active sport fishery in the area. The fact that fish in the St. Lawrence River are exposed to estrogenic compounds suggests that people, as well as fish-eating mammals consuming these fish, may be exposed to the same endocrine-disrupting compounds. Little information exists on the transfer of endocrine-disrupting effects through the food chain. Exposure to environmental contaminants during critical periods of development may represent a far greater risk to animals and humans than exposure as adults. The reproductive systems of mammals undergo substantial development both in utero and after birth, prior to puberty. In rats, the male reproductive tract undergoes substantial development in the first three weeks of life. Thus the period of lactation, when the mother can pass along contaminants to her offspring, represents a critical period of reproductive development for the male pups. As such, this critical period is among the most vulnerable to xenobiotics, including endocrine disruptors. Therefore we wanted to determine whether the maternal consumption of fish from a xenoestrogen-contaminated environment results in adverse reproductive consequences to weaning male pups, and if so, whether such effects become apparent only when the pup reaches

adulthood. This information will assist us in developing a better understanding of the exposure and risks associated with eating fish from environments contaminated with estrogenic compounds.

Spermatogenesis requires direct intercellular communication between Sertoli cells, which is mediated by gap junctions (Roscoe WA et al.,2001). An important role of gap junctions is to regulate cell growth and differentiation by controlling the passage of small molecules, including secondary messengers, between adjacent cells (Bruzzone R et al.,1996). Gap junctions are composed of intercellular pores that allow the passage of small molecules between adjacent cells (<1 kDa). These pores are composed of hexomeric connexins from each cell, which are themselves formed by the oligomerization of connexins. Cx43 is present in many tissues, including the testis, and is localized between adjacent Sertoli cells, Sertoli cells and germ cells, and between Leydig cells (Risley MS et al.,1992; Tan IP et al.,1996; Batias C et al.,2000; Perez-Armendariz EM et al.,2001). Several lines of evidence indicate that Cx43 is essential for normal testicular function (Roscoe WA et al.,2001, Batias C et al.,2000, Juneja SC et al.,1999; Plum A et al.,2000; Batias C et al.,1999). Given the importance of Cx 43 in spermatogenesis, we wanted to determine the mechanism by which nonylphenol, the major contaminant in specific sites in the St. Lawrence river, affects male reproductive system. Our observation in the second part of the study, that Cx 43 in the Sertoli cells can be affected, led us to determine whether nonylphenol could alter intercellular communication in Sertoli cells and to determine if this effect was mediated via an estrogenic pathway.

2.0 LITERATURE REVIEW

2.1 ENDOCRINE DISRUPTING COMPOUNDS IN THE AQUATIC SYSTEM

This chapter has been divided into three main parts and will summarize the current knowledge of the effects of Endocrine disrupting compunds (EDC's) on male reproductive function by (1) focussing on the source of EDC's in the aquatic system and its effects on fish (2) consequences of eating xenoestrogen contaminated fish on the function of male reproduction (3) the mechanism(s) of action of EDC's, and will attempt to identify the gaps in our current knowledge of EDC's and male reproductive function. Since both synthetic and natural estrogens are widely believed to be an important source of EDC in the aquatic sytem, and the findings that the St. Lawrence river along the island of Montreal is a potential source of alkylphenol ethoxylates (Sabik et al., 2003), there will be greater emphasis on the estrogens and alkylphenol ethoxylates in the sections below.

2.1.1 SOURCES OF ENDOCRINE DISRUPTING CHEMICALS

2.1.1.1 MUNICIPAL SEWAGE DISCHARGE

The production of steroids by humans can be substantial. The synthesis and excretion of estrogens in women of reproductive age vary during menstrual cycles and pregnancy until menopause (Johnson et al., 2000; Frandsen and Lundwall, 1966). Synthetic estrogens, primarily in the form of birth control pills and estrogen replacement therapies, represent important sources

of estriol, estrone and E2 in sewage. Approximately 65% of E2 and 15% of estrone from orally administered drugs are excreted in the urine and faeces. Oral contraceptives contain EE2, which is found primarily as sulfate conjugates (80 %) in plasma shortly after administration. A large proportion is then excreted as a non-metabolized conjugated form (Schubert et al., 1994). This is, of course, compounded by the number of people living in a given local municipality who are serviced by a sewage treatment facility. For example, in the city of Montreal (Québec, Canada), the sewage from 1.2 million people is treated at a single plant, while outlying suburbs have other smaller treatment plants. The end result is sewage discharge from approximately 3.5 million people entering the St. Lawrence River over a relatively short distance.

In addition to naturally occurring steroids and oral contraceptives, sewage effluent is also a transporter for estrogen-mimicking compounds that are generated by industrial processes and which are released into sewage. Different civic industrial bases will likely result in having different types or proportions of chemicals present in their sewage effluents. Yet certain chemicals such as alkylphenolethoxylates, plasticizers, such bisphenol A and phthalates, as well as organochlorines and metals, all have been shown to be important contributors to the endocrine disputing capacity of sewage effluents.

2.1.1.2 ALKYLPHENOLS

Commercially available alkylphenols are generally mixtures of alkylphenols with different degrees of branching but with the same number of C-atoms in the alkyl chain (Nimrod and Benson, 1996). Alkylphenols are used primarily in the production of alkylphenol ethoxylates

(APnEO), tris(nonylphenyl)phosphite and alkylphenol-formaldehyde condensation resins (Nimrod and Benson, 1996). However, unreacted alkylphenols can be used as plasticizers in the production of plastics. Alkylphenol ethoxylates are easily degraded to alkylphenols and, therefore, represent an important source of alkylphenols in sewage effluent (Nimrod and Benson, 1996). Nonylphenol is the most commercially prevalent member of the alkylphenol family, representing approximately 85% of the alkylphenol market. Nonylphenol is not used as such, but usually is further reacted to produce nonylphenol ethoxylates. The remaining 15% alkylphenols are generally assumed to be octylphenol (White et al., 1994). These persistent industrial chemicals are used as surface-active agents in industrial cleaning/washing agents, paints, cosmetics, and even as spermicides (Sonnenschein and Soto, 1998). Nonylphenol has also been found in the preparation of lubricating oil additives, plasticizers, and polyvinyl chloride (PVC) used in the food processing and packaging industries.

Studies have shown that alkylphenols can accumulate in the sediments of the receiving waters downstream of municipal effluents along the St. Lawrence river (Sabik et al., 2003). The accumulation of these alkylphenols, and particularly nonylphenol, is likely to be a significant source of exposure for aquatic organisms that live in the receiving waters.

Alkylphenols are readily taken up by fish either through the gills or from benthic organisms that are eaten by fish. For example, studies on juvenile rainbow trout exposed to 4-tert-octylphenol (4 μg/l; octylphenol) for 10 days, indicated that octylphenol was detected in bile, faeces, pyloric caeca, liver and intestine after 4 days of exposure. Octylphenol accumulated as the parent compound in fat with a bioconcentration factor (BCF) of 1190, and in brain, muscle,

skin, bone, gills, and eye with BCFs of between 100 and 260. This indicates that important concentrations of alklphenols, such as octylphenol, can accumulate in a variety of fish tissues, making them potential targets for these chemicals (Ferreira-Leach and Hill, 2001).

2.1.1.3 OTHER SOURCES

Other sources of EDC's include bisphenol A, phthalates, chlorinated hydrocarbons, pesticides, pulp and paper mill effluents, heavy metals and steroids from agricultural wastes.

2.1.1.3.1 BISPHENOL A

Bisphenol A is used in the production of epoxy resins and polycarbonate plastics. It has recently been shown that bisphenol A has estrogenic potency (Toppari et al., 1996). Levels of less than 1 ng to 6.2 μg/l have been reported, but levels below 1 μg/l are most frequently reported (Staples et al., 1998; Klecka et al., 2001). It has been shown that bisphenol A in water samples are degraded within 3-5 days, with a half-life of between 2.5 - 4 days (Staples et al., 1998). Anaerobic biotransformation of bisphenol A has, however, shown no loss of bisphenol A when incubated for 162 days under conditions promoting either methanogenesis, sulfate-reduction, iron(III)-reduction, or nitrate-reduction (Voordeckers et al., 2002). This suggests that there is a potential for bisphenol A to accumulate in anoxic sediments.

2.1.1.3.2 PHTHALATES

'Phthalates' is the generic name given to a class of chemical esters of 1,2-benzenedicarboxylic acid. The majority of phthalates in water comes from industrial effluents from rubber and plastic companies (Thomas and Thomas, 1984). Reported levels of di-2-ethylhexyl phthalate (DEHP) in water samples from rivers in the United Kingdom (UK), Sweden, United States (US) and the Netherlands are all within the range 0.3-1.6 μg/l, with levels in coastal, marine and estuarine waters ranging between <2 ng/l and 335 ng/l (Mackintosh et al., 2004; Scholz, 2003). Dioctylphthalates (DOPs) are the most hydrophobic of the phthalate esters and have been detected in riverbed sediments at concentrations between 0.1-1700 mg/kg, depending on whether the samples were collected from clean or contaminated sites. (Preston and Al-Omran, 1989; Al-Omran and Preston, 1987).

Phthalate esters do not appear to bioaccumulate considerably. BCFs for phthalate esters in three different aquatic species, the oyster (Crassostrea virginica), the brown shrimp (Pennies aztecus) and sheepshead minnow (Cyprinodon variegates) ranged from only 6.9 (oyster) to 16.6 (shrimp), when exposed for 24 hrs to concentrations of 0.1 and 0.5 mg l-1 (DEHP; Wofford et al., 1981). However, in long term studies, an average BCF of 2496 for mussels (total soft tissues) was recorded, following 28 days of exposure to concentrations of 0.004 and 0.042 mg/l (Brown, 1982).

2.1.1.3.3 CHLORINATED HYDROCARBONS

Organochlorines comprise a large group of substances, e.g. 1,1,1-trichloro-2 (p-chlorophenyl) 2-(o-chlorophenyl) ethane (DDT) and its metabolites, gamma-hexachlorocyclohexane (gamma-HCH), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-p-dioxins (PCDDs). The concentrations of persistent chemicals such as dioxins and PCBs in river water are low, but these chemicals concentrate in tissue and accumulate exponentially as they move from phytoplankton or zooplankton up to fish through the food chain. Through the process of biomagnification, the concentrations of PCBs in fish can be a few million times greater than the levels in the surrounding water (Debruyn et al., 2004). Experimentally determined bioconcentration factors of various PCBs in aquatic species (fish, shrimp, oyster) range from 200 to 70 000 or more. In the ocean, there is bioaccumulation of PCBs in higher trophic levels with an increased proportion of higher chlorinated biphenyls in higher-ranking predators (Gelsleichter et al., 2005). The substantial quantities of PCBs in aquatic sediments can therefore act as a reservoir of PCBs for organisms.

2.1.1.3.4 PESTICIDES

Since World War II, the number of pesticides used has dramatically increased. Chemicals such as Mirex, fenitrothion, pyrethroids, and others, are often used in areas for specific purposes, such as insecticides and fungicides for certain crops, but they pose a considerable risk for non-target organisms, including aquatic species, because these chemicals often enter the waterways

as agricultural runoff. Chemicals originating from agricultural activity enter the aquatic environment through atmospheric deposition, surface run-off or leaching, and frequently accumulate in soft-bottom sediments and aquatic organisms (Milers and Pfeuffer, 1997). A wide variety of pesticides have been detected in both water and sediments of many lakes and rivers throughout the world (Milers and Pfeuffer, 1997).

2.1.1.3.5 PULP AND PAPER MILL EFFLUENTS

There is abundant data suggesting that paper mill effluents have endocrine disrupting effects on fish but the identities of the responsible compounds for these effects have not been unequivocally identified (Van der Kraak et al., 1992). Androgenic steroids have been indirectly implicated as the causal agents for masculinization of fish downstream of paper mills in the southern US. Three coastal streams in Florida (Elevenmile Creek, Fenholloway River, and Rice Creek) which received effluent from paper mills contained populations of masculinized female mosquitofish. Using a toxicity identification and evaluation approach, androstenedione was identified in the water of the Fenholloway River (Jenkins et al., 2001). Pine tree pulp and paper mill waste effluent contains an abundance of phytosteroids, most notably ß-sitosterol (Conner et al., 1976). It has been shown that ß-sitosterol in paper mill effluent has a role in masculinizing resident fish populations (MacLatchy and Van Der Kraak, 1995). The masculinizing effects of paper mill effluent on embryonic development of the viviparous fish - the eelpout (*Zoarces viviparous*) has been demonstrated (Larsson and Forlin, 2002). These examples suggest that androgenic compounds are more abundant in aquatic environments than previously suspected.

2.1.1.3.6 HEAVY METALS

Heavy metals have been known form many years to be implicated in the modulation of the endocrine system. Several studies have shown that Cd, Cu, Hg Pb, and tin, for example, can all affect testicular function and the endocrine functions of the testis as well as other endocrine systems. Organotins, including TBT, are widespread in the aquatic environment, especially because of the use of TBT as an anti-fouling agent for boat exteriors. Organotins can accumulate in mollusks where they have been reported to alter male development (Gibbs et al., 1987). As such they can enter the food chain and bioaccumulate up the food chain (Ebdon et al., 1989). Mercury (Hg) contamination of water by mine tailings remains significant in certain parts of the world (Barrios-Guerra, 2004). Methylmercury is considered more toxic than elemental Hg and has been shown to exert endocrine disrupting activity in both fish and mammals (Wester and Canton, 1992; Dufresne and Cyr, 1999; Homma-Takeda et al., 2001). The release of Cd into the environment as a result of anthropogenic activity is ten times greater than contamination from natural sources (Cossa, 1989). Free Cd can readily be absorbed into fish via the gills or can enter the food chain. Cd accumulates in tissues such as kidney and liver bound to intracellular metallothioneins (Nordberg et al., 1992).

2.1.1.3.7 STEROIDS FROM AGRICULTURAL WASTE

Livestock wastes represent a potentially important source of EDC's in the aquatic environment. Manure from bovine, porcine, poultry and other domestic animal production can contribute significant quantities of steroid hormones to the aquatic environment (Raman et al., 2004; Thacker, 2004; Lorenzen et al., 2004; Hanselman et al., 2003; Tashiro et al., 2003).

Animal manures are a potentially significant source of sex hormones in the environment because they are directly applied to land in relatively high amounts and can enter the aquatic ecosystem by runoff (Callantine et al., 1961; Yin et al., 2002; Shore et al., 2004).

Significant concentrations of estrogens and androgens have been reported in ponds or streams receiving runoff from fields fertilized with chicken litter (Hanselman et al., 2003; Nichols, 1997). In fact, depending on application rate, concentrations in runoff have been measured as high as 1280 ng/l (Nichols, 1997). In addition to steroids, a number of pharmaceutical agents used to promote growth also accumulate in animal waste (Schiffer, 2001). In general, however, there is still limited information on the potential of manure as a potential risk source for estrogens to the aquatic environment.

Clearly, the preceding information, although by no means comprehensive, illustrates the varied and widespread sources of EDC contamination in the aquatic ecosystem.

2.2 BIOMARKERS OF EDC CONTAMINATION

Establishing exposure is critical to demonstrating that a class or mixture of chemicals is responsible for inducing toxic effects. As such, there has been considerable attention in toxicology to the use of specific cellular and biochemical responses of target organs, most often the liver, as an indicator of exposure to a given class of toxicants. Often these indicators, or biomarkers, are either enzymes that are implicated in the metabolism and detoxification of a class of toxicants, or cellular binding proteins. The use of biomarkers in ecotoxicology can also offer a form of an early-warning system, as it may become possible to establish exposure and potential risk before toxicants have irreversible effects on the animals inhabiting a given ecosystem. Several biomarkers have been used successfully for environmental monitoring.

Example of these include hepatic cytochrome P4501A (EROD, Fent, 2004; Desantis et al., 2005) for organic contaminants, hepatic and renal metallothionein for heavy metals (Tom et al., 2004; Mourgaud et al., 2002), and brain acetyl cholinesterase activity for insecticides (Gruber and Munn, 1998; Ferrari et al., 2004).

2.2.1 VITELLOGENIN AS A BIOMARKER:

It had been well established that the VTG gene in fish is regulated by E2. Furthermore, since VTG is not sex-linked, it can be expressed in both males and females, mature and immature. Since immature fish and males either do not express VTG due to low or non-detectable circulating levels of E2, VTG has become a widely used biomarker for environmental estrogens. Other estrogen-dependant proteins such as choriogenins, hepatic proteins that are transported to the ovary to become part of the zona radiata, have also been used, as has the ER, whose expression can be upregulated by estrogens (Purdom et al., 1994; Lee et al., 2002a; Lee et al., 2002b; Kleinkauf et al., 2004; Li and Wang, 2005; Meucci and Arukwe, 2005). Its practicality and the wealth of information on the regulation of VTG have resulted in its use as the primary indicator of estrogen exposure in fish.

2.2.2 OTHER RELEVANT BIOMARKERS

The development of other biomarkers in fish for other endocrine systems has been slow. It has been proposed that hepatic deiodinase would represent a good marker for thyroid hormone disruptors, but this has received limited use thus far (Eales et al., 1999; Adams et al., 2000; Brown et al., 2004). The expression of spiggin in stickleback (*Gasterosteus aculeatus L*) has been proposed as a biomarker of androgen action. Spiggin is an androgen-dependant renal glue

protein used by stickleback to build their nests (Katsiaddaki et al., 2002a, b). Its synthesis is induced by methyltestosterone, dihydrotestosterone and by pulp and paper mill effluent (Katsiaddaki et al., 2002b). With the advent of fish genomics, it is likely that a variety of new and reliable biomarkers will become available for the different endocrine systems (Koskinen et al., 2004; Larkin et al., 2003). Microarrays that encompass these markers may prove to be valuable in terms of understanding the effect of mixtures on multiple endocrine systems.

The expression of biomarkers is not necessarily without effect. In male and immature fish, the consequences of prolonged VTG production have also been questioned in terms on inducing toxicity. High levels of VTG in male fish have been reported to cause acute renal failure due to excessive accumulation of protein in the renal tubules (Folmar et al., 2001a). Chronic exposure to lower concentrations of estrogenic chemicals might not cause the extensive liver and kidney pathologies which have been observed with high dose exposures, but it has been proposed that a milder effect on the liver and kidney might result in a reduced ability to metabolize xenoestrogens or to resist diseases (Folmar et al., 2001a). Further, it has been suggested that VTG production in males may decrease or alter energy stores that are destined for growth and spermatogenesis (Korsgaard et al., 2002).

2.3 FIELD STUDIES ON ENDOCRINE DISRUPTING CHEMICALS

2.3.1 FEMINIZATION OF FISH

The induction of both masculinization and feminization of fish by environmental contaminants have been reported over the past decade. Feminization of fish has been more frequently reported than masculinization, and appears to be widespread throughout the world

(Folmar et al., 1996; Knudsen et al., 1997; Harshbarger et al., 2000; Hashimoto et al., 2000; Folmar et al., 2001b; Jobling et al., 2002a; Hecker et al., 2002; Sole et al., 2002). In the mid-1990s, Purdom et al. (1994) reported, for the first time, a connection between the estrogenic compounds present in sewage effluent and the feminization of fish observed in the wild.

The first evidence that fish had been exposed to estrogenic compounds was shown by the presence of the female-specific yolk precursor protein VTG in male fish (Sumpter et al., 1995; Tyler et al., 1996). Further evidence that fish had been exposed to estrogenic endocrine disruptors was the induction of intersex, a condition in which oocytes are present in the testis (Jobling et al., 1998; Harshbarger et al., 2000; Van Aerle et al., 2001; Vigano et al., 2001). An extensive study done throughout the UK demonstrated that roach (Rutilus rutilus), a cyprinid, collected from eight rivers upstream and downstream of sewage treatment works, were found to have a high frequency of intersex (Jobling et al., 1998). Intersex in males was observed in a proportion ranging from 16 to 100% at downstream sites and between 12 and 44% at upstream sites. In comparison, the frequency of intersex at the reference sites ranged from 4 to 18%. The severity of intersex followed a gradient from only a few primary oocytes interspersed among normal testicular tissue to other individuals, in which more than 50% of the testis had become ovarian tissue. In these individuals, the sperm duct was absent and had been replaced by an ovarian cavity. Surprisingly, the degree of feminization of the testis varied between individuals even within a population of fish collected at the same site.

The levels of plasma VTG provided strong evidence that these populations of roach were in contact with estrogenic compounds, and that higher concentrations of VTG were present

downstream of the sewage effluent site as compared to levels found in fish upstream and from the reference site (Jobling et al., 1998). Intersex fish were found to have intermediate plasma levels of VTG as compared to the low levels observed in males and high levels present in females during vitellogenesis. The average gonadosomatic index (GSI) of the males was significantly decreased at the downstream sites as compared to fish from the reference site. Subsequent studies have shown that these observations were not limited to the roach and that other species of fish were also impacted by the sewage effluent.

A second study conducted in UK rivers on another cyprinid species, the gudgeon (Gobio gobio), indicated that fish had intersex at all sites studied, but that the frequency varied from site to site, with the highest levels occurring in fish from the Aire River (Van Aerle et al., 2001). Unlike in the roach, intersex in the gudgeon did not follow a gradient of testicular feminization, but rather the fish could be separated into two separate categories. The first group contained fish in which the testis contained only primary oocytes that were interspersed throughout the testis. In the second group, the intersex gonad was characterized by a high proportion of female tissue. Elevated plasma levels of VTG corroborated the impact of estrogenic substances present in these rivers (Van Aerle et al., 2001).

More recent studies on the intersex roach captured downstream of municipal effluents in the UK indicated that gamete maturation in both males and females was altered (Jobling et al., 2002a). When fish were examined in autumn, the mid-point of the reproductive cycle in the roach, spermatogenesis was delayed in intersex and in male fish captured in rivers receiving sewage effluent. In the most severely intersexed fish, the growth of the testes was inhibited and

fish had lower circulating levels of sex steroids. When fish were examined in the spring, around the time of spermiation, only half of the males from the two rivers receiving sewage effluent were able to release sperm, as compared to 100% spermiation in fish from the reference site. Furthermore, the intersex fish which did spermiate had a reduced milt volume and a reduced sperm density. The lack of ability to spermiate could, in some males, be explained by observations of abnormalities in the sperm duct, which could prevent the release of gametes. They reported no relationship between the proportion of motile sperm and the degree of feminization, although curvilinear velocity of the spermatozoa was reduced with the degree of feminization. Furthermore, lower reproductive success rates (68%) were achieved when sperm from intersex fish were used to fertilize the eggs from effluent-exposed females, as compared to 93% reproductive success in reference fish. Female roach at this time had already begun to spawn and so these observations indicate that the males and females had become asynchronous with respect to gamete maturation, suggesting reduced reproductive success among the roach population from these rivers (Jobling et al., 2002a).

The effects in UK rivers are not limited to freshwater species. Allen et al. (1999) reported an induction of VTG in flounder (*Platichthys flesus*) which displayed ovo-testis. While the frequency of intersex was low (7-9 %) relative to what had been reported in freshwater species, VTG levels in certain male flounders from two estuaries, the Tees and Mersey, were higher than levels measured in vitellogenic females. Extensive studies, such as those done in UK, indicate that the estrogenic contamination resulting from sewage effluent is widespread and can affect both freshwater and marine environments.

2.4 EFFECTS ON FISH SPERMATOGENESIS

Studies by Jobling et al. (1996) have shown that the effects of these chemicals on spermatogenesis are both dose- and time dependent. In the eelpout, Christiansen et al. (1998) reported that injections of high doses of nonylphenol and E2 for 25 days results in a decrease in gonadosomatic index and caused degeneration of the seminiferous lobules of the testis. Similarly, Gronen et al. (1999) reported that octylphenol exposure for 21 days resulted in an inhibition of spermatogenesis in Japanese medaka. More recently Le Gac et al. (2001) reported that nonylphenol diethoxylate and the fungicide prochloraz could partially inhibit spermatogenesis after 3 weeks of treatment.

Other estrogenic chemicals such as bisphenol A have also been reported to inhibit, or delay, spermatogenesis in fathead minnows exposed for either 71 or 164 days (Sohoni et al., 2001). In adult guppies exposed to high doses of bisphenol A, testes contained fewer spermatogenic cysts (500 μg/l) than controls while at the highest dose (5000 μg/l) there were no spermatogenic cysts (Kinnberg and Toft, 2003). Similar effects were observed with octylphenol, and with the anti-androgens p,p'-DDE and flutamide. These results suggest an inhibition of mitosis by the spermatogonia, as well as a blockage of the transformation of spermatogonia into spermatocytes.

Schultz et al. (2003) exposed rainbow trout to different doses of EE2 for 62 days leading up to spermiation. At EE2 concentrations of 10 and 100 ng/l, there was an increase in sperm density. Testis weight was significantly reduced in the 100 ng/l group. Milt from fish exposed to 10 and 100 ng/L EE2 resulted in a 50% reduction in the number of fertilized eggs reaching the

eyed stage of embryonic development. Plasma levels of 17α,20-β-dihydroxy-4-pregnen-3-one (17,20-βP) in exposed fish were roughly twice the level of the controls, while levels of 11-ketotestosterone were significantly reduced in fish exposed to 100 ng/l EE2.

2.5 EFFECTS ON FERTILIZATION

The end product of spermatogenesis is the development of quality spermatozoa. Several studies have reported the effects of EDC's on gamete quality. In zebrafish, exposure of males and females to concentrations ranging from 5-25 ng/l EE2 resulted in a 70% decrease in the rate of fertilization (Van den Belt et al., 2001). In addition, the number of non-exposed females, which spawned successfully when paired with exposed males, was below the expected breeding success of non-exposed pairs. Therefore, both the fertilization success and sexual behaviour of the exposed male zebrafish seemed to be impaired. At exposure levels of 10 and 25 ng/l EE2, significant reductions in the GSI, signalling altered spermatogenesis, were observed. Interestingly, the effects observed on the females were much more dramatic than those observed on the male. This suggests that for certain species, observations of altered testicular growth and development may be indicative of reduced fertility. A lower fertilization rate was reported when male medaka exposed to octylphenol (2-50 μg/l) or E2 (100 ng/l) were paired with unexposed females (Knorr and Braunbeck, 2002).

Male medaka exposed to either E2, bisphenol A, nonylphenol or DEHP for two weeks and paired with unexposed females resulted in chemical-induced alterations in fertilization rates. In fish exposed to E2, while there was no direct effect on fertilisation, at doses of 10-100 nmol/l

there was a significant decrease in the number of hatchlings as compared to controls. Similar effects were observed with bisphenol A, but only at the highest dose of 10 μmol/l. Nonylphenol exposure caused variable effects at all doses and no clear effects could be established; while DEHP did not cause any of these effects (Shioda and Wakabayashi, 2000). In a similar study in which male medaka were exposed to between 20-280 μg/l octylphenol, unexposed females paired with these males produced approximately 50 % fewer eggs than the control group (Gronen et al., 1999). Exposing both male and female rainbow trout to nonylphenol (10 μg/l) for 10 days in each of 4 consecutive months prior to spawning resulted in reduced hatching rate of the F1 offspring due to high mortality of eggs before the eyed stage (Schwaiger et al., 2002).

These studies indicate that exposure of fish to endocrine disrupting compunds cannot only affect spermatognesis, but can also affect subsequent fertilization capability.

2.6 CONSEQUENCES OF EATING FISH EXPOSED TO EDC'S

2.6.1 ABNORMALITIES IN HUMAN REPRODUCTIVE HEALTH

Conclusions from a meta-analysis have shown a decrease in sperm quality of 40% worldwide since the 1940s (Carlsen et al., 1992). Olsen et al., 1995 has questioned these results, but reanalysis by Swan et al., 1997 confirmed the originally described time trend and pointed out that, at least, regional differences exist in sperm quality and/or concentrations. This has been substantiated by mono- and multilaboratory studies in e.g. Belgium (Van Waeleghem et al., 1996), Finland (Pajarinen et al., 1997), France (Auger, and Jouannet, 1997) and Denmark

(Andersen et al., 2000). The regional differences are suggestive of the involvement of environmental factors, which is corroborated by a number of exposure studies. Bibbo et al., 1978 recorded a decrease of ejaculate volume, of sperm concentration and of the percentage of normal motile spermatozoa in sons of women, who were treated with diethylstilbestrol during pregnancy. A recent study found an inverse correlation between the concentration of PCB metabolites in blood and seminal plasma and sperm motility as well as concentration (Dallinga et al., 2002). Guo et al., 2000 concluded that heavy exposure to PCBs resulted in negative effects on sperm morphology and motility, but not on sperm concentration. It became obvious from these studies that, in a number of cases, negative effects could be linked to exposure to environmental contaminants (beside genetic predisposition), when the latter occurred during a well-defined sensitive life stage, the so-called "critical window of exposure" (Andersen et al., 2000).

There are other reports in the literature which also suggest a possible decline in human semen quality during the past 50-60 years (Nelson and Bunge, 1974; Bostofte et al., 1983). Reports were from many different countries, for example the Danish investigation by Carlsen et al. (1992) and the study from the USA by MacLeod & Wang (1979). In a French study a 2.1% decrease in sperm concentration per year was found (Auger et al., 1995) and similar results were obtained in a Scottish study (Irvine, 1994). Deterioration of sperm count and motility was also observed in Belgium (Van Waeleghem et al., 1994) and areas of London (Ginsburg et al., 1993). A study in Belgium showed that over 40% of candidate donors since 1990 exhibited subnormal sperm characteristics compared to only 5% of the group investigated before 1980 (Comhaire et

al., 1995). Data from Swan et al. (1997) have confirmed the existence of a significant decline in sperm density in Europe (3% per year), but also in the United States (1.5% per year).

Humans are exposed to environmental oestrogens in many ways. Diet, drinking water, air, skin and consumption of contaminated fish are the routes through which xeno-oestrogens (environmental chemicals known to possess oestrogenic activity) enter the body. The major groups of environmental chemicals are the organochlorine pesticides, polychlorinated biphenyls, dioxins, alkylphenol polyethoxylates, phyto-oestrogens, and other xeno-oestrogens. Alkylphenols are relatively persistent and bioaccumulate in the lipids of living organisms (Ahel et al., 1993). Alkylphenols have considerable industrial applications and they also appear as pollutants in the environment (Ahel et al., 1993). para-Nonylphenol (p-NP) is used in the preparation of lubricating oil additives, resins, plasticizers and surface active agents. It has also been found in polyvinyl chloride (PVC) used in the food processing and packaging industries and is reported to contaminate water flowing through PVC pipes. In river water it has been shown to be oestrogenic in fish, birds and mammals (White et al., 1994).

While it is not clear what factors or changes in lifestyle may be responsible for these changes, it has been suggested that exposure to certain environmental contaminants may be contributing to these effects (Sharpe et al.,1993). Among the different classes of reproductive toxicants present in the environment, those that act as endocrine-disrupting chemicals have been singled out as contributing to male reproductive dysfunction (Aitken et al.,2004; Brody et al.,2003; Sharpe et al.,2004).

There is growing concern that abnormalities in male reproductive health are becoming more frequent. This is evidenced by an increasing incidence of testicular cancer during the past decades, as well as an increase in occurrence of cryptorchidism and hypospadias (Carlsen et al., 1992; Toppari et al., 1996). The most fundamental change has been the decline in sperm counts and semen quality.

2.6.1.1 INCREASED INCIDENCE OF TESTICULAR CANCER

In Western countries, testicular cancer is the most common malignant tumour in young males. Testicular cancer arises from carcinoma in situ (CIS) cells, which should have their origin in fetal life, whereby subnormal androgen and/or an increased estrogen exposure are potentially important factors. The main risk factor for testicular cancer is cryptorchidism, followed by hypospadias (Sharpe, 2003). Ninety five per cent of the malignant tumours, arising in the testis, are classified as seminomatous or non-seminomatous, reflecting their origin in primordial germ cells (Bosl, and Motzer, 1997). During recent decades, there has been a significant increase in the prevalence of testicular cancer, albeit with clear racial and geographical differences (Dearnaley et al., 2001). The obvious regional differences in incidence and the association with birth cohorts suggest a possible involvement of environmental factors in the development of testicular cancer. Ohlson, and Hardell, 2000 reported, with some reservation regarding the study design, a significantly increased risk of seminoma among plastic workers exposed to polyvinyl chloride (PVC). The mycotoxin ochratoxin A naturally occurs as a contaminant of cereals, pigmeat, and other foods and is a known genotoxic carcinogen in animals. Schwartz, 2002 hypothesizes that ochratoxin A could be a cause for the development of testicular cancer.

2.6.1.2 INCREASE IN CRYPTORCHIDISM AND HYPOSPADIAS CASES

Cryptorchidism is a disorder whereby the testis fails to descend into its normal position in the scrotum. It is the most common congenital condition in babies (Akre et al., 1999). Prevalence values of cryptorchidism are difficult to compare due to differences in screening techniques (Toppari et al., 2001). Two English studies (one in the late 1950s and one in the 1980s), using the same diagnostic parameters, reported a prevalence of cryptorchidism of 1 and 5% respectively (Jensen et al., 1995). In exposure studies, the risk for cryptorchidism was higher in sons of women, working with pesticides (Weidner et al., 1998), while significantly higher concentrations of hexachlorbenzene and heptachlorepoxide were found in adipose tissues of boys with testicular maldescent, compared to those of a control group (Hosie et al., 2000).

Hypospadias is a displacement of the urethral meatus onto the underside of the shaft of the penis. As is the statistics of cryptorchidism, there are differences in the methods of analysis and in the definitions of this disorder (Toppari et al., 2001). Although these differences hamper cross-study comparisons, there are clear indications of a rise in incidence in a number of European countries, the United States and Japan (Dearnaley et al., 2001). In addition, Klip et al., (2002) reported an increased transgenerational risk of hypospadias in sons of women that were exposed in utero to diethylstilbestrol (DES). Since registry data for cryptorchidism and hypospadias cases are highly unreliable, due to different diagnostic approaches, there is a need for prospective studies to make trustworthy conclusions (Sharpe, 2003).

2.6.1.3 ALTERATION IN SEX RATIO

Under "normal" conditions, the ratio of newborn boys to girls is higher than one. Several studies reported a small but significant decrease in this sex ratio in Canada and the United States (Davis et al., 1998), the Netherlands, Denmark and several other European countries (Martuzzi et al., 2001). The most important explanatory variables are probably related to the highly changed socio-economic situation in the Western countries since World War II. Certain studies describe a negative influence of environmental pollution on the percentage of newborn boys. In Turkey, mothers exposed to high concentrations of hexachlorbenzene, beared a lower proportion of boys during their fertile period (Jarrell et al., 2002). Del Rio-Gomez et al., (2002) noticed a remarkable decrease in the number of sons of fathers who were exposed to PCBs before (but not after) the age of 19 during the Yu-Cheng disaster. Dioxin exposure during the Seveso accident resulted in a dose-dependent decrease in the sex ratio of the offspring of males that were younger then 19 years of age at the moment of exposure (Mocarelli et al., 2000).

2.6.1.4 TESTICULAR DYSGENESIS SYNDROME (TDS)

In 1993, Sharpe and Skakkebaek suggested fetal exposure to environmental estrogens to be the common aetiological factor for the observed rise in incidence of testicular cancer, cryptorchidism and the downward trend in sperm quality (Sharpe, and Skakkebaek, 1993). Several scientists have analysed the mutual relationship between different testicular disorders. Forman, and Moller, (1994) identified cryptorchidism as a risk factor for the development of testicular cancer. Petersen et al., (1998) concluded that clinical data suggested a common

aetiology for testicular cancer, male infertility and cryptorchidism, with a possible role of hormonal factors. Møller et al., (1998) studied a similar population and recorded a possible link between the increase in testicular cancer, the decreased male fertility and the altered sex ratio over time. In spite of the fact that several epidemiological studies have identified a simultaneous evolution of the above-described defects and that some of these could be correlated with exposure to environmental contaminants, the detailed mechanisms through which these agents sort their effects remain largely unknown. To further investigate this, it seems important to no longer focus on exclusively fertility disorders, but to integrate all aspects of the so-called testicular dysgenesis syndrome (TDS), incorporating sperm quality, hypospadias, cryptorchidism and testicular cancer (Skakkebaek et al., 2001). In addition, one should be aware that the relationships between exposure and disorder only appears after a sufficient interval of latency time following exposure, and after inclusion of only those persons who have been exposed in a sensitive developmental life stage (Skakkebaek et al., 2001).

2.6.2 ABNORMALITIES IN WILD LIFE REPRODUCTIVE HEALTH

There is evidence that prenatal or early postnatal exposure to endocrine-disrupting actions of various environmental contaminants, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), can adversely affect wildlife populations (de Solla et al., 1998; Giesy et al., 1994; Guillette et al., 1994, 1996). Studies have shown that bald eagles nesting along the shores of the Great Lakes and feeding primarily on contaminated Great Lakes fish have lower reproductive success (Bowerman et al., 1995; Colborn 1991). The most direct evidence for adverse human health effects from environmental pollution is found in a series of

studies linking PCB exposure to consumption of contaminated fish (Fein et al., 1984; Jacobson et al., 1984; Jacobson and Jacobson 1988). These studies were able to associate the maternal consumption of contaminated fish with adverse health effects in their children who were exposed to the PCBs via lactation.

2.7 BIOACCUMULATION OF CHEMICALS

A study was conducted in 1999 to determine the occurrence of alkylphenol polyethoxylates in the St. Lawrence River. This study analysed the levels of 4-tert-octylphenol (4-t-OP), 4-n-nonylphenol (4-n-NP), nonylphenol polyethoxylates (NP(1-16)EO), nonylphenol-mono and di-ethoxycarboxylic acids (NP(1)EC and NP(2)EC), and octylphenol-mono and di-ethoxycarboxylic acids (OP(1)EC and OP(2)EC). High levels of nonylphenol were detected downstream of the sewage effluent (Sabik et al., 2003). Mussels (Elliptio complanata) taken from a reference lake were placed in cages and submerged for 62 days at two sites in the St. Lawrence River, 1.5 km upstream and 5 km downstream of the outfall of a municipal wastewater treatment plant. The results showed that many of the target chemicals were present in all matrices studied: in water, at ppt and ppb levels, and reaching ppm levels in sediments and mussels. Concentrations of these contaminants were higher in matrices sampled at the downstream site than in those drawn at the site upstream of the Montreal effluent outfall, especially in sediments. Likewise, the slight, but not significant, bioconcentration of certain alkylphenol polyethoxylates (AP(n)EO) in the mussels was more noticeable at the downstream site than at the upstream site. (Sabik et al., 2003). If xenoestrogens can bioaccumulate in aquatic organisms, this brings forth the question whether they can be passed up the food chain.

2.8 CAN THE XENOESTROGEN EFFECTS BE PASSED UP THE FOOD CHAIN?

Kosatsky et al. (1999a) reported that in the Montreal area, sport fishers eat their catch as often as three times weekly and can consume in excess of 18 kg of St. Lawrence River–caught fish annually. Heavy consumers of these fish have higher hair mercury levels and higher circulating levels of PCBs and dichlorodiphenyldichloroethylene (DDE) than do infrequent consumers. Studies also suggest that Montrealers of Asian origin consume more fish from the St. Lawrence River than other sport fishers do and, in turn, that they have higher levels of contaminants than median levels found in other sport fishers (Kosatsky et al., 1999b). The fact that fish in the St. Lawrence River are exposed to estrogenic compounds suggests that people, as well as fish-eating mammals consuming these fish, may be exposed to the same endocrine-disrupting compounds, assuming that these chemicals can bioaccumulate.

Exposure to environmental contaminants during critical periods of development may represent a far greater risk to animals and humans than exposure as adults. Endocrine-disrupting chemicals are particularly problematic to developing animals, because the timing of endocrine-mediated events may be deregulated, resulting in permanent physiological defects to the immune, nervous, or reproductive system (Arukwe 2001; Jobling and Tyler, 2003; Rothcell and Ostrander, 2003). The reproductive systems of mammals undergo substantial development both in utero and after birth, prior to puberty. In rats, the male reproductive tract undergoes substantial development in the first three weeks of life. During this period, cells of the testis and epididymis differentiate into cells resembling those of the adult (Pelletier, 2001; Rodriguez et al., 2002). The

blood–testis and blood–epididymal barriers are formed, and the first wave of spermatogenesis is initiated (Cyr, 2001; Cyr et al., 2002; Pelletier, 2001). Thus the period of lactation, when the mother can pass along contaminants to her offspring, represents a critical period of reproductive development for the male pups. As such, this critical period is among the most vulnerable to xenobiotics, including endocrine disruptors.

Organochlorinated compounds are known to pass up the food chain and become biomagnified in top predators, yet we know relatively little about whether endocrine-disrupting compounds can be passed up the food chain and cause endocrine disruption to higher vertebrates. This possibility is particularly relevant, given the increasing number of aquatic ecosystems in which endocrine-disrupting chemicals reportedly affect aquatic organisms. The transfer of these chemicals and their effects to fish-eating predators and humans must therefore be established, in order to evaluate the potential risk of endocrine disruptors in aquatic ecosystems to riverine mammalian species.

Also our personal observations indicate, an active sport fishery in the St. Lawrence River, and other investigators have reported that these fishers often eat their catch and some may consume sizeable quantities of fish (Kosatsky et al., 1999a). We can therefore infer that humans are exposed to contaminants present in fish from the St. Lawrence River and that the chemicals responsible for inducing an estrogenic response in the fish are transferred to humans. Furthermore, other predators such as fish-eating mammals and birds are likely targets for these contaminants, although, at the moment, there is no information regarding possible endocrine-disrupting effects on these species in the St. Lawrence River. Therefore it is important to

determine whether the maternal consumption of fish from the xenoestrogen-contaminated environment in the St. Lawrence river along the island of Montreal results in adverse reproductive consequences to weaning male pups, and if so, whether such effects become apparent only when the pup reaches adulthood. This information will assist us in developing a better understanding of the exposure and risks associated with eating fish from environments contaminated with estrogenic compounds.

2.9 MECHANISMS OF ACTION

2.9.1 ESTROGEN RECEPTOR MEDIATED ACTION

The notion that some endocrine-disrupting substances and the so-called xenoestrogens mediate their estrogenic effects via the estrogen receptor has received considerable support, in studies of both fish and other vertebrate species. It is well-known that estrogens and estrogenic-like substances bind to ERs, which in turn activate transcription by binding to estrogen response elements (EREs). It is generally believed that xenoestrogens produce their adverse effects by acting on the estrogen receptor. Estrogens exert pleiotropic effects in wildlife and humans. They regulate development and growth by inducing cell proliferation and differentiation. Observations of developmental abnormalities in wildlife exposed to chemicals and rising incidences of hormone-dependent cancers in humans have raised concerns in the public and the scientific community (Colborn 1991). These concerns were supported by experimental data on chemicals like diethylstilbestrol (DES) (Newbold and McLachlan., 1996) that confirmed the potential of

xenobiotics to impair the endocrine system, especially estrogen function and to cause developmental abnormalities and cancer.

The estrogenic or antiestrogenic activity of any chemical is due to the compounds capability of interacting with the estrogen receptor (ER), and the ER plays a pivotal role in development and neoplasia as a ligand-inducible transcription factor that regulates genes that are involved in cell proliferation and differentiation. Since the ER is an important transcription factor in cell proliferation and differentiation, any disruption of the ER signaling pathways may contribute to infertility, developmental abnormalities, or endocrine cancer seen in wildlife and humans. Accordingly, observed adverse health effects might be linked to the exposure of chemicals with estrogenic or antiestrogenic activities (Colborn 1991). Regulatory agencies and the scientific community have therefore put a lot of effort into identifying the estrogenic potential of synthetic and natural compounds (Colborn 1991).

The best documented endocrine disruptor is diethylstilbestrol (DES), a synthetic estrogenic hormone, which was administered until the 1970s in order to prevent miscarriage and to suppress lactation. The intake of DES during pregnancy has resulted in an increased incidence of malformations of the testes, the development of epididymal cysts and an impaired sperm quality in the male offspring (Klip et al., 2002). More recently, the widespread use of powerful hormone receptor agonists, such as 17- α -ethinylestradiol (EE2) in oral contraceptives, could be a cause for concern. Indeed, free EE2 is released in surface waters through the metabolisation of EE2-glucuronides by bacteria in sewage water treatment plants. In contrast to the natural 17-ß-estradiol, which is inactivated within 24 h, the reactivated 17-α-ethinylestradiol is slowly broken

down in the environment, and thus maintains its estrogenic activity for several weeks. In Germany, Kuch and Ballschmiter, 2001 investigated the presence of several estrogenic active compounds, including steroid hormones such as estrone, 17-ß-estradiol and 17- α - ethinylestradiol, in surface and drinking water. In all river water samples, they found bisphenol A (500 pg/l - 16 ng/l), 4-nonylphenol (6-135 ng/l) and steroids (200 pg/l - 5 ng/l). In drinking water, bisphenol A (300 pg/l- 2 ng/l), 4-nonylphenol (2–15 ng/l), 4-tert-octylphenol (150 pg/l-5 ng/l) and steroids (100 pg/l - 2 ng/l) were present. They concluded that environmental estrogens are not completely eliminated during sewage treatment and can enter the aquatic environment. They also could not exclude the possibility that these compounds can be eventually found in drinking water. Nevertheless, the biggest problem of the presence of estrogens in surface water, is their effects on fish populations, namely intersexuality, for example female sexual characteristics in the reproductive tract of male fishes (Matthiessen et al., 2002) and masculinization of female molluscs exposed to tributyltin (TBT) (Sumpter, 1998).

HPTE (2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane), a metabolite of the proestrogenic pesticide methoxychlor (MTX), displays an estrogenic activity (in vitro) that is up to 100 fold stronger than that of its precursor. However, in vivo, MTX acts an estrogen agonist in the uterus and as an antagonist in the ovary (Gaido et al., 1999). Alkylphenols, for example p-nonylphenol, which are used as antioxidants and plasticizers in the production of plastics can bind to the estrogen receptor and can stimulate growth of estrogenic sensitive breast cancer cells (Soto et al., 1991). Similarly, estrogenic effects have been described for a number of phthalates, generally used as plasticizers, and for a number of polychlorinated biphenyls (PCBs) as well as their hydroxylated metabolites (Layton et al., 2002).

2.9.2 OTHER RECEPTOR INTERACTION MECHANISMS BY ENDOCRINE DISRUPTORS

Several endocrine disruptors, such as dioxins and PCBs, mediate some of their effects through binding to the arylhydrocarbon receptor (AhR), a ligand-activated transcription factor (Fischer, 2000). Dioxins are predominantly formed during incomplete burning processes and are released into the environment from exhaust pipes of household and industrial waste incinerators and cars. In addition, an important source of dioxins seems to be domestic incinerations (Fischer, 2000). Several studies suggest a correlation between exposure to dioxins and the increased risk of endometriosis. Results of a study by Rier et al., (1993), whereby rhesus monkeys were exposed for four years to 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD), showed a highly significant, dose dependent correlation between exposure and the grade of endometriosis. Kuchenhoff et al., 1999 reported that the arylhydrocarbon receptor is expressed in the endometrium.

PCBs containing two para and at least two meta chlorine atoms, also referred to as the non-ortho or planar PCBs, resemble 2,3,7,8 TCDD in their affinity for the Ah receptor. The hydroxylated metabolites of PCBs in the body do not only exhibit estrogenic properties, but are also neurotoxic and interfere with the thyroid system, especially with transthyretin, a protein that is responsible for the transport of thyroxine in the blood (Meerts et al., 2002). Thyroid hormones are essential for brain development because they regulate the proliferation and differentiation of neuronal cells in the fetal brain (Meerts et al., 2002). Certain phthalates are suggested to display

anti-androgenic properties in vivo. This seems not to be caused by an interaction with the androgen receptor, but rather to be related to their effect on the peroxisome proliferator activated receptor a (PPAR-a) (Gazouli et al., 2002).

2.9.3 CHEMICALS INTERACTING WITH HORMONE SYNTHEIS AND METABOLISM

Several chemical compounds do not only exert (anti-)estrogenic or (anti-)androgenic properties through their interaction at the receptor level, but they can also interfere with enzymes involved in the steroidogenesis or metabolism of hormones. Particular attention has been given to the enzyme aromatase that catalyses the conversion of androgens to estrogens (testosterone to 17-ß-estradiol and androstenedione to estrone). For example, the phytoestrogen enterolactone (a lignan) has a low binding capacity to the estrogen receptor α, but exerts a mainly anti-estrogenic effect through the inhibition of aromatase (Makela et al., 2000).

17-ß-estradiol is rapidly converted in the liver to estrone and subsequently metabolised into 2-, 4- and/or 16-a-hydroxyestrone. 2-Hydroxyestrone (2-OHE) exerts anti-estrogenic effects, whereas 16a-hydroxyestrone (16a-OHE1) is a potent estrogen that exhibits genotoxic characteristics. It is suggested that a high urinary 16a-OHE1-to-2-OHE1 ratio is a biomarker of increased mammary cancer risk. The phytoestrogens daidzein and genistein reduce the urinary 16a-OHE1-to-2-OHE1 ratio without increasing the cytochrome P-450 content of hepatic microsomes or liver weight. This suggests that the soy isoflavonoids genistein and daidzein may display a cancer-preventive effect by shifting metabolism away from the production of genotoxic

metabolites toward the production of inactive metabolites (Kishida et al., 2000). In contrast, sulphotransferases, which are involved in the inactivation of estrogens, can also be inhibited by certain isoflavones and by hydroxylated PCBs (Kirk et al., 2001), thus possibly enhancing the estrogenic effects of endogenous estrogens at the cellular level.

These data suggest that different chemicals exert their effects by different mechanisms and further detailed studies are necessary to elucidate their mechanisms.

2.10 SPERMATOGENESIS AND GAP JUNCTIONS

There has been speculation that certain phenolic plasticizing agents, such as p-nonylphenol, which are now prevalent in the environment, may affect Sertoli cell development and function, because males have much lower levels of estradiol than females (Toppari et al.,1996; Soto et al.,1991). Studies have reported that severe testicular abnormalities, including poor germ cell differentiation and reduced sperm counts, are observed following gestational, lactational, or direct exposure of male rats to moderate levels of several alkylphenols (Sharpe et al.,1995; de Jager et al.,1999). Similar adverse effects on testicular structure and function have been observed following exposure to artificial estrogens, such as diethylstilbestrol or ethinylestradiol in rodents (Atanassova et al.,1999). It has been suggested that the toxic effects of alkylphenols are mediated via estrogen receptors (Laws et al.,2000). Of interest, alkylphenols have also been shown to induce apoptosis in a wide variety of cells (Roy et al.,1997), including rat primary germ and Sertoli cell cultures, while 17ß-estradiol was without effect (Raychoudhury et al.,1999). Another report has shown that hCG-stimulated steroidogenesis in cultured mouse Leydig tumor cells was inhibited by octylphenol in an estrogen receptor-independent manner

(Nikula et al.,1999), suggesting that the biological effects of alkylphenols may not be mediated entirely through direct interaction with estrogen receptors.

Spermatogenesis requires direct intercellular communication between Sertoli cells, which is mediated by gap junctions (Roscoe et al.,2001). Gap junctions consist of plasma-membrane-spanning channels that permit the intercellular exchange of ions and low molecular weight molecules (Roscoe et al., 2001). Gap-junctional intercellular communication (GJIC) is therefore believed to be involved in cell growth and differentiation. An important role of gap junctions is to regulate cell growth and differentiation by controlling the passage of small molecules, including secondary messengers, between adjacent cells (Bruzzone et al.,1996). Gap junctions are composed of intercellular pores that allow the passage of small molecules between adjacent cells (<1 kDa). These pores are composed of hexomeric connexins from each cell, which are themselves formed by the oligomerization of connexins. Cx43 is present in many tissues, including the testis, and is localized between adjacent Sertoli cells, Sertoli cells and germ cells, and between Leydig cells (Risley et al.,1992; Tan et al.,1996; Batias et al.,2000; Perez-Armendariz et al.,2001). Several lines of evidence indicate that Cx43 is essential for normal testicular function (Roscoe et al.,2001, Batias et al.,2000, Juneja et al.,1999; Plum et al.,2000; Batias et al.,1999).

The mechanisms that regulate GJIC are not yet fully understood, although there is evidence that post-translational alterations of the connexins are involved (Roscoe et al., 2001). Connexin 43 (Cx43) is a widely expressed gap-junction protein found in many animal organs and many recent investigations into the relationships between Cx43 phosphorylation and events

in gap-junction assembly (channel gating) suggest that several protein kinases are capable of mediating both Cx43 phosphorylation and GJIC inhibition (Musil et al., 1990; Warn-Cramer et al., 1996).

The mitogen-activated protein (MAP) kinase belongs to an important family of protein kinases that act by phosphorylating specific amino acids on their target substrates. Previous investigations have shown that activation of p38 MAPK is an important element in the regulation of both GJIC and Cx43 (Warn-Cramer et al., 1996).

Therefore in the third part of my study, I was interested in determining whether nonylphenol could alter intercellular communication in Sertoli cells and to determine if this effect was mediated via an estrogenic pathway.

3.0 CONSEQUENCES OF XENOESTROGEN EXPOSURE ON MALE REPRODUCTIVE FUNCTION IN SPOTTAIL SHINERS (*NOTROPIS HUDSONIUS*)

3.1 ABSTRACT

There is limited information on the physiological consequences associated with exposure to xenoestrogens under field conditions. The objectives of this study were to determine the presence of estrogenic chemicals in the St. Lawrence River and their effects on male reproduction in the spottail shiner (*Notropis hudsonius*). Hepatic vitellogenin (VTG) mRNA levels in immature shiners indicate extensive estrogenic contamination spanning almost 50 km both upstream and downstream from the island of Montreal. Stages of spermatogenesis were assessed in fish captured at sites having varying levels of estrogenic contamination. In control fish, 95% had testis of either stage IV (50%) or stage V (45%) of spermatogenesis. At Île Dorval, where VTG mRNA levels are moderate, fish had testes of stage III (38%) and IV (45%) and only 15% of fish were at spermatogenic stage V. In contrast, at Îlet Vert and Île Beauregard, located in the sewage effluent plume from the City of Montreal and where hepatic VTG mRNA levels are high in fish, none of the fish were at stage V and 8% of fish at Îlet Vert were at stage II of development. Sperm concentration and various motility parameters were significantly lower in shiners from Îlet Vert as compared with those from Îles de la Paix (reference). Histological analyses of testes revealed that more than one-third of the fish captured at sites with the highest estrogenic contamination displayed intersex, a condition in which ovarian follicles were

developing within the testis. These data indicate that there is significant estrogenic contamination in the St. Lawrence River that is associated with impaired reproductive function in male fish.

3.2 INTRODUCTION

The potential adverse effects of xenoestrogens on the endocrine function of fish have been a growing concern. Feminization has been reported in male fish exposed to effluents from industrial and sewage treatment plants in the UK (Harries et al., 1997; Jobling et al., 1998, Jobling et al., 2002; Purdom et al., 1994). The threat posed by xenoestrogens to fish populations has been met with intensive efforts to develop biomarkers suitable for screening chemicals for estrogen-mimicking capacity (Routledge and Sumpter, 1996; Sumpter and Jobling, 1995; White et al., 1994). Estrogenic exposure of either male or immature fish usually can be determined by measuring the egg yolk protein, vitellogenin (VTG). VTG is normally secreted into the bloodstream by the liver of female fish during ovarian follicular development and incorporated into oocytes as yolk. The expression of the VTG gene is under estrogenic regulation and is not sex-linked; therefore male and immature fish, which do not normally express VTG, can be stimulated to produce VTG via injections of estradiol or exposure to chemicals that can act via the estradiol receptor (Arukwe et al., 2000; Sumpter and Jobling, 1995). A number of anthropogenic compounds have been identified as possessing estrogen-mimicking activity. These include industrial chemicals (e.g., alkylphenols, bisphenol-A, phtalates, polychlorinated biphenyls), and pesticides (e.g., DDT, methoxychlor, chlordecone, lindane, dieldrin, toxaphene, endosulfan; Soto et al., 1995; Yamamoto et al., 1996). While several studies have identified the presence of estrogenic substances in aquatic ecosystems, there is limited information on the

physiological and population consequences associated with exposure to low levels of estrogenic compounds under field conditions.

Several studies have reported abnormalities in the male reproductive system of fish exposed to xenoestrogens (Harries et al., 1997; Hassanin et al., 2002; Jobling et al., 1996; Lye et al., 1997). These include effects on sperm counts, spermatogenesis, and atrophy of germ cells (Christiansen et al., 1998; Gimeno et al., 1998; Haubruge et al., 2000; Zaroogian et al., 2001). A three-month exposure to a sublethal dose of 4-tert-pentylphenol or 17ß-estradiol causes progressive disappearance of spermatozoa and spermatogenic cysts, and reduces the seminiferous tubule diameter in mature male carp (Gimeno et al., 1998). Furthermore, it has been reported that that sperm motility may be decreased in male fish exposed to xenoestrogens, although other studies have failed to observe an effect of ethinyl estradiol exposure on sperm motility (Jobling et al., 2002; Schultz et al., 2003). In a study of a wide range of rivers throughout the British Isles, Jobling et al. (2002) demonstrated a high incidence of intersex, a condition in which female reproductive tissue is present in the testis, which was correlated with induced levels of VTG. Several studies have shown that intersex can be induced by exposing fish to estradiol (Koger et al., 2000; Krisfalusi and Nagler, 2000).

Municipal sewage effluent, industrial effluents, and agricultural runoff remain major sources of xenoestrogen pollution. The Montreal Urban Community (MUC) sewage treatment facility discharges all of its effluent at a single site near the eastern tip of the Island of Montreal, where it enters the St. Lawrence River. Thus, the sewage created by a population of approximately 1.8 million people enters the St. Lawrence at a single discharge point. Chemical

analyses of sediments around the Island of Montreal suggest that contaminant levels are generally low, with the exception of zinc in sewage effluent and alkyl phenols, nonylphenol ethoxylates and octylphenol that are present downstream from the Montreal Urban Community (MUC) discharge point (Bennie et al., 1998; Gagnon and Saulnier 2003). Sabik et al. (2003) reported the presence alkylphenol ethoxylates in the sediments of the St. Lawrence River at many sites around the Island of Montreal and found levels to be low at Île de la Paix, high at Île Dorval, and much higher at Îlet Vert and Île Beauregard (see Fig. 3.1). Ethinyl estradiol is also present in these effluents (MUC Treatment Plant, personal communication).

The objective of this study was to determine whether or not estrogenic substances were present in the St. Lawrence River in proximity of the Island of Montreal and to assess the effects of such contaminants on male reproductive function in the spottail shiner (*Notropis hudsonius*). This species was selected to examine the impact of sewage effluent on fish in the St. Lawrence because it was common at all the sites and could be readily studied for site-specific effects. Spottail shiners are minnows that usually live for approximately 5 years and mature by 1-2 years of age. In the St. Lawrence River, spawning occurs in June. It is an important foraging species for gamefish and is distributed widely throughout eastern North America (Jenkins and Burkhead, 1994).

3.3 MATERIALS AND METHODS

3.3.1 Study area

We established sampling sites upstream and downstream of the MUC municipal discharge area (Fig. 3.1). The downstream sites (Îlet Vert, Île Beauregard and Île St. Ours) and

the upstream sites (Ottawa River, Îles de la Paix, Île Dorval, Îles de Boucherville) receive varying concentrations of effluents from different sources. Îlet Vert is located 4 km downstream from the sewage outfall, Île Beauregard 10 km, and Île St. Ours approximately 35 km. Two other sites (Bout de l'île and Île au Bois Blanc) are located downstream of Montreal but outside the city's sewage effluents, and thus any effluents received are from other sources. With the exception of those fish captured in the Ottawa River, all fish were captured in the St Lawrence River near the city of Montreal (Quebec, Canada).

3.3.2 Animal sampling

Spottail shiners were captured in June (1999-2002) from each site using a beach seine. Fish were placed in aerated river water and transported to the laboratory. Fish were euthanized using a solution of 0.1% tricaine methansulfonate (MS222, Boreal, Ontario, Canada) and tissue samples were collected. All protocols used in this study were done according to the guidelines of the Canadian Council for Animal Care Committee.

3.3.3 Condition factor

Fork length and body weight were recorded for each fish. Condition factors were calculated using the equation: K = [body weight (g) x 100]/fork length3 (cm).

3.3.4 VTG mRNA levels

Spottail shiners were captured at Îles de la Paix, Île Dorval, Îles de Boucherville, Îlet Vert, and Île St. Ours in June 1999 and at all nine sites in June 2000. Total RNA was extracted from livers of immature spottail shiners using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Following RNA isolation, RT-PCR was performed to determine VTG mRNA levels. The primers used for RT-PCR of VTG were 5'-AGCATCCAGGCAGACAACG-3'(forward) and 3'-AAACTCGAGACACCACGTTAG-5' (reverse). Primers specific for the 28S rRNA, 5'-GTGCAGATCTTGGTGGTAAGTAGC-3' (forward) and 3'-AGAGCCAATCCTTATCCCGAAGTT-5' (reverse) were used as internal controls. A 2 μg aliquot of total RNA from immature shiners provided template for the reverse transcription reaction (RT). RT was performed in a 20 μl reaction volume with a final concentration of 1.5 mM 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 15 mM MgCl2), 3.5 mM MgCl2, 1 mM dNTP, 1 U RNase inhibitor, 20 U reverse transcriptase, 0.75 mM of the 3'- VTG primer, and 0.75 mM 3'-28S primer at room temperature for 10 min and then at 42°C for 60 min. PCR was performed in a 50 μl reaction volume with a final concentration of 1.5 mM 10X PCR buffer, 0.5 mM MgCl2, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase, 0.5 μM forward, and 0.5 μM reverse primer. Amplification was carried out with 28 cycles (linear amplification) at 94°C for 5 min, 55°C for 1 min 30 s, and 72°C for 2 min for VTG and 11 cycles (linear amplification) at 94°C for 5 min, 55°C for 1 min 30 s, and 72°C for 2 min for the 28S. Aliquots of 10 μl of the reaction products were then analyzed on a 1.2% agarose gel containing ethidium bromide. Gels were scanned using a Bio-Rad Fluor Image analyzer and quantified using the integrated area under the curve for each band.

The PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, Burlington, Ontario, Canada) and used to transfect Escherichia coli. Plasmids were amplified and purified from individual colonies using the Qiagen Plasmid Purification Kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The insert was digested from the plasmid with EcoR1 restriction enzyme (Amersham Biosciences, Baie d'Urfe, Quebec, Canada) and sequenced using an automated sequencer (Sheldon Biotechnology Centre, McGill University, Montreal, Quebec, Canada).

3.3.5 Spermatogenesis

Male spottail shiners were collected with a beach seine at different sites in June 2002 along the St. Lawrence River: Îles de la Paix, the reference site (n = 38) and at sites with xenoestrogen contamination: Île Dorval (n = 13), Îlet Vert (n = 13), and Île Beauregard (n = 11). Weight and fork length of the fish were recorded. Testes were removed, fixed in Bouin's solution, dehydrated through a graded series of ethanol, cleared with xylene, and embedded in paraffin. Sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin. The stage of spermatogenesis (I through V) for each fish was determined according to the classification scheme of Schulz (1994) for rainbow trout.

3.3.6 Sperm motility analysis

For sperm motility analysis, sexually mature male shiners (n = 10) were collected with a beach seine in June 2001, kept alive in aerated river water, and transported to the laboratory. All

the milt samples used were from adult spottail shiners of approximately the same size (40-50 g). Since spottail shiners do not readily release milt by manual stripping, the fish were killed and the testes were removed. Testicular sperm were then gently squeezed into a 1.5-ml plastic centrifuge tube. Care was taken to avoid contamination of milt with water, urine, or fecal matter.

A two-step sperm dilution procedure was adopted. An aliquot of fresh milt was immediately diluted 100-fold with buffer (30 mM Tris-HCl, 200 mM KCl). At this initial dilution, the spermatozoa remained immotile. Spermatozoa were then activated by 1:100 dilution in Tris- HCl (30 mM) alone. All fluid movement was stabilized within 5 s after activation, allowing precise measurement immediately after activation. Spermatozoa were motile for approximately 1 min. Sperm motility was analyzed using a Hamilton-Thorne-IVOS Semen Motility Analyzer.

Using computer assisted semen analyses (CASA) we were able to determine the sperm concentration, the number of motile spermatozoa in a sample, and various motility parameters for any single spermatozoa. Among those motility parameters measured were: Percentage motility—the percentage of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the analysis field; Path Velocity (VAP)—the average velocity of the smoothed cell path in microns/s); Progressive Velocity (VSL)—the average velocity measured in a straight line from the beginning to the end of the track; Curvilinear Velocity (VCL)—the sum of the incremental distances moved in each frame along the sampled path divided by the time taken for the sperm to cover the track; Beat Cross Frequency (BCF)—the frequency with which the sperm track crosses the sperm path; Straightness (STR)—the departure

of the cell path from a straight line; Linearity (LIN)—the departure of the cell track from a straight line.

3.3.7 Intersex

Histological preparation of spottail shiners testes was completed as described above for staging of the testes. Testes were analyzed for the presence of developing oocytes. Oocytes were identified according to staining, size of the cell, and the presence of multivesicular bodies in the cytoplasm.

3.3.8 Statistical analysis

The data were tested for normality using the Kolmogorov-Smirnov test while the Levine median test was done for equal variance. Statistical differences between sites were determined by ANOVA followed a posteriori by a Student-Newman-Keuls test for multiple comparisons between experimental groups. A Dunn's test was done if normality failed. Correlations between intersex and stages of spermatogenesis were determined using a Pearson Product Moment Correlation Tests. Significance for each of these tests was established at $p < 0.05$. All analyses were done using the SigmaStat computer software (Jandel Scientific Software, San Rafael, CA). Analyses of covariance for length, weight, and condition factor of the fish between sites were determined using the Statistica software (Statsoft, Tulsa, OK). All data are presented as mean ± SEM, unless otherwise indicated.

3.4 RESULTS

3.4.1 Size and Condition Factor

External examination of the fishes showed no overt abnormalities such as fin erosion, gross malformation of the spine, external lesions, or visible tumors. The length, weight, and condition factor were not significantly different among sites (data not shown). Analyses of covariance indicate that there were no differences between the three variables for each of the collection sites. The fact that there was no decrease in the condition factor suggests that the fish were not overtly affected by exposure to xenoestrogens (at least with respect to somatic growth) and were not moribund.

3.4.2 Hepatic VTG mRNA Levels

The amplified cDNA product for spottail shiner VTG was 556 bp long (GenBank access number AY365131). The VTG transcript was 95% homologous with rainbow trout (Oncorhynchus mykiss) VTG. RNA from a mature female rainbow trout was used as a positive control in RT-PCR reactions (Fig. 3.2). VTG mRNA levels were standardized using a 28S RNA internal standard which resulted in the synthesis of a 524 bp amplicon, which was then used to standardize mRNA loading. The linearity of the PCR amplifications was determined experimentally and the number of cycles used for both VTG and the 28S rRNA was done within the linear range of the assay (Fig. 3.3).

In 1999, VTG mRNA levels were significantly higher at Île Dorval, Boucherville, Îlet Vert, and Île Saint-Ours as compared with the reference site at Îles de la Paix (Fig. 3.4A). This led us to hypothesize that xenoestrogens may be originating from the Ottawa River. Therefore we expanded our sampling effort in 2000 to encompass new sites in the St. Lawrence River and a site at the mouth of the Ottawa River (Fig. 3.1). In the year 2000 sampling campaign, the highest concentrations of hepatic VTG mRNA were observed in fish from Îlet Vert, Île Beauregard, and Île Saint-Ours, all of which are downstream sites of the MUC wastewater discharge (Fig. 3.4B). VTG mRNA concentrations were also significantly higher in fishes captured upstream of the MUC wastewater discharge, including the Ottawa River, as compared with the reference site at Îles de la Paix.

3.4.3 Effects on Spermatogenesis

Testes of spottail shiners consist of two dorsal elongated structures. Each testis contains a sperm duct that runs lengthwise. Examination of histological sections of the testis by light microscopy indicates that the testis is surrounded by a continuous layer of connective tissue and is composed of cysts containing germ cells with interstitial spaces containing myoid cells present at all the stages of the reproductive cycle. Representative stages of development are shown in Figure 3.5.

Stages II through IV represent the process of spermatogenesis, during which the cysts increase in size and the spaces between the cysts become narrow. The number of spermatogonia gradually decreases in stage II to IV due to their development into spermatocytes (Fig. 3.5A-C).

Spermatocytes undergo meiosis to produce spermatids (stage III, Fig. 3.5B), and eventually spermatozoa (stage IV, Fig. 3.5C). Spermiation (stage V, Fig. 3.5D) is the release of sperm cells from the cysts into the tubules; the tubules are filled with spermatozoa and the interstitium becomes very narrow. Following stage V, the testes regress.

In order to determine if spermatogenesis was altered by exposure to estrogenic contaminants in the St. Lawrence River, stages of spermatogenesis were determined for spottail shiners captured at different sites along the St. Lawrence River (Fig. 3.6). Histological analyses of shiner testes from the reference site (Îles de la Paix) indicated that fish had testes of stage IV (50%) and stage V (45%) of spermatogenesis, indicating that spermatogenesis was either nearly completed or completed (only 5% of the fish were still at stage III of development). At Île Dorval, where VTG mRNA levels were moderate, fish had testes at stage III (38%) and stage IV (45%), and only 15% of testes were at stage V of development. In contrast, at Îlet Vert and Île Beauregard, where hepatic VTG mRNA levels were high, none of the testes were at stage V, and 8% of testes at Îlet Vert were still at stage II of development. The majority of fish testes from these sites were at stages III and IV of development (Fig. 3.6). These data indicate that spermatogenesis is markedly delayed at sites where shiners were exposed to xenoestrogens.

3.4.4 Effect of Xenoestrogen Exposure on Fish Spermatozoa Motility

Milt from male spottail shiners at Îlet Vert were compared with fish captured at Îles de la Paix. Motility parameters were generated by computer assisted semen analysis. The spermatozoa appeared bright against a dark background and their movement was clearly visible. Sperm

concentrations were significantly lower in shiners from Îlet Vert as compared with Îles de la Paix shiners (Fig. 3.7). Progressive motility was significantly reduced in sperm from fish captured at Îlet Vert (Fig. 3.8). Spermatozoa were classified according to their swimming velocity: static spermatozoa (<5 μm/s), slow spermatozoa (5-10 μm/s), medium motile spermatozoa (10-20 μm/s), and rapid spermatozoa (>20 μm/s). Results show that xenoestrogen exposure had a significant effect on certain sperm motility parameters. Analysis of data based on speed indicated a significant decrease in the percentage of rapid cells and a significant increase in the percentage of static cells in the milt of spottails from Îlet Vert (Fig. 3.9). Path velocity, progressive velocity, and curvilinear velocity were also lower in fish from Îlet Vert (Fig. 3.10). Beat cross frequency did not differ between shiners from the two sites. Average straightness and linearity was also significantly reduced in the milt from Îlet Vert (Fig. 3.11). These results indicate that in fish exposed to municipal effluent and which had induced VTG mRNA levels, sperm concentrations and most sperm motility parameters were significantly affected.

3.4.5 Intersex

The presence of intersex in male fish was determined for fish sampled at Îles de la Paix, Île Dorval, Îlet Vert, and Île Beauregard (Fig. 3.12). Intersex testes are comprised primarily of testicular tissue, but with one or more oocytes located randomly within the testicular tissue. At Îles de la Paix, our reference site, a single fish had an oocyte in its testis (2.6% of fish sampled; n = 38). At Île Dorval, 15% (n = 13) of male fish exhibited intersex, while 31% (n = 13) of fish at Îlet Vert and 27% (n = 11) of fish at Île Beauregard exhibited intersex (Fig. 3.12). There was a significant inverse correlation between the presence of intersex testes and stage V of

spermatogenesis. Together these data indicate that at sites where VTG mRNA were elevated, there was also an increase in the percentage of fish with intersex testes.

3.5 DISCUSSION

Experiments involving long-term exposure are required in order to fully understand the physiological consequences associated with chronic, life-long environmental exposure to xenoestrogens. Our results indicate that water from within the municipal effluent plume from the city of Montreal contains estrogenic compounds, which can be monitored using VTG mRNA levels in immature spottail shiners. Unexpectedly, the results indicate that fish sampled upstream from the Montreal sewage discharge point also had induced VTG mRNA levels, for example at Île Dorval. While this contamination may be due to other industries and sources, sewage overflow outlets are present along the Island of Montreal and these can release effluent into the St. Lawrence River upstream from the main discharge point when the system is saturated or following heavy rainfall. Therefore, we cannot rule out the possibility that sewage effluents along the Island of Montreal are responsible for the sizeable estrogenic contamination of the St. Lawrence River. Furthermore, the Ottawa River also appears to contribute xenoestrogens to the St. Lawrence River as indicated by significantly higher VTG mRNA levels in fish sampled at the mouth of the Ottawa River.

There is evidence that environmental estrogens can alter testicular function in fish. Our results indicated a marked delay in the stages of spermatogenesis in shiners from sites containing significant levels of xenoestrogens. In fact, at sites where VTG mRNA levels are high, none of

the spottail shiner testes were at stage V of spermatogenesis, compared with 45% of the testis in fish from our reference site. These findings are supported by a study on the effects of estrogen on spermatogenesis in male trout. Estrogen treatment via diet resulted in a marked decrease in spermatogenesis in rainbow trout (Billard et al., 1981) compared with fish exposed to natural estrogens. On the other hand, another study on the effects of alkylphenol on spermatogenesis in fully mature trout reported no effect on spermatogenesis (Jobling et al., 1996). This may be explained by the fact that the period of spermatogenesis, in which germ cells develop, is more sensitive to estrogenic treatment and may be associated with failure of Sertoli cells to develop (Billard et al., 1982). One of the differences between laboratory and field studies is that fish in the wild are likely exposed to estrogenic contaminants over the entire reproductive cycle, a situation which can be difficult to reproduce under laboratory conditions with seasonal spawners.

Estradiol receptors have been identified in fish testis. Wu et al. (2001) reported that the α- and ß-isoforms of the estradiol receptor are localized in secondary spermatocytes and spermatids of channel catfish. Furthermore, Bouma and Nagler (2001) reported the localization of the estrogen receptor- in precursors of Leydig cells in the testis of rainbow trout during the early stages of the reproductive cycle, and in fully differentiated Leydig cells. Whether or not the effects of xenoestrogens present in the St. Lawrence River on spermatogenesis in spottail shiners is the result of a direct effect on developing germ cells, inhibition of androgen synthesis or action via the hypothalamo-pituitary-gonadal axis (Christiansen et al., 1998) remains to be established.

While other environmental factors such as temperature are known to alter spermatogenesis, the water temperature in the present varied by less than 2°C between collection

sites and all fish were collected within 4 days of each other. Furthermore, other factors such as dissolved oxygen levels did not vary between sites in the St. Lawrence, a large river system whose water is well oxygenated. While in a field study such as this, it is impossible to limit all variables, the weight of evidence strongly suggests that the effects observed on spermatogenesis are the result of effects related to the presence of xenoestrogens.

The sperm motility data of the fish from sites exposed to xenoestrogens were compared with those obtained from a nonestrogenic reference site using CASA. This approach has been used to monitor the effects of heavy metals on sperm quality (Rurangwa et al., 1998), to improve the efficiency of cryopreservation and storage (McNiven et al., 1993; Rurangwa et al., 2001), and to optimize conditions for fertilization (Duplinsky, 1982; McMaster et al., 1992; Morisawa et al., 1983; Toth et al., 1995). Sperm concentrations in fish from Îlet Vert were significantly lower than those in fish from Îles de la Paix (reference site).

A recent study of roach (Rutilis rutilis) in the UK, indicated that sperm concentrations ranged from 6,000,000-8,000,000/μl (Jobling et al., 2002). While sperm concentrations or sperm density vary among species of fish, the higher sperm concentration observed in shiners is likely attributable to the different method of collection (manual stripping after administration of pituitary extract versus the removal of testis and extracting sperm). Differences in the time of sampling or age of the fish may also contribute to interspecies differences.

This decreased gamete concentration in shiners from Îlet Vert was well correlated with the high VTG mRNA levels, the high incidences of intersex fish, and the observed delay in

spermatogenesis. Studies have shown that exposure to estrogens or xenoestrogens which induce VTG in fish (Christiansen et al., 1998; Hill and Janz, 2003) have inhibitory effects on fish spermatogenesis (Hassanin et al., 2002; Jobling et al., 2002), sperm production (Haubruge et al., 2000; Jobling et al., 2002), and sperm motility (Jobling et al., 2002; McMaster et al., 1992).

In this study, shiners from Îlet Vert exhibited a lower percentage of progressively motile sperm when compared to our reference site. To further evaluate sperm motility, sperm were classified into four different classes according to speed (rapid, medium, slow, or static). Our observations suggest that different subpopulations of spermatozoa based on sperm velocity coexist within the milt. Distribution of sperm among the different categories of velocities varied between fish from Îles de la Paix and Îlet Vert. There was a significant increase in the number of static spermatozoa and a concomitant decrease in the number of rapid spermatozoa in shiners from Îlet Vert. The results from the present study demonstrate that sperm concentration and percentage of motile spermatozoa may not always tell the whole story, especially if effects cause abnormal sperm motion. Spottail shiner spermatozoa displayed a linear motility pattern, unlike trout spermatozoa, which exhibit distinct temporal phases of swimming starting with a circular path, lasting up to 5 s, followed by a linear path (up to 60 s; Boitano and Omoto, 1992). Velocity parameters (VAP, VSL, VCL) were decreased in spermatozoa of shiners from Îlet Vert. These parameters directly express sperm motion (swimming speed) and decreased low velocities may reduce the probability of the spermatozoa to reach the micropyle. Investigations have revealed that teleost spermatozoa must swim actively into the micropylar channel for successful fertilization (Hart, 1990; Iwamatsu et al., 1993).

Spermatozoal motility in teleost fish is activated when they come in contact with water and the motility lasts for only a few minutes. Although good fertilization rates were obtained from Atlantic salmon and rainbow trout spermatozoa that showed little or no motility (Erdahl and Graham, 1987; Levanduski and Cloud, 1988), these studies are confounded by the limitations of the traditional subjective scoring methodology in defining sperm motility and the low sensitivity of fertilization tests with excessive spermatozoa. A relationship between motility and the capacity for fertilization for teleost spermatozoa has been confirmed by several other authors (Billard and Cosson, 1992; Ohta et al., 1995). Though the efficiency of fertilization was not determined in this study, it stands to reason from the motility studies that spermatozoa in shiners from Îlet Vert will be at a disadvantage, considering the shorter distance of displacement due to its decreased velocity; thereby reducing the probability of spermatozoa meeting with the micropyle. Also given that the sperm activation conditions in the present study were maintained between fish, and sperm motility parameters were examined immediately after activation, the results from this study indicate that spermatozoa from fish captured at Îlet Vert have an initial "poor" capacity to move.

Histological examination of spottail shiner testes revealed that fish with induced VTG mRNA levels had a high incidence of intersex. While intersex male fish were found at all sites, the incidence of intersex ranged from 2.6% for the reference site to 31% at Îlet Vert where VTG mRNA levels were high (Fig. 3.12). This finding is similar to a recently published field study of intersex in the roach (Jobling et al., 2002). In these experiments, fish were sampled both upstream and downstream of sewage treatment works in the UK. They reported that intersex was also found at all sites and that the incidence of intersex ranged from 4% (at two reference sites)

and increased to as much as 100% in fish downstream of the sewage treatment (Jobling et al., 1998). The low proportion of intersexuality at the reference site seen our studies is comparable with the reported level of intersexuality in roach (Jobling et al., 1998) and carp of 5% (Billard et al., 1981). Interestingly, in the roach, gamete production, milt release, and sperm motility were all reduced in fish living in aquatic habitats receiving input from sewage effluent (Jobling et al., 2002). These results are similar to observations from the present study on spottail shiners in the St. Lawrence River. Further studies will be needed to determine potential effects on fecundity and development of the F-1 progeny, both of which were reported to be altered in the roach.

In conclusion, exposure of immature and male spottail shiners to xenoestrogens in the St. Lawrence River was widespread, as indicated by the induced levels of VTG mRNA. Furthermore, this exposure was shown to have marked effects on their reproductive function in males. Exposure to xenoestrogens was linked to delayed spermatogenesis, reduced spermatozoal production, decreased sperm motility, and high incidence of intersexuality. This is among the first studies which demonstrate that exposure of wild fish to xentoestrogens is correlated with a reduction in male reproductive function, and suggests that fish populations may be affected in the St. Lawrence River as a result of altered reproductive functions.

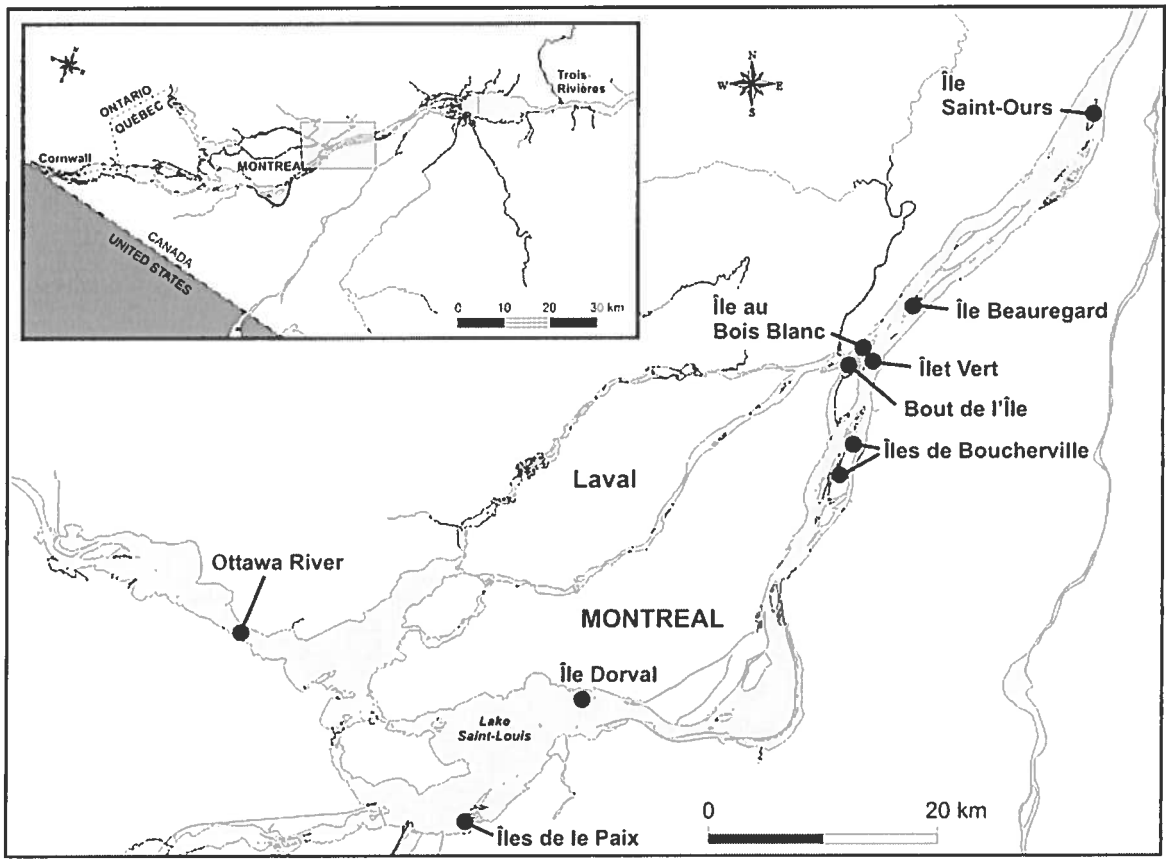

FIG. 3.1. Schematic map depicting the location of sampling sites along the St. Lawrence River in the vicinity of the island of Montreal.

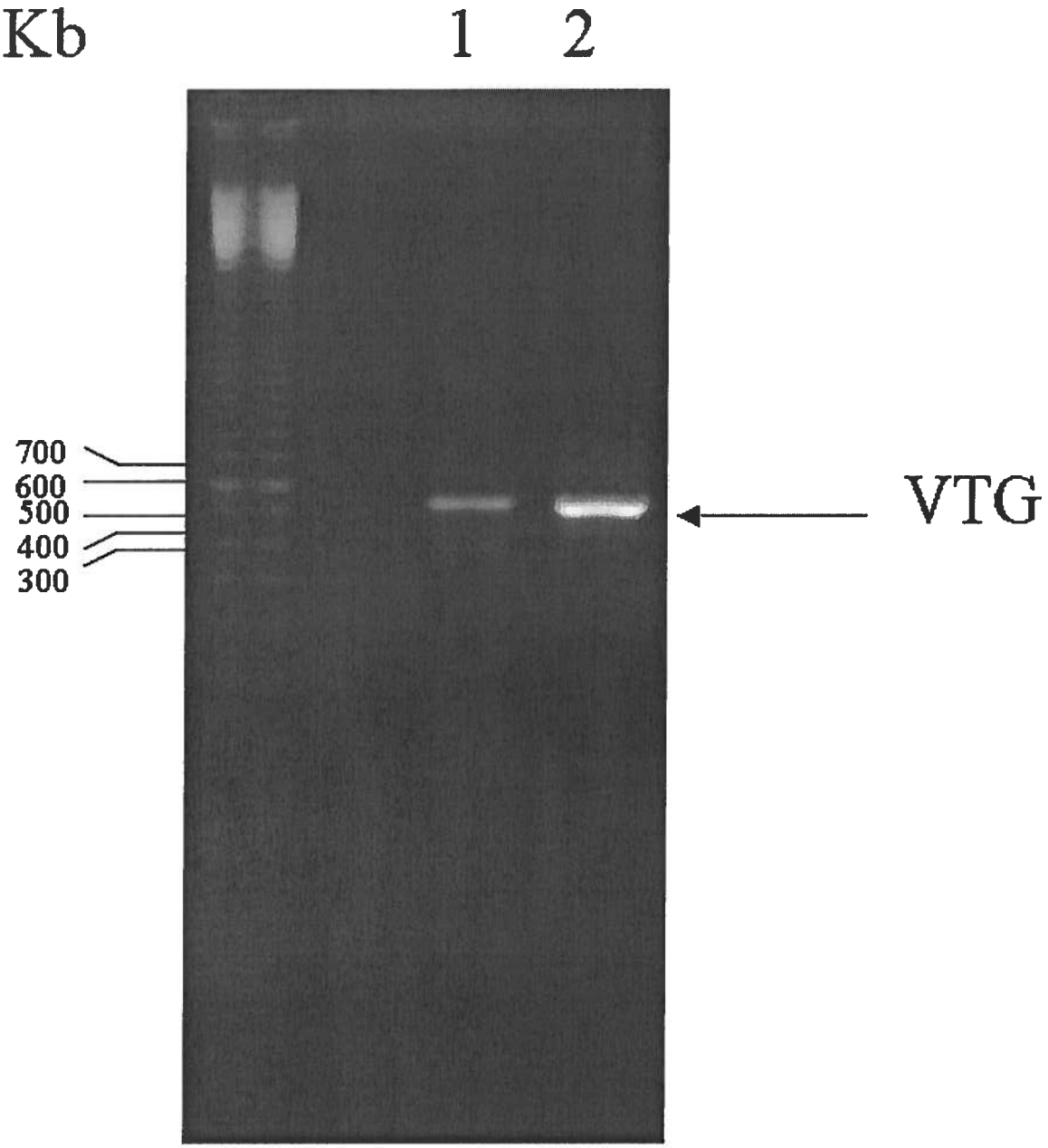

FIG. 3.2 RT-PCR of hepatic VTG in a mature vitellogenic female spottail shiner and rainbow trout (positive control). Lane 1 contains the molecular ladder; Lane 2 contains the RT-PCR product obtained for spottail shiner VTG cDNA; and Lane 3 contains amplified rainbow trout VTG cDNA. The DNA was visualized by staining the gel with ethidium bromide (0.1% w/v).

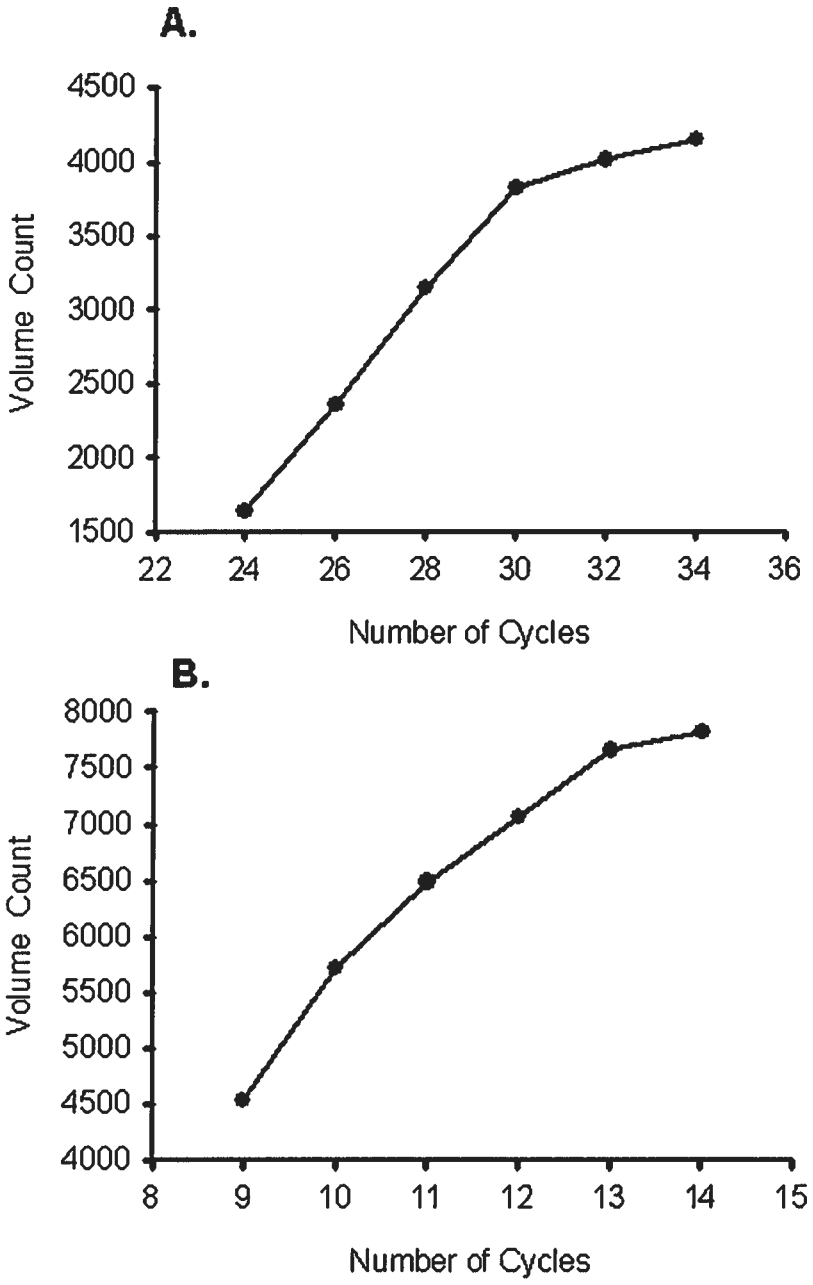

FIG. 3.3. Linear PCR amplification of spottail shiner VTG (A) and 28S rRNA (B). RNA was isolated from the liver of mature female spottail shiners and reverse transcribed as described in the Material and Methods. Using specific primers, VTG or the 28S rRNA were amplified over a range of PCR cycles. PCR products were electrophoresed on an agarose gel and stained with ethidium bromide. The volume of the PCR products were determined by measuring the optical density of the DNA bands using a Bio-Rad Fluor Image analyzer. VTG amplification was linear from 26 to 30 cycles while the 28S rRNA was linear from 10 to 13 cycles.

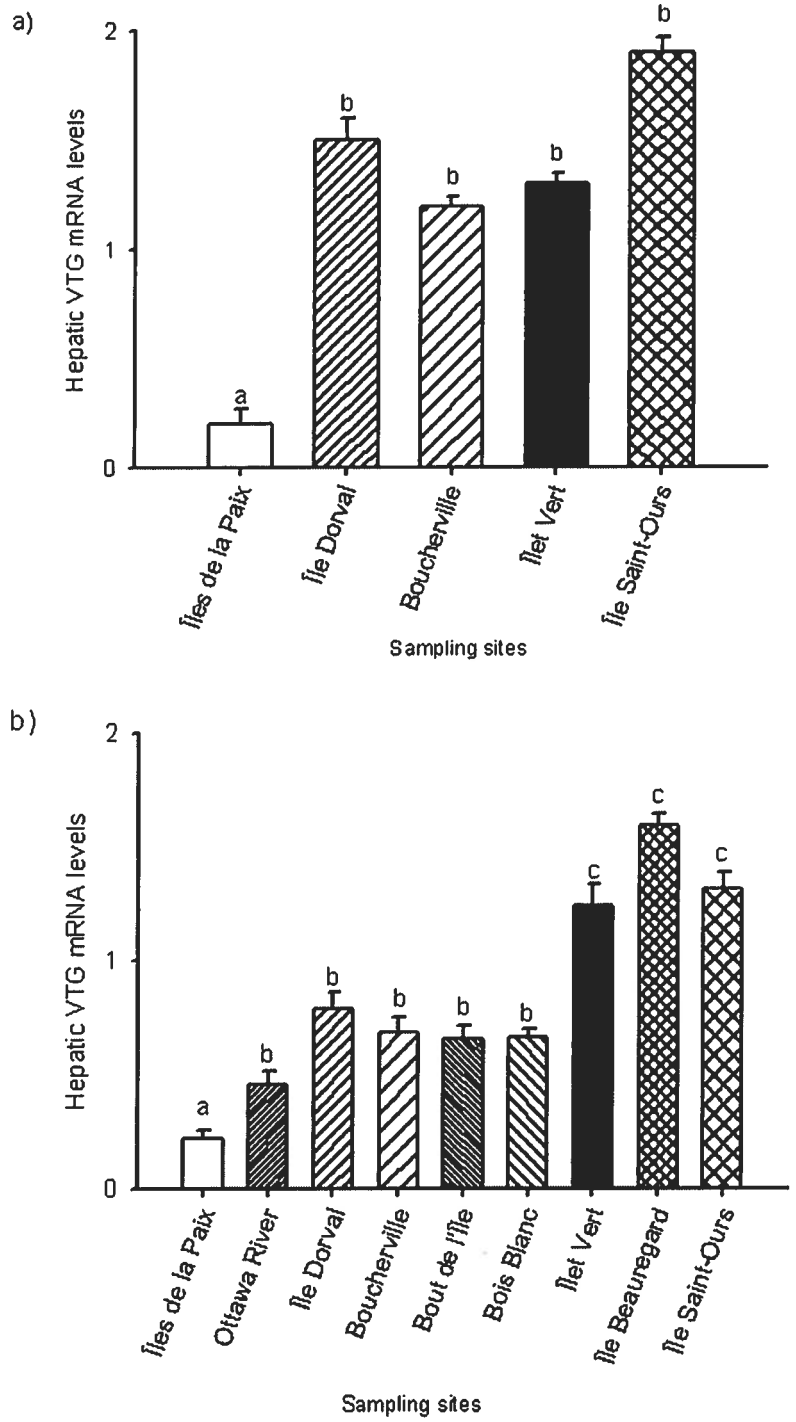

FIG. 3.4. Hepatic VTG mRNA levels in immature spottail shiners captured at different sites along the St. Lawrence River during June 1999 (a) and 2000 (b). Total cellular RNA was isolated from fish liver (n = 10) and subjected to RT-PCR with specific VTG primers. Data was standardized by amplification of the 28S rRNA. Each reaction was within the linear range of the assay. Data are expressed as the mean ± SEM. Different superscripts show significant differences between groups ($p < 0.05$).

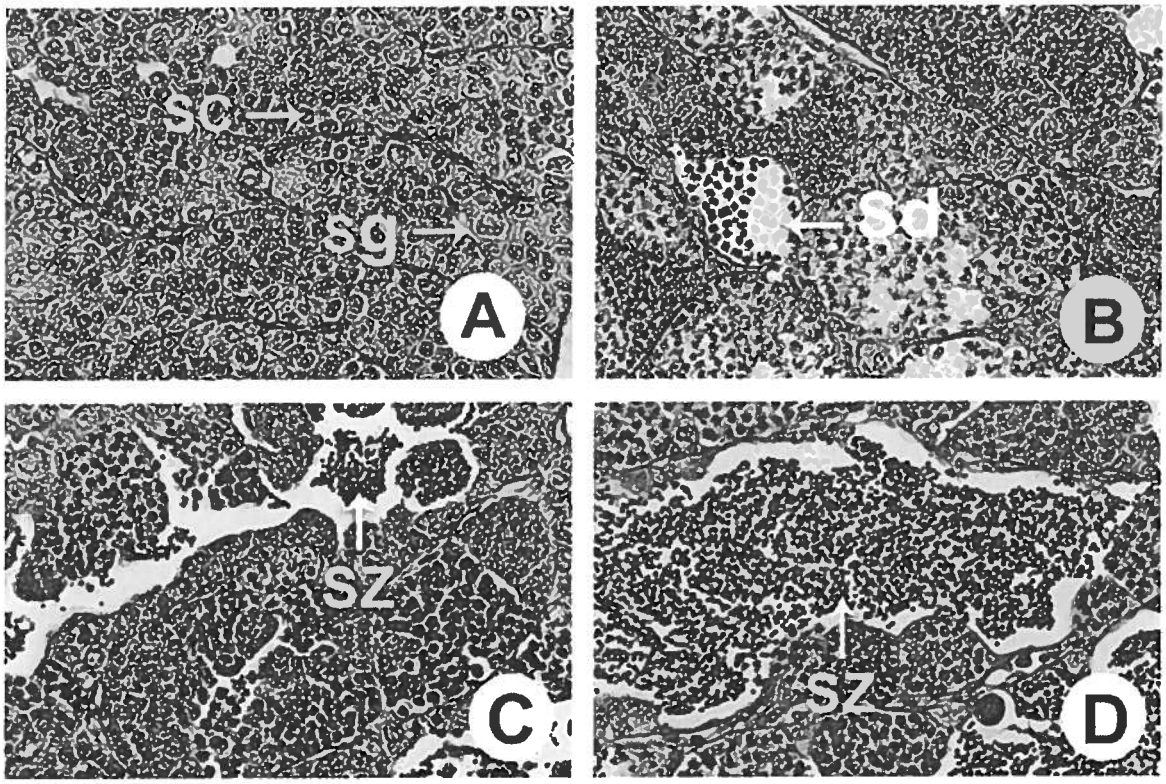

FIG. 3.5. Photomicrographs of four representative stages of spermatogenesis from spottail shiner testes. (A) Stage II, represents early spermatogenesis characterized by cysts containing spermatogonia (sg) and spermatocytes (sc). (B) Stage III testes have cysts containing spermatogonia, spermatocytes, and spermatids (sd). (C) Stage IV, in which spermatozoa are present in the testis. (D) Stage V is the stage of spermiation in which the tubules are filled with spermatozoa (sz).

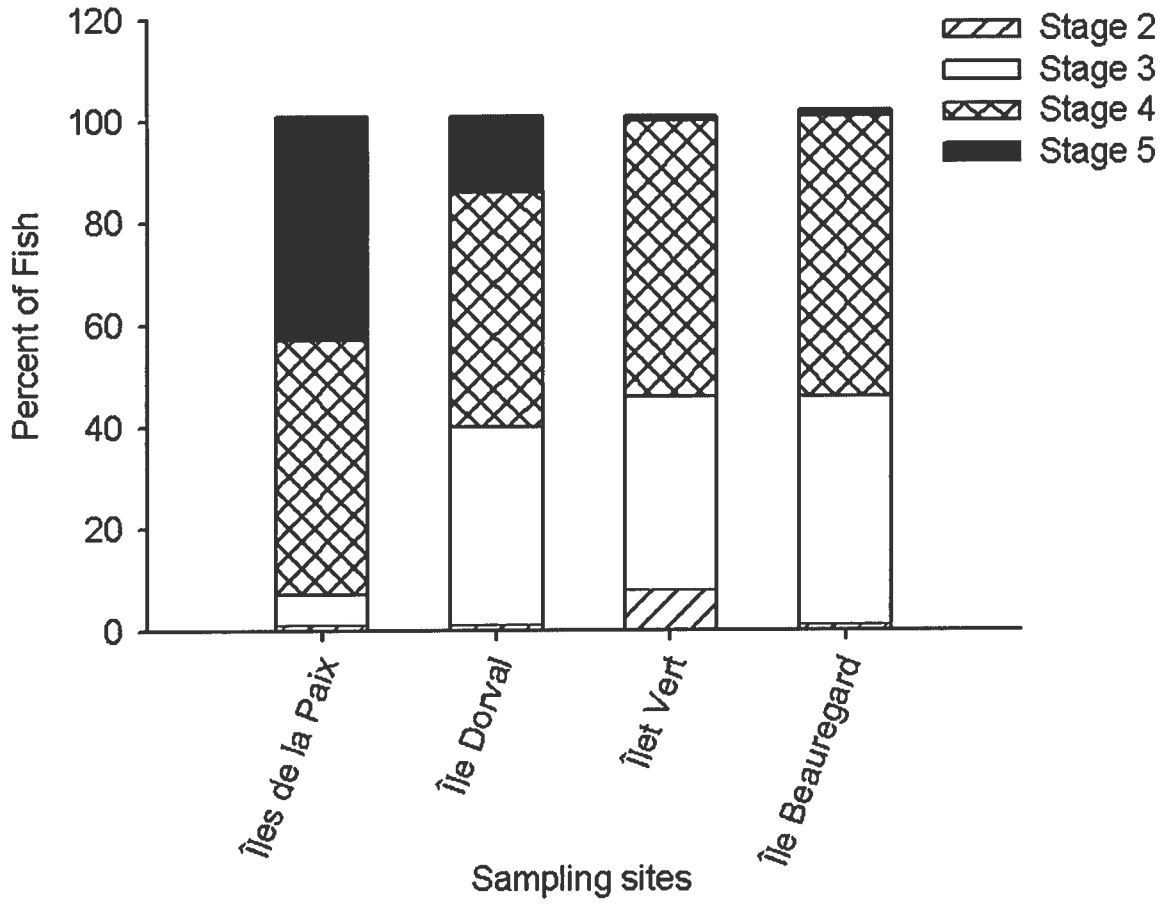

FIG. 3.6. Stages of spermatogenesis in spottail shiner testes from different sites along the St. Lawrence River. Testes were removed, fixed, and embedded in paraffin. Sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin. The stage of spermatogenesis for each fish was then determined.

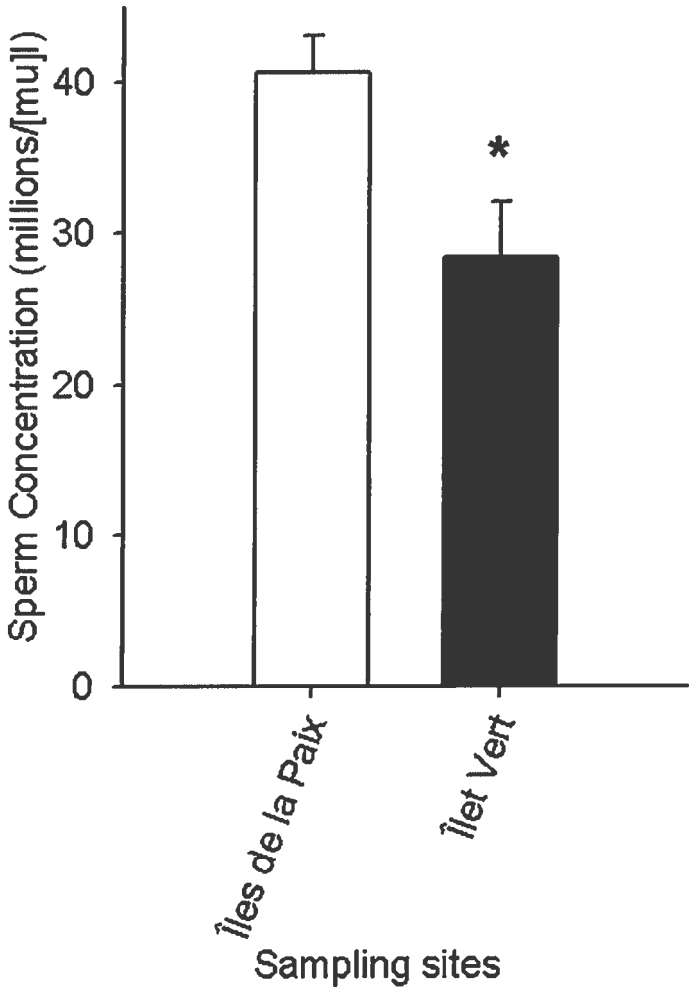

FIG. 3.7 Sperm concentration (mean spermatozoa concentration in millions/μl) in spottail shiners captured at Îles de la Paix and Îlet Vert. Milt was obtained from isolated testes and diluted 100-fold with dilution buffer. Spermatozoa were then activated by a further 1:100 dilution in Tris-HCl (30 mM) alone. Sperm concentration was assessed using the IVOS semen analyzer. Data are expressed as the mean ± SEM. Asterisks indicate significant differences between groups ($p < 0.05$).

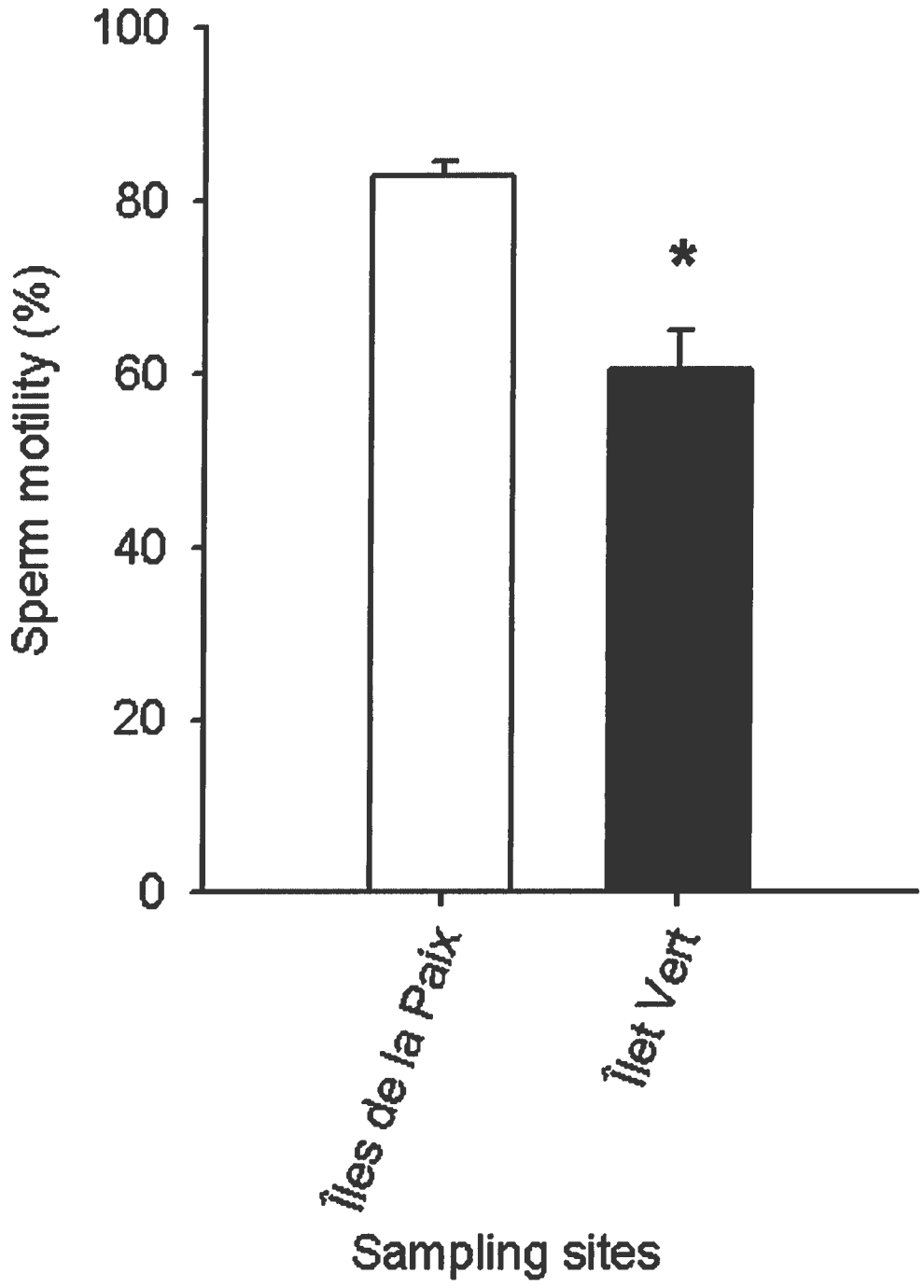

FIG. 3.8 Mean percentage sperm motility from spottail shiners captured at Îles de la Paix and Îlet Vert. Data are expressed as the mean ± SEM. Asterisks indicate significant differences between groups ($p < 0.05$).

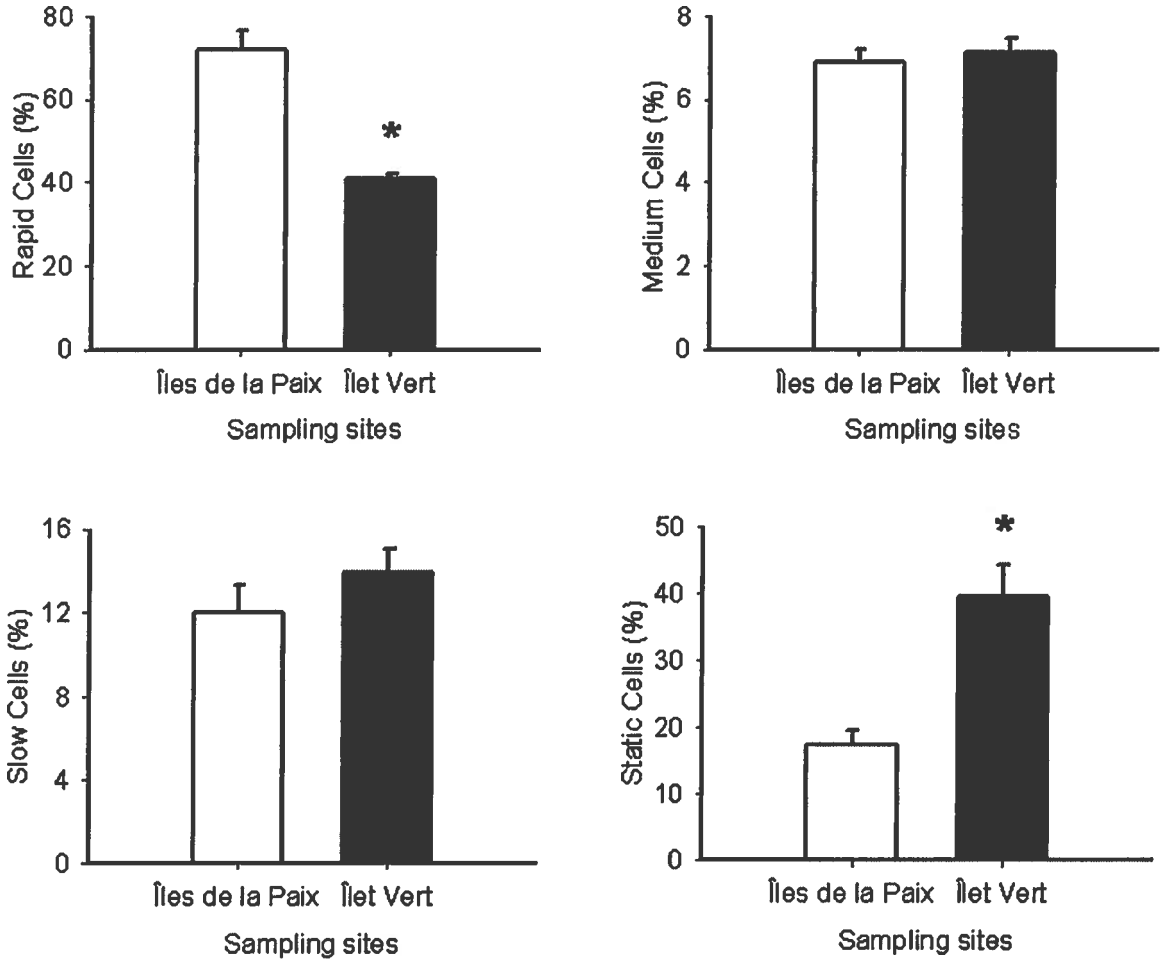

FIG. 3.9 Mean percentage of (a) rapid, (b) medium, (c) slow, and (d) static spermatozoal cells from spottail shiners captured at Îles de la Paix and Îlet Vert. Data are expressed as the mean ± SEM. Asterisks indicate significant differences between groups ($p < 0.05$).

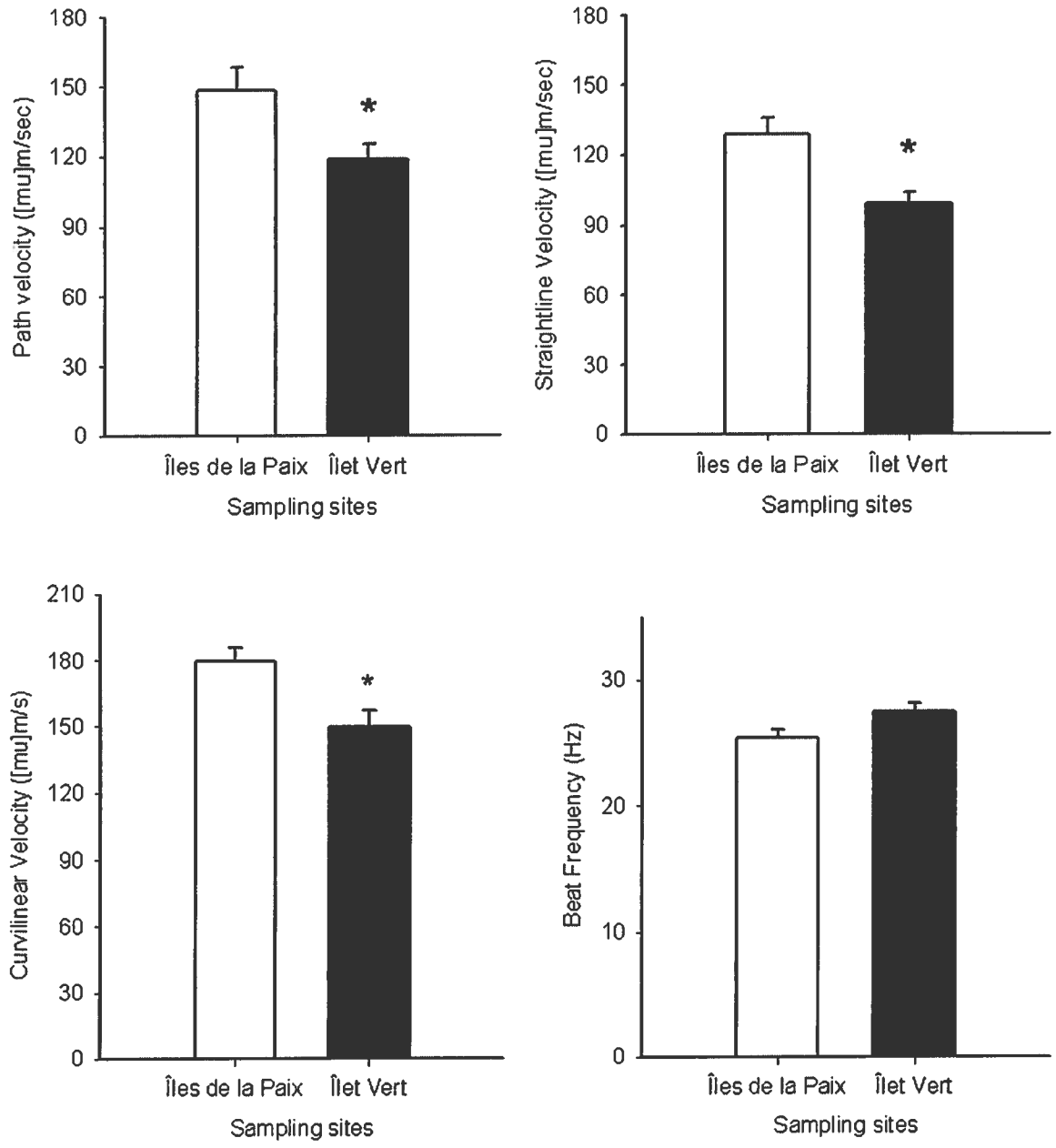

FIG. 3.10 Sperm motility parameters from spottail shiners captured at Îles de la Paix and Îlet Vert. Mean (a) path velocity, VAP; (b) progressive velocity,VSL; (c) curvilinear velocity, VCL; (d) beat frequency, BCF, were determined using the IVOS semen analyzer. Data are expressed as the mean ± SEM. Asterisks indicate significant differences between groups ($p < 0.05$).

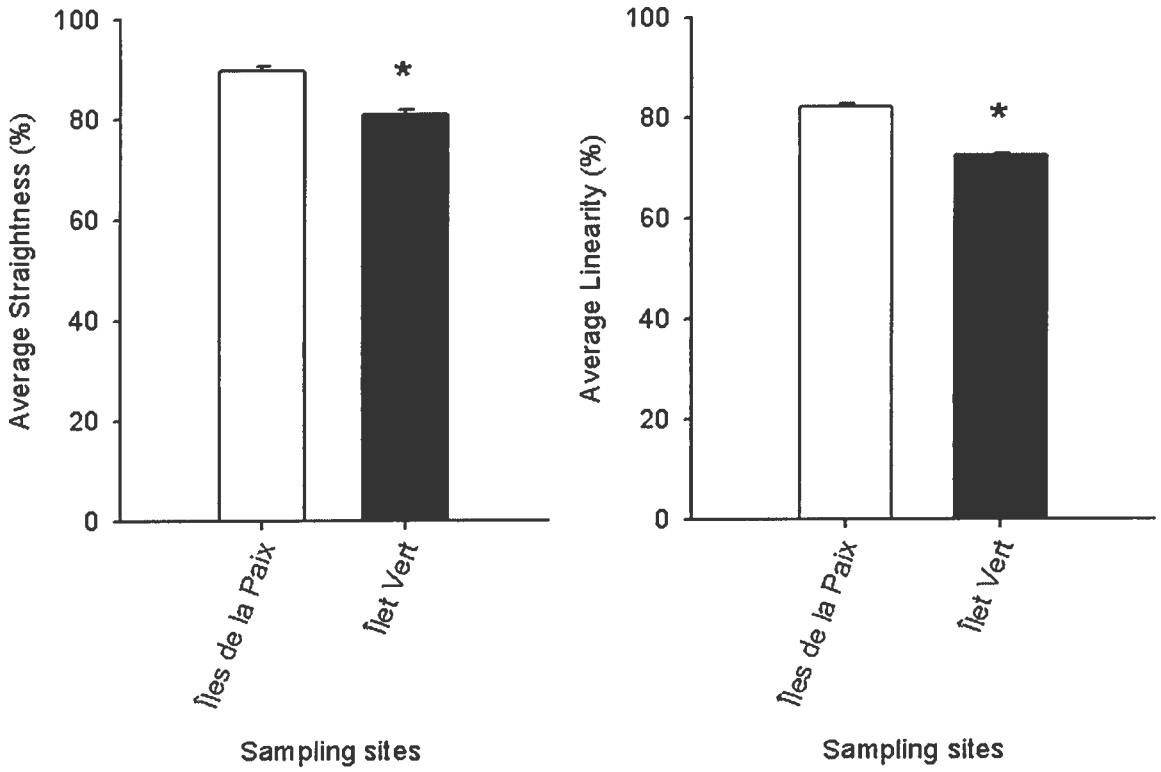

FIG. 3.11 Sperm motility parameters from spottail shiners captured at Îles de la Paix and Îlet Vert. Mean (a) Straightness (b) linearity was determined using the IVOS semen analyzer. Data are expressed as the mean ± SEM. Asterisks indicate significant differences between groups ($p < 0.05$).

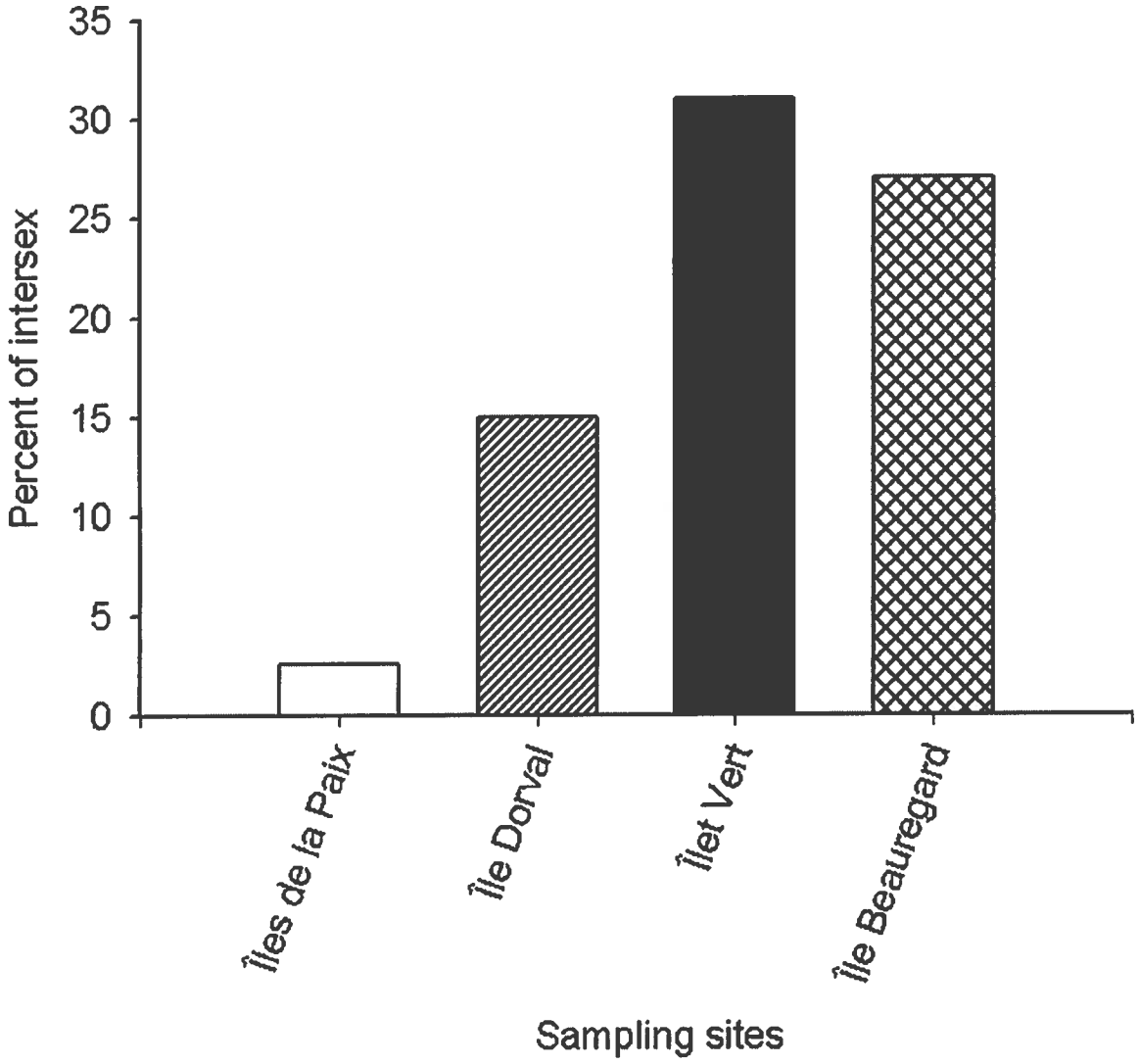

FIG. 3.12 Intersex in male spottail shiners captured at different sites along the St. Lawrence River. Testes were fixed in Bouin's solution and mounted in paraffin. Sections (5 μm section) were mounted on glass slides and stained with hematoxylin and eosin. Sections were examined under the light microscope. Results are expressed as percentage of fish exhibiting intersex.

4.0 CONSUMPTION OF XENOESTROGEN –CONTAMINATED FISH DURING LACTATION ALTERS ADULT MALE REPRODUCTIVE FUNCTION

4.1 ABSTRACT

Little information exists on the transfer of endocrine-disrupting effects through the food chain. The transfer of chemicals, particularly from the aquatic ecosystem, that can cause such effects on fish-eating predators must be established. Fish from the St. Lawrence River are exposed to xenoestrogens causing male reproductive dysfunction. The objective of this study was to determine if lactational exposure to contaminated fish could alter the development of the male reproductive system in rats. Three experimental groups were used: rats (dams) gavaged with (a) distilled water (control), or (b) homogenized fish from a reference site (Îles de la Paix) or (c) homogenized fish from a xenoestrogen-contaminated site (Îlet Vert). Pups were exposed via lactation and sampled on either day 21 (weaning) or day 91 (adults). There was no effect on the body weights or in the male reproductive organ weights between groups except for adult epididymal weight, which was significantly decreased in the xenoestrogen group. Adult sperm concentrations and sperm motility parameters were all significantly decreased in the xeonestrogen group as compared to the reference and control groups. Furthermore, the distribution of stages of spermatogenesis was altered in the xenoestrogen group, indicating an effect on the kinetics of spermatogenesis. Immunoreactivity of connexin43 (Cx43), a gap-junctional protein, was markedly decreased in the seminiferous epithelium of the xenoestrogen group, suggesting that the intercellular coordination of testicular function may be affected. These

data indicate that contaminants from xenoestrogen environments may pass through the food chain and exert effects on male reproductive functions.

4.2 INTRODUCTION

There is substantial evidence that prenatal or early postnatal exposure to endocrine-disrupting actions of various environmental contaminants, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), can adversely affect wildlife populations (de Solla et al., 1998; Giesy et al., 1994; Guillette et al., 1994, 1996). Studies have shown that bald eagles nesting along the shores of the Great Lakes and feeding primarily on contaminated Great Lakes fish have lower reproductive success (Bowerman et al., 1995; Colborn 1991). The most direct evidence for adverse human health effects from environmental pollution is found in a series of studies linking PCB exposure to consumption of contaminated fish (Fein et al., 1984; Jacobson et al., 1984; Jacobson and Jacobson 1988). These studies were able to associate the maternal consumption of contaminated fish with adverse health effects in their children who were exposed to the PCBs via lactation.

Contaminants such as 17--ethinyl estradiol, bisphenol A, and alkyl phenols that are present in treated sewage effluent can act as estrogen-mimics and induce a wide range of effects on fish. These effects include feminization and hermaphroditism in males (Arukwe 2001; Jobling and Tyler, 2003; Rothcell and Ostrander, 2003). In fact, fish can serve as "barometers" of the effects of xenoestrogen contamination in the aquatic ecosystem. One index of exposure is the presence of vitellogenin (Vtg), an egg yolk protein, in male fish. Normally, females synthesize

Vtg in the liver in response to estradiol. Several reports have shown an upregulation of Vtg in male and immature fish collected near sewage effluents (Aravindakshan et al., 2004a; Jobling and Sumpter, 1993; Tyler et al., 1996) and other industrial effluents.

The effluent generated by the municipal sewage treatment facility of the City of Montreal, Quebec, Canada, is discharged into the St. Lawrence River at a single site near the eastern tip of the Island of Montreal. We have shown that spottail shiners exposed to this effluent exhibit induced Vtg levels, delayed spermatogenesis, reduced spermatozoal production, decreased sperm motility, and a high incidence of intersexuality (Aravindakshan et al., 2004a).

We noted (while sampling fish in the St. Lawrence River to study the presence and effects of endocrine-disrupting chemicals) the presence of a sport fishery in this area, suggesting that people eat fish from sites where we have reported estrogenic effects on fish (Aravindakshan et al., 2004a). Kosatsky et al. (1999a) reported that in the Montreal area, sport fishers eat their catch as often as three times weekly and can consume in excess of 18 kg of St. Lawrence River–caught fish annually. Heavy consumers of these fish have higher hair mercury levels and higher circulating levels of PCBs and dichlorodiphenyldichloroethylene (DDE) than do infrequent consumers. Studies also suggest that Montrealers of Asian origin consume more fish from the St. Lawrence River than other sport fishers do and, in turn, that they have higher levels of contaminants than median levels found in other sport fishers (Kosatsky et al., 1999b). The fact that fish in the St. Lawrence River are exposed to estrogenic compounds suggests that people, as well as fish-eating mammals consuming these fish, may be exposed to the same endocrine-disrupting compounds, assuming that these chemicals can bioaccumulate.

Exposure to environmental contaminants during critical periods of development may represent a far greater risk to animals and humans than exposure as adults. Endocrine-disrupting chemicals are particularly problematic to developing animals, because the timing of endocrine-mediated events may be deregulated, resulting in permanent physiological defects to the immune, nervous, or reproductive system (Arukwe 2001; Jobling and Tyler, 2003; Rothcell and Ostrander, 2003). The reproductive systems of mammals undergo substantial development both in utero and after birth, prior to puberty. In rats, the male reproductive tract undergoes substantial development in the first three weeks of life. During this period, cells of the testis and epididymis differentiate into cells resembling those of the adult (Pelletier, 2001; Rodriguez et al., 2002). The blood–testis and blood–epididymal barriers are formed, and the first wave of spermatogenesis is initiated (Cyr, 2001; Cyr et al., 2002; Pelletier, 2001). Thus the period of lactation, when the mother can pass along contaminants to her offspring, represents a critical period of reproductive development for the male pups. As such, this critical period is among the most vulnerable to xenobiotics, including endocrine disruptors.

Organochlorinated compounds are known to pass up the food chain and become biomagnified in top predators, yet we know relatively little about whether endocrine-disrupting compounds can be passed up the food chain and cause endocrine disruption to higher vertebrates. This possibility is particularly relevant, given the increasing number of aquatic ecosystems in which endocrine-disrupting chemicals reportedly affect aquatic organisms. The transfer of these chemicals and their effects to fish-eating predators and humans must therefore be established, in order to evaluate the potential risk of endocrine disruptors in aquatic ecosystems to riverine

mammalian species. The objective of the present study was to determine whether the maternal consumption of fish from a xenoestrogen-contaminated environment results in adverse reproductive consequences to weaning male pups, and if so, whether such effects become apparent only when the pup reaches adulthood. This information will assist us in developing a better understanding of the exposure and risks associated with eating fish from environments contaminated with estrogenic compounds.

4.3 MATERIALS AND METHODS

4.3.1 Collection of fish

Previous studies from our laboratory established the presence of xenoestrogens in the St. Lawrence River in proximity to the Island of Montreal by monitoring Vtg mRNA levels in spottail shiners (*Notropis hudsonius*; Aravindakshan et al., 2004a). Based on these results, a reference site (Îles de la Paix) and a site with a high level of xenoestrogen-contamination (Îlet Vert) were identified. For the present study, immature spottail shiners of the same age and size were captured at each of these sites using a beach seine. Fish were returned to the laboratory and euthanized. The livers of a subset of immature fish were dissected, immediately frozen in liquid nitrogen, and stored at –80°C. The rest of the fish were subsequently stored at –80°C.

4.3.2 Vtg mRNA levels

Total RNA was extracted from livers of immature spottail shiners captured at both the reference site and the xenoestrogen-contaminated sites using the guanidinium thiocyanate-

phenol-chloroform method (Chomczynski and Sacchi, 1987). After RNA isolation, reverse transcriptase polymerase chain reaction (RT-PCR) was performed to determine Vtg mRNA levels as described previously (Aravindakshan et al., 2004a). The resulting Vtg amplicons were separated on an agarose gel, stained with ethidium bromide, and their relative levels determined by densitometry using a Bio-Rad Fluor Image analyzer (Bio-Rad Laboratories, Mississauga, ON). The 28S rRNA was used as an internal standard. Data were expressed as the intensity of the Vtg amplification product relative to that of the 28S rRNA.

4.3.3 Experimental protocol

Timed-pregnant Sprague-Dawley rats (250 ± 5 g; 6–8 weeks old) were purchased from Charles River Canada, Ltd (St. Constant, Montreal, QC) one week prior to parturition and kept under standard controlled temperature (22°C) and lighting (12 h light, 12 h darkness). On the day of birth, 120 male pups were randomly mixed, and 10 male pups were placed with a lactating dam. All rats were fed Purina rat chow and given water ad libitum. Three experimental groups (n = 40 rats per group) were used: (1) control, (2) rats fed fish from the reference site; (3) rats fed fish from the xenoestrogen-contaminated site. Fish from either the reference site or the contaminated site were homogenized in distilled water and fed to the dams in each of the respective experimental groups by gavage. The control group received distilled water alone with no fish. The dams were gavaged three times a week at a dosage of 1% of their body weight, starting from the day of parturition to the time the pups were weaned (day 21). Therefore, an average 250 g female received 2.5 g of fish (wet weight) three times per week, or an average of 1.43 g of fish/kg/day. We based this dose on previous studies around the Island of Montreal that

indicate that fishers consume as much as 18 kg of fish per year on average, but that consumption can be higher depending on ethnic background. There is virtually no ice fishing on the St. Lawrence around the Island of Montreal; therefore fishing is limited to the months from late spring until early autumn. If we assume an average person weighing 65 kg eating 18 kg of fish over a 6-month period, then the daily consumption averages approximately 0.76 g fish/kg/day. The US average fish consumption is 0.25 g/kg/day and the Canadian average is 0.17 g/kg/day for the general population, although fish consumption is greater among certain aboriginal populations (Boyer et al., 1991; Chan et al., 1999).

After weaning, the pups were subsequently maintained on a standard diet of rat chow and water ad libitum. The dams and pups were weighed three times each week. The pups were sampled on either day 21 or day 91 (adult). All animal protocols used in the present study were approved by the University Animal Care Committee.

Male rats (21 or 91 days of age) were anaesthetized with an intraperitoneal injection of ketamine/xylazine (50:10 mg/kg). The animals were weighed, bled, and euthanized. Paired testis, epididymis, seminal vesicle (empty), and ventral prostate weights were recorded. Tissues were then frozen in liquid nitrogen and stored at –80°C or fixed in Bouin's solution for further histological analyses.

4.3.4 Testosterone radioimmunoassays

Blood samples from adult rats (day 91; n = 25 per group) were obtained by puncturing the dorsal aorta prior to euthanasia. Blood samples were allowed to clot overnight at 4°C and were subsequently centrifuged to obtain the serum. Serum samples were frozen at –80°C until the time of assay. Serum testosterone levels were determined by means of a commercial assay kit used according to the manufacturer's protocol (ImmuChem Double Antibody Testosterone RIA kit, ICN Biomedicals, Costa Mesa, CA). Intra-assay variability was assessed to be less than 2.9%, and inter-assay variability was calculated at 6.3%.

4.3.5 Sperm motility parameters

Functional analysis of male reproduction was determined by measuring sperm motility of adult rats (n = 10 per group), using an IVOS semen analyzer (Hamilton-Thorne Research, Beverly, MA). The cauda epididymidis was clamped both proximally and distally, removed from the epididymis, and rinsed in Medium 199 (with Hank's salts supplemented with 0.5% w/v BSA, pH 7.4; GIBCO, Mississauga, ON) in a 35-mm plastic Petri dish at 37°C. The cauda epididymidis was punctured with a surgical scalpel blade (No. 11; Fisher Scientific, Ottawa, ON) allowing the sperm to flow out. The cauda epididymidis was removed from the media and the Petri dish was returned to the incubator kept at 37°C in a 5% CO2 atmosphere for 5 min, to allow the sperm to disperse. The sperm were diluted 1:10 in medium prior to analyses for motility parameters. Among those motility parameters measured were the following: Percent motility—the percent of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the analysis field; Path Velocity (VAP)—the average velocity of the smoothed cell path, expressed in microns per second); Progressive Velocity (VSL)—the average

velocity measured in a straight line from the beginning to the end of the track; Curvilinear Velocity (VCL)—the sum of the incremental distances moved in each frame along the sampled path divided by the time taken for the sperm to cover the track; Beat Cross Frequency (BCF)—the frequency with which the sperm track crosses the sperm path; Straightness (STR)—the departure of the cell path from a straight line; Linearity (LIN)—the departure of the cell track from a straight line.

The other cauda epididymidis was dissected and frozen (–20°C) for subsequent analysis of sperm concentration. Briefly, the cauda epididymis was thawed and homogenized in a 50 ml conical tube containing 20 ml of distilled water. The "IDENT fluorescent dye" (Hamilton-Thorne Research) was resuspended in 100 μl of distilled water in a small 1.5 ml microcentrifuge tube. A 100 μl aliquot of the homogenized sample was added to the resuspended IDENT solution and incubated at room temperature for 2 min. The solution was mixed and a 5 μl aliquot was placed on a 20 μm sperm analysis chamber (2X Cel; Hamilton-Thorne Research) slide and analyzed with the IVOS semen analyzer under ultraviolet light.

4.3.6 Morphological analyses

At day 21 (n = 15 per group) and at day 91 (n = 15 per group), testes and epididymides were fixed by immersion in Bouin's fixative for 24 h and subsequently transferred to 70% ethanol until processing. Fixed tissues were then dehydrated in graded ethanol, cleared in xylene, and mounted in paraffin for light microscope and immunocytochemical analysis.

To identify the stages of spermatogenesis in the testis, sections of adult testes were stained with periodic acid–Schiff (PAS) stain (Sigma-Aldrich Canada Ltd, Mississauga, ON) according to the manufacturer's instructions. Anatomical and morphological changes in the testis and epididymis were assessed at the light microscopic level. The diameters of 50 round seminiferous tubules were measured, and staging of spermatogenesis was accomplished by observing 200 tubules from each testis according to the criteria described by Leblond and Clermont (1952).

4.3.7 Immunohistochemistry

Immunoperoxidase staining of testicular sections (n = 5 rats per group) was performed according to previously published protocols (Oko and Clermont, 1989). A Heat Induced Epitope Retrieval (HIER) step was included, using citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate). Sections in citrate buffer were heated in a microwave for 15 min. Immunolocalization of connexin 43 (Cx43) was performed using rabbit polyclonal antisera raised against Cx43 (100 $\mu g/\mu l$; Zymed Laboratories, South San Francisco, CA). The DAKO CSA System HRP kit (DAKO, Carpinteria, CA) was then used to localize Cx43 according to the manufacturer's instructions. Negative control slides, in which the primary antibody was replaced with normal goat serum, were also performed concurrently.

4.3.8 Statistical analysis

The data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov (K-S) test. All data were analyzed using one-way analysis of variance (ANOVA; SigmaStat, Version 2.0, Jandel Corporation, San Rafael, CA). If the effects were significant, the Student-Newman-Keuls test was used for post-ANOVA multiple comparisons ($p < 0.05$). All data are presented as the mean ± standard error of the mean (SEM), unless otherwise indicated.

4.4 RESULTS

4.4.1 Vitellogenin Induction in Spottail Shiners

Semi-quantitative RT-PCR results indicate that Vtg mRNA levels were significantly higher in fish captured at Îlet Vert (xenoestrogen-contaminated site) as compared to the reference site at Îles de la Paix (Fig. 4.1). These results confirmed that fish from Îlet Vert were exposed to xenoestrogens.

4.4.2 Body and Tissue Weights and Testosterone Concentrations

The body weights of the dams and the pups did not differ significantly between treatment groups throughout the experimental period. At 21 days, paired testis, epididymis, seminal vesicles, and ventral prostate weights were not altered by treatment (Table 4.1). Morphologically, the diameter of the seminiferous tubules of control rats and of rats whose mothers were fed fish from the reference site and the xenoestrogen-contaminated site were not

statistically different. Testes from rats in all experimental groups displayed a well-defined lumen by 21 days of age.

When the pups reached adulthood (91 days), however, there was a significant decrease in the weight of the epididymis in pups whose mothers were fed fish from the xenoestrogen-contaminated site, as compared to either the control or reference site group (Table 4.1). Paired seminal vesicle weights and ventral prostate weights were not altered by treatment, suggesting that biologically active androgen levels were not altered by treatment (Table 4.1). Further analyses of serum testosterone levels confirmed that they were not altered by treatment (Table 4.1).

4.4.3 Sperm Motility Parameters

Indicators of sperm concentrations and their motility were assessed for the three experimental groups. Cauda epididymidis spermatozoal concentrations were significantly decreased in the xenoestrogen-exposed group as compared to both the controls and the group whose mothers were fed fish from the reference site (Fig. 4.2). In the xenoestrogen-exposed group, the percentage of motile spermatozoa was decreased by approximately 20% (Fig. 4.3a) and the percentage of motile spermatozoa with progressive motility was significantly decreased as compared to the controls and the group exposed to reference site fish (Fig. 4.3b). The VAP, VSL, and VCL (Figs. 4.4a, 4.3b, and 4.3c) were all reduced in the xenoestrogen-exposed group, BCF was not different from the control group (Fig. 4.4d). Straightness and linearity were also lower in the xenoestrogen-exposed group than in both the controls and the group exposed to

reference fish (Fig. 4.5a and 4.5b). When spermatozoa were classified according to their velocity, there was a reduction in the percentage of rapidly moving cells and an increase in the percentage of static cells in the xenoestrogen-exposed group, as compared to the controls and the reference group (Fig. 4.6).

4.4.4 Effects on Spermatogenesis

Because sperm concentrations and motility were reduced, we wanted to determine whether their reduction was due to an effect on spermatogenesis. Histological examination of the testes indicated that there were no significant differences in the diameter of the seminiferous tubules in any of the three experimental groups (data not shown). When tubules were staged according to the different stages of spermatogenesis, shifts in the frequencies of stages were observed in adult rats. There were no differences in the stages of spermatogenesis between the control group and the group whose mothers were fed reference site fish. In the xenoestrogen-exposed group, however, the frequency of tubules at stages I through VIII was decreased. In addition, a significant decrease in the incidence of both stages VII and XIV in the xenoestrogen-exposed group as compared to the two other experimental groups was noted (Fig. 4.7). Stage XIV is the stage at which the second meiotic division occurs, whereas sperm are released into the lumen of the seminiferous tubule between stages VII and VIII. These results suggest that these two critical stages of development are delayed as a result of lactational exposure to contaminants from the fish captured in the xenoestrogen-contaminated site.

4.4.5 Connexin 43 in the Testes

The process of spermatogenesis requires highly coordinated intercellular communication between developing germ cells and Sertoli cells. This is accomplished by gap junctions. In the testis, gap junctions are predominantly composed of Cx43 (Batias et al., 2000). We therefore wanted to determine whether Cx43 was altered in the testis of rats whose mothers were fed fish from the xenoestrogen-contaminated site. In controls and in rats whose mothers were fed fish from the reference site, Cx43 was localized at the base of the tubules between adjacent Sertoli cells. The immunoperoxidase reaction resembled discrete ribbon-like strands localized in the area of the Sertoli–Sertoli junctional complex (Fig. 4.8A). A sparse punctate immunoreaction was also observed higher up in the seminiferous tubule between developing germ cells and Sertoli cells. The immunoreaction in some of the tubules from stage II to VII was more intense than in tubules at stages IX–XIV. Although this finding is consistent with previously published studies (Riley et al., 1992; Tan et al., 1996; Lablak et al., 1998), the mechanisms that drive this stage-specific expression of Cx43 is not known.. Immunostaining was also observed outside the seminiferous tubule between Leydig cells (Fig. 4.8B). There were no differences in the immunoperoxidase staining pattern between the control group and the Site I group (data not shown). In testes from rats whose mothers had been fed xenoestrogen-contaminated fish, Cx43 immunostaining along the plasma membrane was less pronounced than in either the control group or the reference group (Fig. 4.8C and 4.8D). In some tubules the staining between adjacent Sertoli cells appeared to be completely absent. Likewise, the immunoreaction between adjacent Leydig cells appeared to be less intense, although the effect was not as pronounced as in Sertoli cells. There was no immunoperoxidase reaction observed in sections incubated without primary antibody, which were used as negative controls (data not shown).

4.5 DISCUSSION

The long-term adverse effects resulting from the consumption of fish captured in xenoestrogen-contaminated environments are an important consideration in assessing the risk of endocrine-disrupting chemicals. We have shown, both in this study and in a previous study, that xenoestrogens are present in the St. Lawrence River over a distance exceeding 75 km (Aravindakshan et al., 2004a). Our observations indicate an active sport fishery in the St. Lawrence River, and other investigators have reported that these fishers often eat their catch and some may consume sizeable quantities of fish (Kosatsky et al., 1999a). In fact, heavy eaters of these fish have elevated levels of mercury in hair, and elevated circulating levels of DDE and PCBs (Kosatsky et al., 1999a). We can therefore infer that humans are exposed to contaminants present in fish from the St. Lawrence River and that the chemicals responsible for inducing an estrogenic response in the fish are transferred to humans. Furthermore, other predators such as fish-eating mammals and birds are likely targets for these contaminants, although, at the moment, there is no information regarding possible endocrine-disrupting effects on these species in the St. Lawrence River.

The body weights of the dams and the pups in our rat population did not differ among any of the treatment groups in this study, suggesting that fish consumption did not have an effect on the growth rates of the rats. This finding also suggests that there do not appear to be any differences in the nutritive content of fish from the reference site and those from the xenoestrogen-contaminated site, a finding in accordance with our previous studies, which

showed no differences in the condition factors of the fish between these two sites (Aravindakshan et al., 2004a).

In 21-day-old rats, there were no effects on the weights of male reproductive organs or in their histological appearance. However, when the rats reached adulthood, there was a significant decrease in the weight of the epididymis. Other tissue weights were unaffected. Sperm production occurs in the testis, but the epididymis is where sperm acquire the ability to swim and fertilize and where they are stored until the time of ejaculation (Yeung and Cooper, 2002). The lower epididymal weight may result from a decrease in the concentration of spermatozoa that are stored in the cauda region of the epididymis. Previous studies with estrogenic compounds have shown that the time required for sperm transport through the epididymis can be altered (Hess, 1998; Klinefelter and Suarez, 1997). It is possible that an increase in transit time of the sperm through the caput/corpus region of the epididymis may account for a decrease in epididymal weights. Epididymal weight is also dependent on circulating levels of testosterone. However, serum testosterone levels were not different between experimental groups, and since both ventral prostate and seminal vesicle weights also did not differ, this indicates that circulating androgen levels and activity were not affected in rats whose mothers were fed xenoestrogen-contaminated fish.

Sperm concentration, progressive motility, and linearity were significantly reduced in rats exposed to xenoestrogen-contaminated fish. It has been reported that in humans, there is a significant correlation between fertilization rates and linearity (Hirano et al., 2001). The reduction in the sperm motion (manifested as decreases in the percentage of motile sperm) and

linear velocity may be significant factors in the onset of infertility. Furthermore, cells that were designated as slow and static were not included in the average calculated for our velocity parameters. The high incidences of slow and static cells, the decreased velocity parameters, and the decreased linearity seen in the rats exposed to xenoestrogens all suggest that the sperm in rats from this group may have reduced fertilizing potential. Goyal et al. (2003) reported that the administration of the potent estrogen, diethylstilbestrol (DES), to rats at 10 μg given on alternate days for the first 12 days neonatally resulted in a decrease in sperm motility and linearity. However, in contrast to the present study, they also observed hypertrophy of both the seminiferous tubule and intertubular cells. The fact that we observed effects on sperm motility parameters without extensive testicular tissue damage suggests that estrogenic chemicals may exert more subtle effects at environmentally relevant doses.

Spermatogenesis is a linear process in which adjacent cellular associations (14 stages in the rat; Leblond and Clermont, 1952) advance from stage to stage in wavelike formation (Parvinen and Vanha Perttula, 1973). There is limited information regarding the 14 stages of rat spermatogenesis after treatment with environmentally relevant doses of xenoestrogens. In the present study, the frequency of the stages of spermatogenesis was altered, an indication of a disturbance in the kinetics of spermato- genesis. These observations raise the questions of whether this alteration is due to certain stages maturing more rapidly or whether certain cell types experience germ cell arrest. Alternatively, the primary effect may have been on the germ cells that are outside the blood–testis barrier during the early postnatal period. Environmental contaminants such as diethylcarbamyl methyl-2–4-dinitropyrrole (Patanelli and Nelson, 1964), 2,5-hexanedione, (Chapin et al.; 1983), and ethylene glycol monomethyl ether (Chapin et al.,

1984; Creasy et al., 1985) have all been shown to alter the stage frequencies of spermatogenesis. The changes in stage kinetics and the absence of gross histopathological effect on germ cells observed in this study suggest that the xenoestrogens could be causing more subtle effects on male reproductive function.

Spermatogenesis is a synchronized and spatially patterned process of cell proliferation and differentiation in which gap-junctional intercellular coupling plays an important role in coordinating these functions. The relatively large number of different connexins and their localization in the testis is suggestive of the importance of intercellular gap-junctional communication in testicular functions (Batias et al., 2000; Risley et al., 1992; Tan et al., 1996). In rodents, both Sertoli–Sertoli cell interaction and germ cell (spermatogonia and spermatocytes)–Sertoli cell communication are mediated by gap junctions containing Cx43 (Batias et al., 2000). Testicular intercellular communication mediated by Cx43 gap junctions is believed to represent an essential process for spermatogenesis, because spermatogenesis is arrested in testes that lack Cx43 (Roscoe et al., 2001). Immunocytochemical localization of Cx43 indicated that in each experimental group, some tubules displayed a more intense Cx43 immunoreaction than others. Previous studies have shown that Cx43 immunostaining is dependent on the stage of the tubule (Batias et al., 2000; Risley et al., 1992; St-Pierre et al., 2003; Tan et al., 1996). Our results indicate that the expression of testicular Cx43 is reduced in rats that were exposed to xenoestrogenic compounds during lactation. The reduced expression of Cx43 may be responsible, in part, for alterations observed in the kinetics of spermatogenesis. Recent studies have shown that lindane can alter the expression and targeting of Cx43 in Sertoli cells (Mograbi et al., 2003). Furthermore, Defamie et al. (2003) reported alterations in the

expression of Cx43 in patients with azoospermia and undifferentiated Sertoli cells, a finding that suggests pathological consequences associated with altered gap-junctional communication.

Although the nature of the chemicals responsible for causing these effects is at present unknown, chemical analyses of sediments around the Island of Montreal suggest that contaminant levels are generally low, with the exception of zinc in sewage effluent and alkyl phenols that are present downstream from the Montreal sewage discharge point into the St. Lawrence River (Gagnon and Saulnier 2003; Sabik et al., 2003). Whether these chemicals or other, unidentified chemicals are responsible for the reproductive toxicity effects observed in this study remains to be established. It is clear, however, either that certain chemicals at the sampling site can be transferred to developing offspring and exert permanent effects on male reproductive development, or that the effects of such chemicals on the mothers can be transferred and cause latent pathological effects in the male offspring.

In summary, the present findings show that exposure of immature rats to fish from a xenoestrogen-contaminated environment during the early postnatal period exerts long-lasting effects on epididymal sperm concentration and motility. This effect appears to result in part from alterations in spermatogenesis that are associated with a decrease in the gap- junctional protein Cx43. Together, these results suggest that the consumption of fish from xenoestrogen-contaminated ecosystems may alter the postnatal development of the male reproductive tract, resulting in permanent effects on male reproductive parameters. These results may be particularly important for both fish-eating humans and other riverine mammalian species and

they raise serious concerns regarding the transmission of endocrine-disrupting effects through the food chain.

Table 4.1 Body and Organ Weights as Well as Circulating Serum Testosterone Levels in Adult Rats Whose Mothers Were Given Either Water or Fish from a Reference Site of Xenoestrogen-Contaminated Fish during Lactation

	Control	Reference group	Xenoestrogen group
Body weight (g)			
Day 21	43.2 ± 0.62	43.1 ± 0.45	43.0 ± 0.34
Day 91	545.7 ± 7.12	542.2 ± 8.35	543 ± 8.23
Testis weight (g)			
Day 21	0.19 ± 0.03	0.18 ± 0.02	0.20 ± 0.04
Day 91	3.55 ± 0.06	3.48 ± 0.05	3.37 ± 0.06
Epididymis weight (g)			
Day 21	0.035 ± 0.004	0.034 ± 0.005	0.031 ± 0.004
Day 91	1.29 ± 0.06^{a}	1.25 ± 0.04^{a}	1.08 ± 0.07^{b}
Prostate weight (g)			
Day 91	0.63 ± 0.06	0.61 ± 0.05	0.60 ± 0.07
Seminal vesicles (g)			
Day 91	0.59 ± 0.05	0.62 ± 0.06	0.56 ± 0.07
Testosterone (ng/ml)			
Day 91	3.1 ± 0.8	3.0 ± 0.9	2.8 ± 0.6

Note. Reproductive tissues were collected in male rats at 21 and 91 days of age. Different superscripts indicate significant differences between groups.

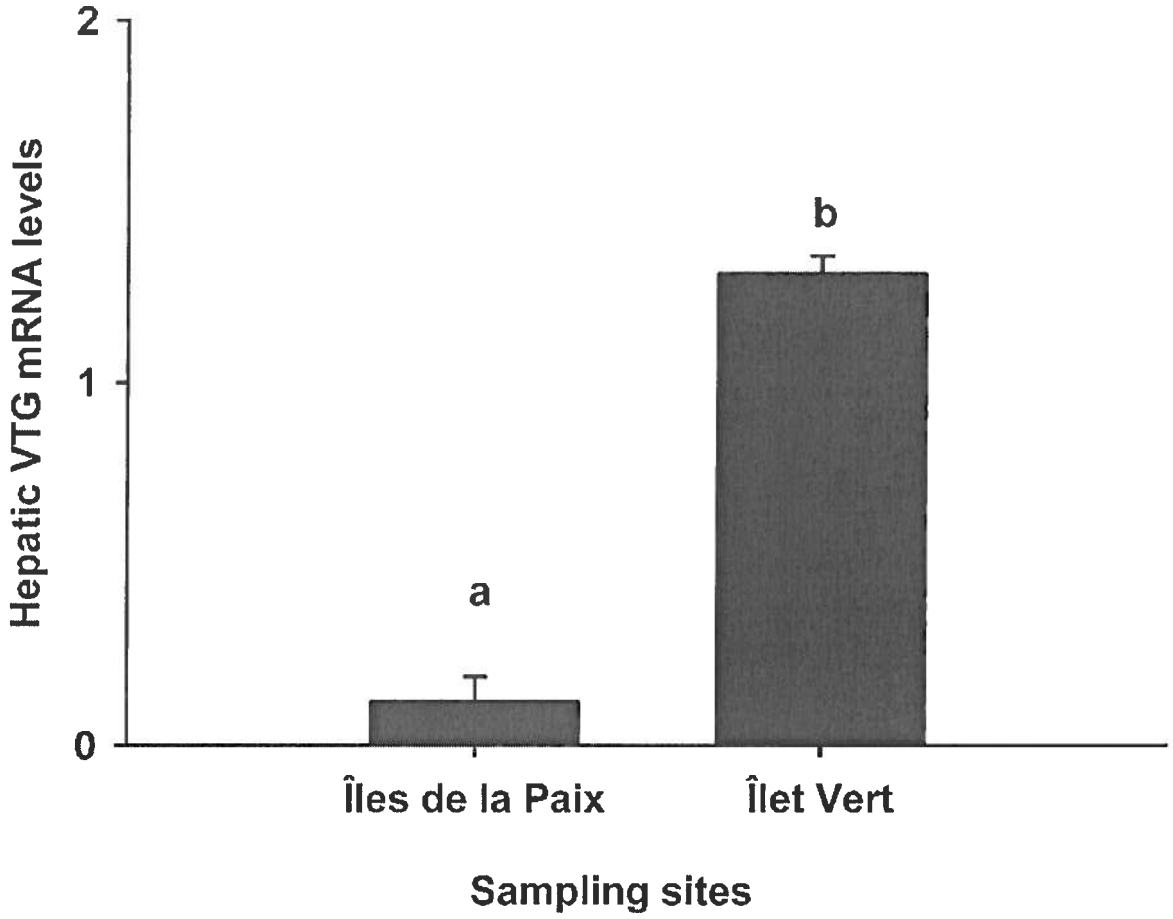

FIG. 4.1. Hepatic Vtg mRNA levels in immature spottail shiners captured at the reference site (Îles de la Paix) and the contaminated site (Îlet Vert) along the St. Lawrence River. Total cellular RNA was extracted from fish liver and subjected to RT-PCR with specific Vtg primers. Data were standardized to the 28S rRNA. Each reaction was done within the linear range of the assay. Data are expressed as the mean ± SEM. Different superscripts indicate significant differences between groups ($p < 0.05$).

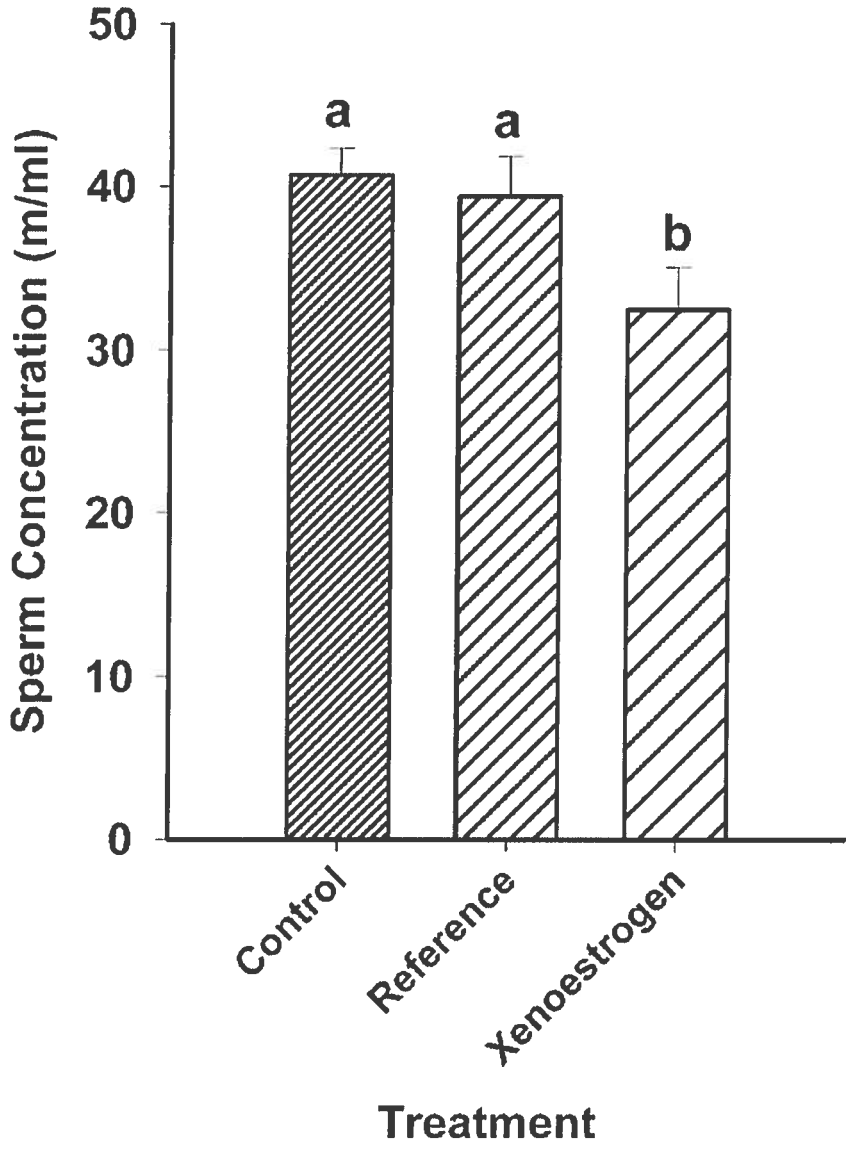

FIG. 4.2. Mean sperm concentration in adult rats (n = 10 per group) whose mothers were given either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Sperm concentrations were assessed using the IVOS semen analyzer, and the data are expressed as the mean ± SEM. Different superscripts indicate significant differences between groups (i.e., "a" is different from "b").

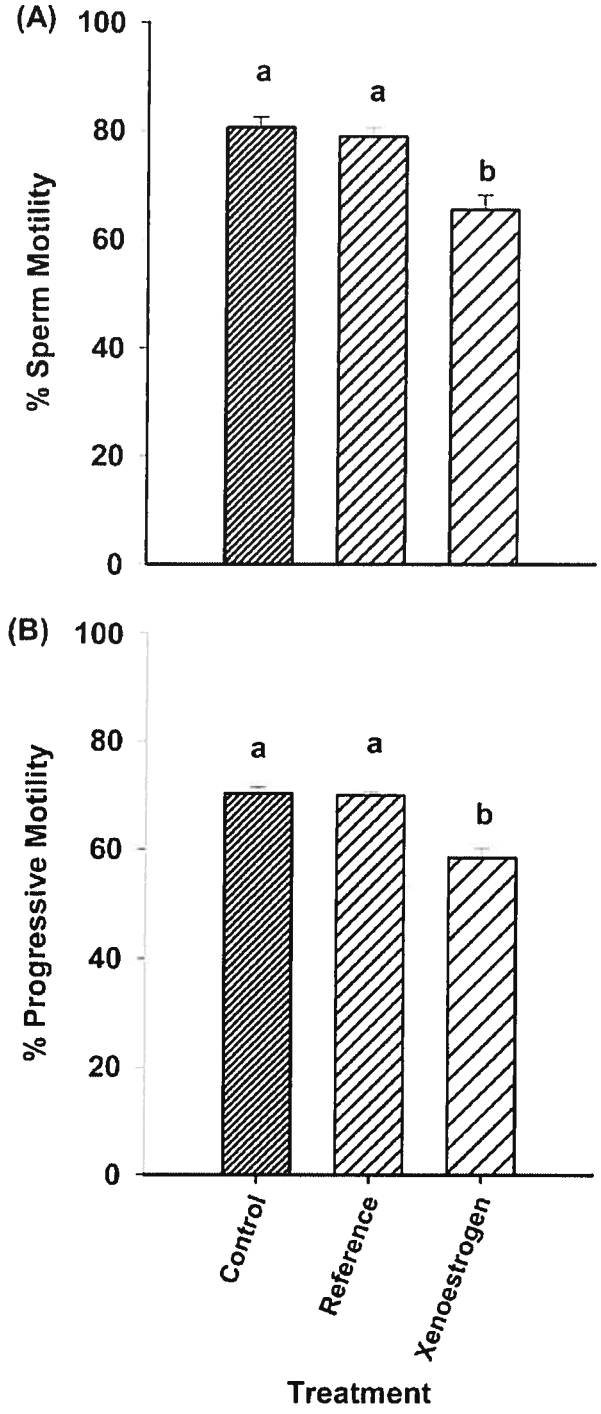

FIG. 4.3 Sperm motility (A) and progressive motility (B) in adult rats (n = 10 per group) whose mothers were give either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Data are expressed as the mean ± SEM. Different superscripts indicate significant differences between groups (i.e., "a" is different from "b").

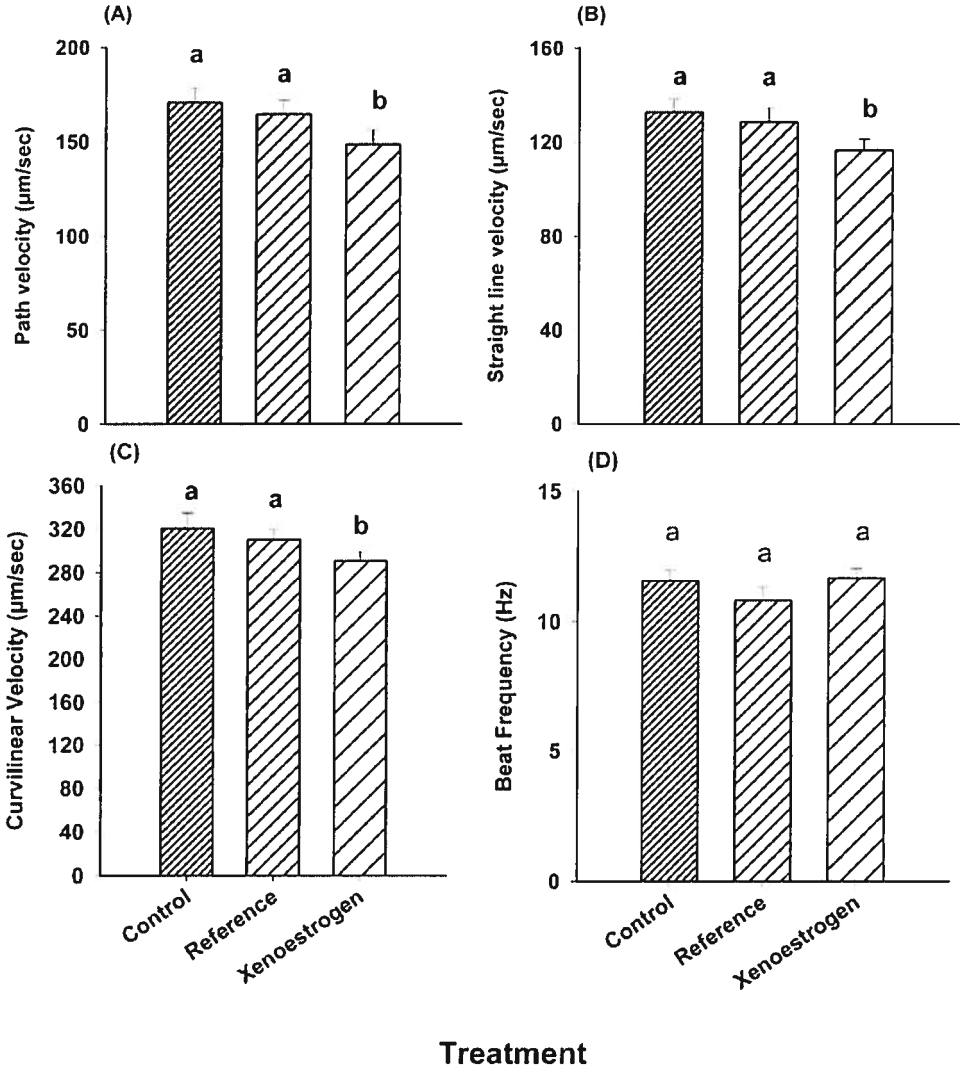

FIG. 4.4 Sperm Path Velocity (VAP; A), Progressive Velocity (VSL; B), Curvilinear Velocity (VCL; C), Beat Frequency (BCF; D) in adult rats (n = 10 per group) whose mothers were give either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Analyses were determined using the IVOS semen analyzer. Data are expressed as the mean ± SEM. Different superscripts indicate significant differences between groups (i.e., "a" is different from "b").

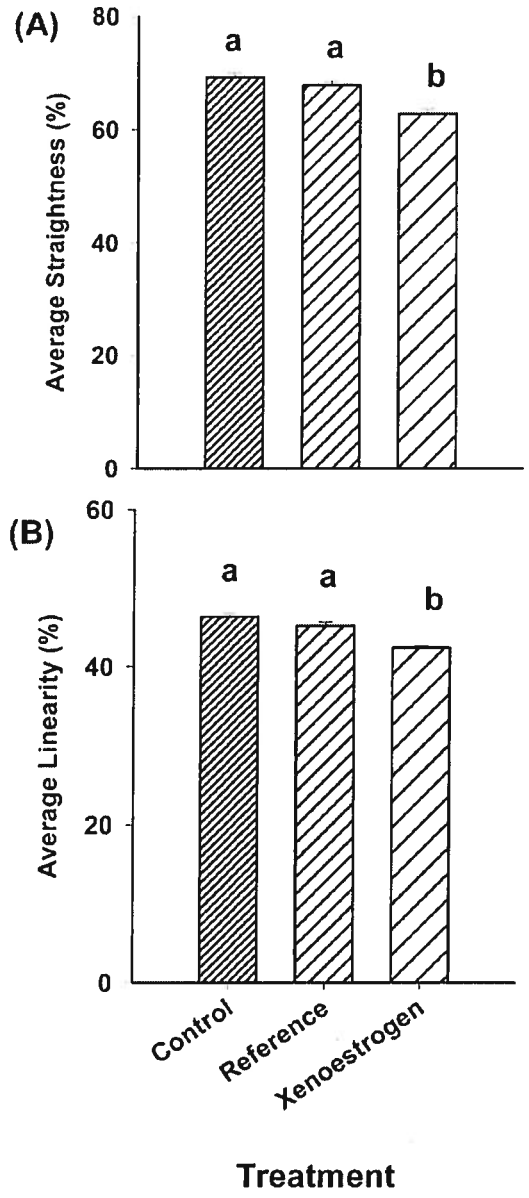

FIG. 4.5 Straightness (A) and linearity (B) of sperm displacement in adult rats (n = 10 per group) whose mothers were give either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Analyses were done using the IVOS semen analyzer, and the data are expressed as the mean ± SEM. Lowercase letters indicate significant differences between groups (i.e., "a" is different from "b").

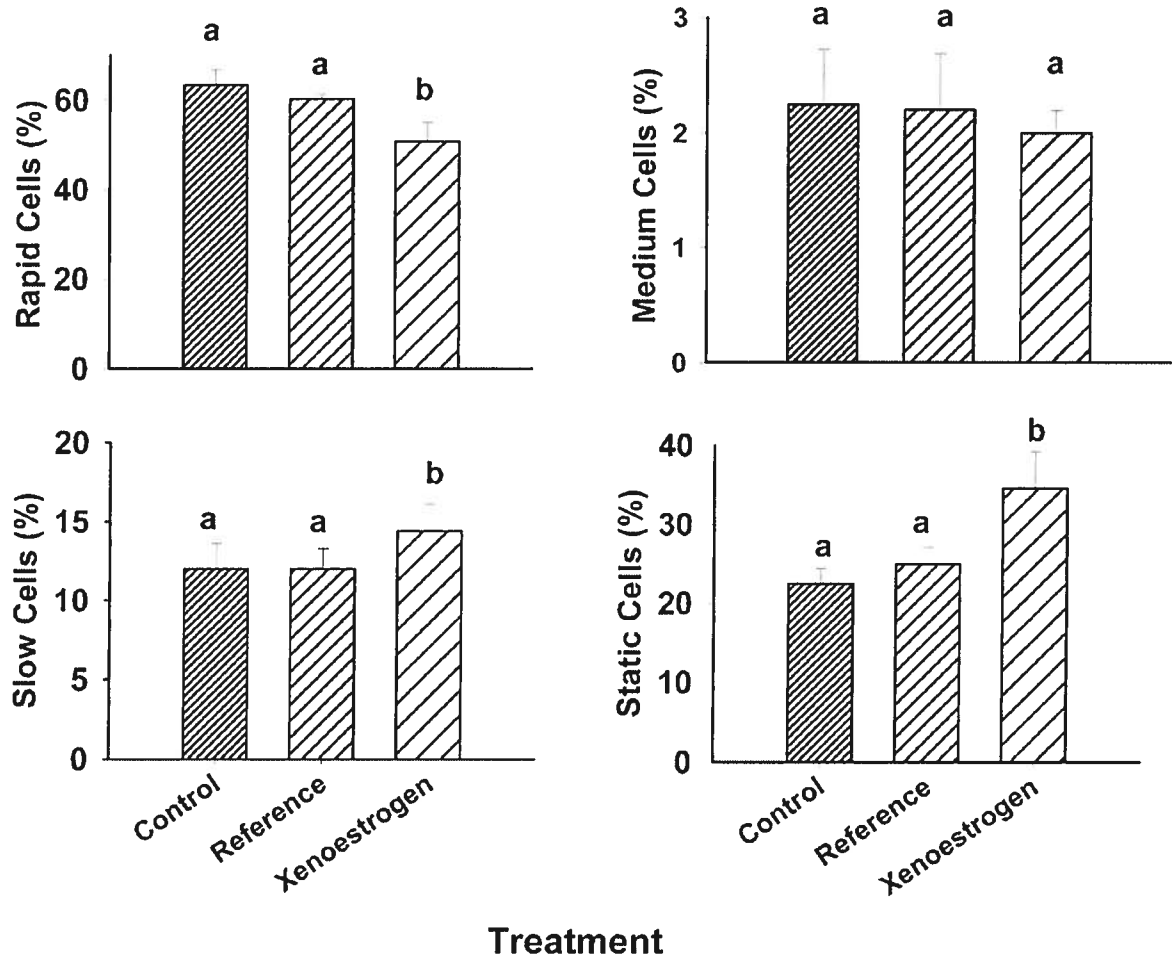

FIG. 4.6 Mean percentage of Rapid, Medium, Slow, and Static cells in adult rats whose mothers were given either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Data are expressed as the mean ± SEM. Different superscripts indicate significant differences between groups (i.e., "a" is different from "b").

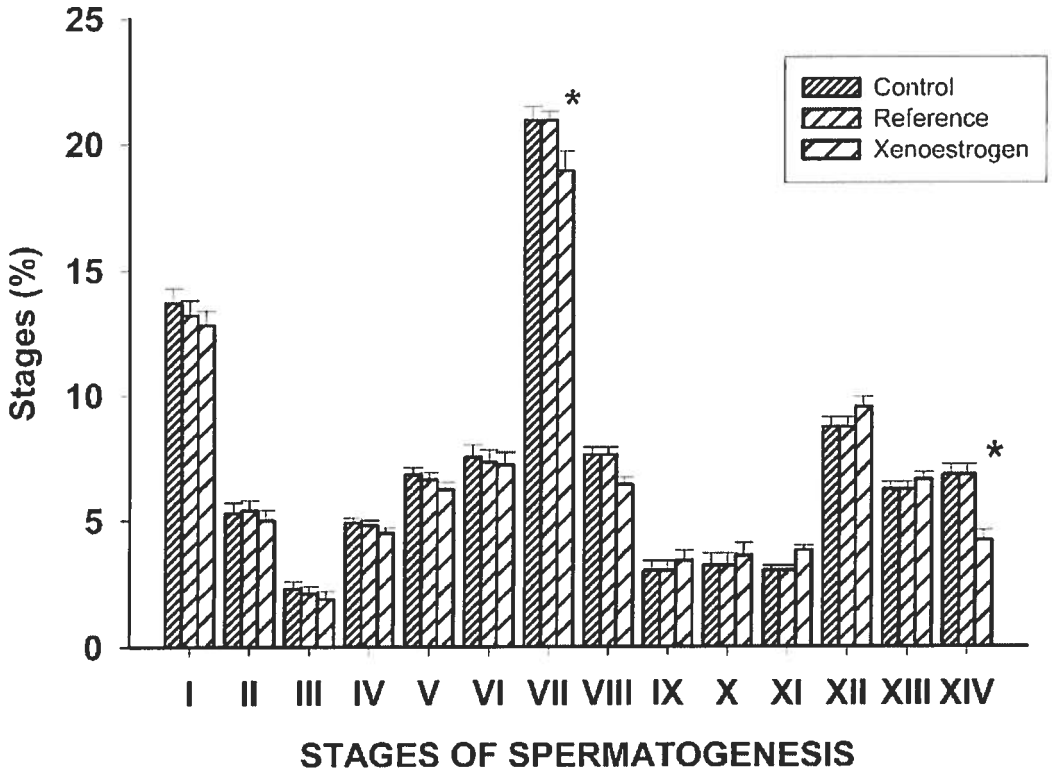

FIG. 4.7 Distribution of the stages of spermatogenesis in adult rats (n = 15 per group) whose mothers were give either water or fish from a reference site of xenoestrogen-contaminated fish during lactation. Testes were removed, fixed in Bouin's solution, and embedded in paraffin. Sections (5 μm) were mounted on glass slides and stained with periodic acid–Schiff (PAS) stain. Staging of spermatogenesis was done by evaluating 200 tubules from each adult rat testis. "a" indicates a significant difference between the xenoestrogen group and both the control and reference group.

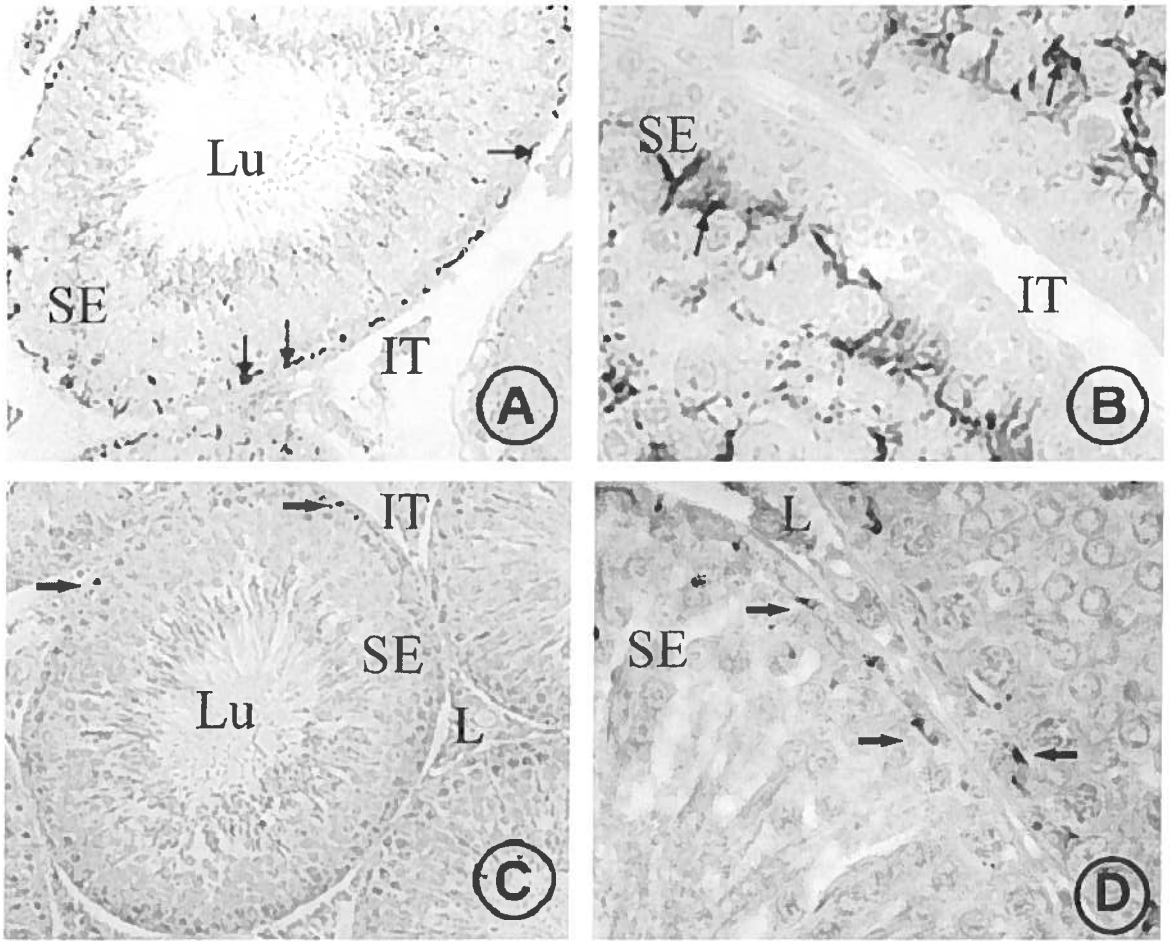

FIG. 4.8 Immunolocalization of Cx43 in testis. Histological sections of adult rat testes (n = 5 per group) whose mothers were give either water (A, B) or fish from a xenoestrogen-contaminated site (C, D) during lactation were immunostained with anti-Cx43 antibody and a horseradish peroxidase-linked secondary antibody. Cx43 immunostaining is indicated by arrows. The micrographs indicate that there is less Cx43 immunostaining in rats exposed to xenoestrogens. Lu = lumen, L = Leydig cells, IT = intertubular space. Magnification x400 (A, C); x640 (B, D).

5.0 NONYLPHENOL ALTERS CONNEXIN 43 LEVELS AND CONNEXIN 43 PHOSPHORYLATION VIA AN INHIBITION OF THE p38-MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

5.1 ABSTRACT

Endocrine-disrupting chemicals are exogenous compounds that mimic or inhibit the action of estrogens or other hormones. Nonylphenol, an environmental contaminant distributed along the St. Lawrence River, has been reported to act as a weak estrogen. Previous studies from our laboratory have shown that rats that were fed fish taken from nonylphenol contaminated sites have altered spermatogenesis and decreased sperm count. The mechanism responsible for this effect is unknown. Gap junctional intercellular communication (GJIC) in the testis is critical for coordinating spermatogenesis. The objectives of the study were to determine the effects of nonylphenol on GJIC and connexin 43 (Cx43) in a murine Sertoli cell line, TM4. Cells were exposed for 24 h to different concentrations (1 to 50 μM) of either nonylphenol or 17ß-estradiol. GJIC was determined using a microinjection approach in which Lucifer yellow was injected directly into a single cell, and GJIC was assessed 3 min postinjection. Nonylphenol exposure decreased GJIC between adjacent cells by almost 80% relative to controls. A significant concentration-dependent reduction in GJIC was observed at nonylphenol concentrations between 1 and 50 μM. Cx43 immunofluorescent staining was reduced at both 10 and 50 μM doses of nonylphenol. Cx43 phosphorylation, as determined by Western blot analysis, was reduced at both 10 and 50 μM concentrations, which may explain, at least in part, the inhibition of GJIC. In contrast, no effect on GJIC or Cx43 protein was observed in cells exposed to 17ß-estradiol at

these concentrations. Cx43 has been reported to be phosphorylated via the p38-mitogen-activated protein kinase (MAPK) pathway. P38-MAPK activity was assessed in both control and nonylphenol-exposed cells. A dose-dependent decrease in p38-MAPK activity was observed in nonylphenol-exposed Sertoli cells. Protein kinase C activity was also measured and was not influenced by nonylphenol. These results suggest that nonylphenol inhibits GJIC between Sertoli cells and that this is modulated via nonestrogenic pathways.

5.2 INTRODUCTION

Several reports suggest a deterioration in the quality of male human reproductive function over the last 50 yr (Carlsen E et al.,1992; Aitken RJ et al.,2004). Male reproductive parameters that have been reported as altered include a decrease in sperm count, increased incidences of congenital malformations of the male reproductive tract, and testicular cancer among young men (Toppari J et al.,1996; Sharpe RM et al.,2001). While it is not clear what factors or changes in lifestyle may be responsible for these changes, it has been suggested that exposure to certain environmental contaminants may be contributing to these effects (Sharpe RM et al.,1993). Among the different classes of reproductive toxicants present in the environment, those that act as endocrine-disrupting chemicals have been singled out as contributing to male reproductive dysfunction (Aitken RJ et al.,2004; Brody JG et al.,2003; Sharpe RM et al.,2004). We have previously shown that spermatogenesis is delayed in fish exposed to xenoestrogens in the St. Lawrence River (Aravindakshan J et al.,2004a). Furthermore, if lactating rats are fed fish from xenoestrogen contaminated sites, the male pups will produce fewer sperm and sperm with decreased motility when they reach adulthood. As well, spermatogenesis appears to be altered,

and a decrease in the expression of the gap junctional protein connexin 43 (Cx43) in the testis has been shown (Aravindakshan J et al.,2004b).

Studies have reported that alkylphenol ethoxylates from municipal sewage effluent are estrogenic chemicals that may contribute to the xenoestrogenic effects observed in fish from the St. Lawrence River (Aravindakshan J et al.,2004a; Sabik H et al.,2003). There has been speculation that certain phenolic plasticizing agents, such as p-nonylphenol, which are now prevalent in the environment, may affect Sertoli cell development and function, because males have much lower levels of estradiol than females (Toppari J et al.,1996; Soto AM et al.,1991). Studies have reported that severe testicular abnormalities, including poor germ cell differentiation and reduced sperm counts, are observed following gestational, lactational, or direct exposure of male rats to moderate levels of several alkylphenols (Sharpe RM et al.,1995; de Jager C et al.,1999; de Jager C et al.,1999). Similar adverse effects on testicular structure and function have been observed following exposure to artificial estrogens, such as diethylstilbestrol or ethinylestradiol in rodents (Atanassova N et al.,1999). It has been suggested that the toxic effects of alkylphenols are mediated via estrogen receptors (Laws SC et al.,2000). Of interest, alkylphenols have also been shown to induce apoptosis in a wide variety of cells (Roy D et al.,1997), including rat primary germ and Sertoli cell cultures, while 17ß-estradiol was without effect (Raychoudhury S et al.,1999). Another report has shown that hCG-stimulated steroidogenesis in cultured mouse Leydig tumor cells was inhibited by octylphenol in an estrogen receptor-independent manner (Nikula H et al.,1999), suggesting that the biological effects of alkylphenols may not be mediated entirely through direct interaction with estrogen receptors.

Spermatogenesis requires direct intercellular communication between Sertoli cells, which is mediated by gap junctions (Roscoe WA et al.,2001). An important role of gap junctions is to regulate cell growth and differentiation by controlling the passage of small molecules, including secondary messengers, between adjacent cells (Bruzzone R et al.,1996). Gap junctions are composed of intercellular pores that allow the passage of small molecules between adjacent cells (<1 kDa). These pores are composed of hexameric connexins from each cell, which are themselves formed by the oligomerization of connexins. Cx43 is present in many tissues, including the testis, and is localized between adjacent Sertoli cells, Sertoli cells and germ cells, and between Leydig cells (Risley MS et al.,1992; Tan IP et al.,1996; Batias C et al.,2000; Perez-Armendariz EM et al.,2001). Several lines of evidence indicate that Cx43 is essential for normal testicular function (Roscoe WA et al.,2001, Batias C et al.,2000, Juneja SC et al.,1999; Plum A et al.,2000; Batias C et al.,1999).

The objective of the present study was to determine whether nonylphenol could alter intercellular communication in Sertoli cells and to determine if this effect was mediated via an estrogenic pathway.

5.3 MATERIALS AND METHODS

5.3.1 Cell Culture

TM4 mouse Sertoli cells were purchased from the American Type Culture Collection (Manassas, VA). TM4 cells were grown in medium containing a mixture of Ham F12 medium

and Dulbecco modified Eagle medium (1:1) with 1.2 g/L sodium bicarbonate and 15 mM Hepes (92.5%), horse serum (5%), and fetal bovine serum (2.5%) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin. For subculturing, the medium was removed, and the cells were rinsed with a solution of 0.25% trypsin and 0.03% EDTA. The solution was removed and an additional 1 to 2 ml of trypsin-EDTA solution was added. The flask was incubated at 37°C until the cells detached. The cells were then washed with fresh culture medium and subsequently aspirated and pelleted by centrifugation. The supernatant was discarded and the cells were then resuspended in fresh culture medium and dispensed into new culture flasks.

5.3.2 Treatment and Cell Viability

To determine whether nonylphenol affects Sertoli cells via an estrogenic pathway, we treated TM4 Sertoli cells with either nonylphenol or 17ß-estradiol. Nonylphenol and 17ß-estradiol were dissolved in ethanol (0.05%), which was used as the vehicle. TM4 cells were passaged at a concentration of 1 x 106 cells/ ml and incubated overnight. The cells were then washed and left untreated (control), exposed to 0.05% ethanol (vehicle), exposed to varying concentrations of nonylphenol (0.1 nM to 50 μM; Schenectady International, Schenectady, NY), or exposed to varying concentrations of 17ß-estradiol (0.1 nM to 1 μM). After 24 h, cells were harvested and cell viability was assessed by trypan blue exclusion.

5.3.3 Immunolocalization of Cx43

Cells were plated on glass coverslips and fixed in ice-cold methanol for 30 min at –20°C. Cells were washed in PBS and blocked with 2% BSA in PBS for 20 min at room temperature. This was followed by three 5-min washes in PBS. Immunocytochemical localization of Cx43 was performed using rabbit polyclonal anti-Cx43 antisera (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were incubated for 90 min in a hydrated chamber with the primary antibody at room temperature. The cells were then washed in PBS and incubated for 45 min with a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (1: 2000; Jackson Immunoresearch, West Grove, PA). The cells were subsequently washed three times in PBS and mounted with Vectashield containing propidium iodide (Vectastain Laboratories, Burlington, ON). The cells were viewed and photographed under a Leica fluorescent microscope.

5.3.4 Estimation of Gap Junctional Intercellular Communication by Dye-Transfer Assay

TM4 Sertoli cells were cultured with 17ß estradiol, nonylphenol, or vehicle alone for 24 h. To determine the functionality of gap junctions, gap junctional intercellular communication (GJIC) was assayed by scoring the number of dye-coupled cells after microinjection of a single cell with Lucifer yellow (Sigma Chemical Co., St Louis, MO) using an Eppendorf pressure injection system. The extent of GJIC was determined by the number of neighboring fluorescent cells, scored with the aid of a fluorescence/ phase-contrast microscope 3 min postinjection. For each experimental point, 10–20 independent injections were carried out.

5.3.5 Western Blot Analysis

Culture medium was removed and the cells were rinsed with PBS at room temperature. The following steps were performed on ice using fresh, ice-cold buffers. A 1-ml aliquot of RIPA buffer (1x Tris-buffered saline (TBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, and 100 μM sodium orthovanadate) was added to the cell culture plate and adherent cells were scraped with a cell scraper. The scraped lysate was transferred to a clean microcentrifuge tube. The plate was washed once again with 0.3 ml of RIPA buffer and then combined with the first lysate. The lysate was passed through a 21-gauge needle to shear the DNA and incubated for 60 min on ice. The lysates were centrifuged at 10 000 x g for 10 min at 4°C. The supernatant was collected and protein concentration determined using the Bradford method (Protein Assay Kit; Bio-Rad Laboratories, Mississauga, ON). An aliquot of 40 μg of the whole cell lysate was mixed with an equal volume of electrophoresis sample buffer (5% v/v glycerol, 2.5% v/v 2-mercaptoethanol, 1% SDS, 125 mM Tris-HCl pH 6.7, and 0.4% w/v bromophenol blue) and loaded on a 10% polyacrylamide gel with a 4% stacking gel. Electrophoresis was carried out at 90 V until the dye front reached the bottom of the gel. The proteins were transferred from the gel onto a polyvinylidene difluoride membrane using an electroblotting apparatus according to the manufacturer's protocols (Bio-Rad Laboratories). To block nonspecific binding, the membranes were incubated in 5% Blotto for 60 min at room temperature. The blocked membrane was incubated with anti-Cx43 antisera (1:200 in Blotto; Santa Cruz Biotechnology) for 1 h at room temperature. The membranes were washed three times for 5 min each with TBST (TBS with Tween-20, pH 7.4). The membrane was then incubated for 45 min at room temperature with an alkaline phosphatase-conjugated secondary antibody (1:2000 in Blotto; Sigma-Aldrich, Mississauga, ON). Following the incubation, the

membranes were once again washed three times for 5 min with TBST and once for 5 min with TBS (pH 7.4). Quantification of Cx43 was performed by scanning the immunoblots using ImageQuant software (Bio-Rad Laboratories).

5.3.6 p38-Mitogen-Activated Protein Kinase Assay

The activity of p38-mitogen-activated protein kinase (MAPK) was analyzed by using a p38 MAPK assay kit (Cell Signaling Technology Inc, Beverly, MA) according to the manufacturer's instructions. Briefly, phosphorylated p38-MAPK was immunoprecipitated from 200 μg of cell lysate with an anti-p38-MAPK phospho-specific antibody. The immunoprecipitated protein pellets were washed thoroughly and resuspended in kinase buffer containing ATP and 1 μg of recombinant activating transcription factor-2 (ATF-2) as a p38-MAPK substrate. The reaction was incubated at 30°C for 45 min and terminated by the addition of 3x SDS sample buffer (187.5 mM Tris-HCl pH 6.8 at 25°C, 6% w/v SDS, 30% glycerol, 150 mM dithiothreitol, and 0.03% w/v bromophenol blue). The kinase reaction (ATF-2 phosphorylation) was detected using anti-phospho-ATF-2 (Thr71) antisera by Western blotting and chemiluminescent detection.

5.3.7 Protein Kinase C Activity Assay

The protein kinase C (PKC) activity of TM4 Sertoli cells was determined using the PepTag PKC assay kit (Promega, Ottawa, ON), according to the manufacturer's instructions. This assay utilizes a florescent peptide substrate that is specific to PKC. Phosphorylation by PKC

changes the net charge of the substrate from +1 to −1, allowing the phosphorylated and nonphosphorylated forms of the substrate to be separated on an agarose gel (1%), because the phosphorylated species migrates toward the anode, while the nonphosphorylated substrate migrates toward the cathode. The samples were separated on an agarose gel at 100 V for 15 min, and the bands were visualized under UV light. The negatively charged phosphorylated bands were excised using a razor blade, placed in a graduated microcentrifuge tube, and heated at 95°C until the gel slice melted. The volume of the solution was adjusted to 250 μl with water. The hot agarose solution (175 μl) was added to a separate tube containing 75 μl of gel solubilization solution, 100 μl of glacial acetic acid, and 150 μl of distilled water. The mixture was vortexed and then transferred to a spectrophotometric cuvette, and absorbency was read at 570 nm. One unit of kinase activity is defined as the number of nanomoles of phosphate transferred to a substrate in 1 ml/min. One phosphorylation site exists on each peptide; therefore, the number of moles of peptide present in the negatively charged, phosphorylated bands is equivalent to the number of moles of phosphate transferred. The number of moles of phosphorylated peptide () was determined by calculating the number of units of kinase activity in each slice of agarose.

5.3.8 Statistical Analysis

Data are expressed as mean ± SEM unless otherwise indicated, of at least three independent experiments performed at different time points. The data were tested for normality and homogeneity of variance using Kolmogorov-Smirnov test. All data were analyzed using one-way analysis of variance (ANOVA) (SigmaStat for Windows, Version 2.0, Jandel Corporation,

San Rafael, CA). If the differences were significant, a Student-Newman-Keuls test was used for post-ANOVA multiple comparisons ($P < 0.05$).

5.4 RESULTS

5.4.1 Effects of Nonylphenol on Cell Viability

To establish whether or not nonylphenol or estradiol altered cell viability, Sertoli cells were exposed for 24 h to different concentrations of nonylphenol (0.1 nM to 50 μM) and viability was assessed by trypan blue exclusion. Concentrations of nonylphenol ranging between 0.1 nM and 25 μM did not exert any significant effects on Sertoli cell viability (Table 5.1). In contrast, when cells were exposed to a 50 μM concentration of nonylphenol, the number of viable cells was decreased by 28% ($P < 0.05$; Table 5.1). Because nonylphenol is at least 1000 times less estrogenic than 17ß-estradiol, we treated the Sertoli cells with doses ranging between 0.1 nM to 1 μM. 17ß-Estradiol did not alter cell viability at any of these doses (data not shown). Nonylphenol exposure at the 10 μM dose did not appear to alter either apoptosis or cell proliferation as determined either by the general morphology of the cells or in the expression of cell cycle or apoptosis genes (data not shown).

5.4.2 Effects of Nonylphenol on GJIC

To assess the effects of nonylphenol on functional GJIC, Sertoli cells were microinjected with Lucifer yellow and GJIC was assessed by examining the transfer of dye between cells. In control cells, baseline coupling varied from 4 to 6 cells, with dye passing to a mean of 5.56 cells

(Fig. 5.1Aa). 17ß-Estradiol treatment did not affect GJIC between adjacent cells and these cells were not different from controls (Fig. 5.1Ab). A significant reduction in GJIC was observed at 10 μM nonylphenol (Fig. 5.1Ac). Following exposure to 10 μM nonylphenol, the number of coupled cells decreased between 0 and 1 cells, an almost 80% reduction relative to controls. This indicated that the functionality of gap junctions was reduced (Fig. 5.1B).

5.4.3 Immunolocalization of Cx43

To assess whether or not the decrease in gap junctional communication was associated with a decrease or alteration in the cellular localization of Cx43, immunolocalization was performed following 24 h of treatment. In control cells (Fig. 5.2a), a punctate immunostaining for Cx43 along the plasma membrane of adjacent cells was observed. Sertoli cells treated with different doses of 17ß-estradiol did not affect Cx43 localization, and the intensity of the immunostaining appeared to be similar to that of control cells (Fig. 5.2b). However, in cells treated with nonylphenol (1–50 μM), the intensity of the immunostaining was markedly decreased at all doses used. The localization of Cx43 in the nonylphenol-treated cells did not appear to be affected by treatment, and the immunostaining was restricted to the plasma membrane of the cells at areas of contact between neighboring cells. However, in cells treated with 10 μM nonylphenol, the intensity of the immunoreaction was substantially decreased (Fig. 5.2c) and was almost completely absent in cells treated with 50 μM (Fig. 5.2d).

The effects of nonylphenol appeared to be both dose- and time-dependent. The decrease in Cx43 immunostaining induced by nonylphenol was evident as early as 6 h after the addition of nonylphenol and decreased gradually until 24 h.

5.4.4 Effects of Nonylphenol on Cx43 Levels in Sertoli Cells

Cx43 levels in Sertoli cells were determined by Western blot analysis. Cx43 is a phosphorylated protein, and its phosphorylation status has been shown to be critical for its function. Western blots of enriched membrane fractions of Sertoli cells revealed two major bands, representing the phosphorylated and nonphosphorylated forms of Cx43 (Fig. 5.3A).

Sertoli cells incubated with nonylphenol expressed significantly less Cx43 (Fig. 5.3B). Furthermore, both the phosphorylated and nonphosphorylated Cx43 levels were significantly lower in nonylphenol-exposed cells (Fig. 5.3, B and C). However, when the levels of phosphorylated Cx43 were compared with those in either nonexposed or vehicle-treated cells, there was a significant reduction in the ratio of phosphorylated to nonphosphorylated Cx43 in the nonylphenol-treated cells (Fig. 5.3D). The decrease in Cx43 phosphorylation occurred as early as 6 h after the start of treatment and continued to decrease until 24 h (Fig. 5.4, A–D). There were no significant differences in the phosphorylation status of Cx43 in the 17ß-estradiol treated cells (Fig. 5.5, A–D). This suggested that nonylphenol not only decreased Cx43 expression levels but also its phosphorylation status.

5.4.5 p38-MAPK and PKC Activity

To understand the mechanisms by which Cx43 phosphorylation was altered by nonylphenol, both p38-MAPK and PKC activity were measured, because these are known to

phosphorylate Cx43 in other cell types (Saez JC et al.,1997; Warn-Cramer BJ et al.,1996; Lampe PD et al.,2000).

We first determined whether or not p38-MAPK represented a biologically active kinase in TM4 Sertoli cells. This was done by testing whether phosphorylated p38-MAPK found in TM4 Sertoli cells could phosphorylate its downstream substrate, ATF-2. Using an in vitro kinase assay we were able to demonstrate that p38-MAPK can phosphorylate ATF-2 and that it is therefore active in TM4 cells (Fig. 5.6A).

Once it had been established that TM4 Sertoli cells possessed p38-MAPK activity we assessed whether or not nonylphenol could alter its activity. Western blot analysis, with ATF-2-phospho-specific antibody, revealed that exposure to nonylphenol resulted in a dose-dependent decrease of ATF-2 phosphorylation by as much as 80%, indicating an inhibition of p38-MAPK activity (Fig. 5.6B). There was no significant change in p38-MAPK activity in cells treated with 17ß-estradiol (Fig. 5.6, C and D).

Because PKC has also been shown to phosphorylate Cx43, we examined the level of PKC activation in TM4 Sertoli cells exposed to either nonylphenol or 17ß estradiol. PKC activity was not altered in cells exposed to either nonylphenol or 17ß-estradiol, compared with that of untreated control cells (Fig. 5.7, A and B).

5.5 DISCUSSION

Nonylphenol is the final biodegradation product of nonylphenol polyethoxylates, a major contaminant in the St. Lawrence River (Ekeland E et al.,1993; Giger W et al.,1984). Rats whose mothers are fed fish from these sites during lactation have reduced testicular Cx43 expression (Aravindakshan J et al.,2004b). In the present study we have shown that TM4 Sertoli cells form functional intercellular gap junctions between adjacent cells by allowing the passage of microinjected Lucifer yellow between cells. Using both immunocytochemistry and Western blot analyses, we demonstrated that TM4 Sertoli cell gap junctions are composed of Cx43. These junctions appear as punctate structures localized to cell-cell contacts between adjacent cells. Gap junctions were shown to be present between adjacent Sertoli cells of the testis (Gilula NB et al.,1976). McGinley et al. (McGinley DM et al.,1979) also reported that gap junctions were present between Sertoli cells and developing germ cells. Tan et al. (Tan IP et al.,1996) first reported that Cx43 was localized between adjacent Sertoli cells, as well as between developing germ cells and Sertoli cells in the adult rat. Other studies have reported the presence of connexins 26, 31, 32, 33, 37, 40, and 45 in the seminiferous tubules of the rat (Risley MS et al.,2000).

While exposure of the cells to estradiol did not alter intercellular communication, exposure to nonylphenol dramatically reduced intercellular communication at all doses tested. This effect suggests that while nonylphenol is considered an estrogenic substance (Tapiero H et al.,2002), its effects on intercellular communication are not mediated via the estradiol receptor

pathway. This is despite the fact that estrogen receptors have been shown to be expressed in TM4 Sertoli cells (Nakhla AM et al.,1984; Nakhla AM et al.,1989).

While there have not been any studies to our knowledge regarding the effects of nonylphenol on intercellular communication, there is a growing list of environmental toxicants that appear to alter intercellular communication. Using the microinjection technique, we were able to demonstrate that nonylphenol almost completely inhibited GJIC at higher exposure doses. Studies in nonreproductive tissues have also been shown to reduce GJIC. Hexachlorobenzene, an organochlorine, can promote gender-specific tumor promotion by blocking intercellular communication between hepatocytes in the rat (Plante I et al.,2002). Other toxicants such as dioxins, polychlorinated aromatic hydrocarbons, and cadmium can also decrease gap junctional communication in cultured hepatocytes (Jeong SH et al.,2000; Krutovskikh VA et al.,1995). Studies by Defamie et al., (Defamie N et al.,2001) have shown that lindane can abolish GJIC between rat Sertoli cells at the 50 μM dose. The importance of Cx43 gap junctions for spermatogenesis is indicated by the severe depletion of germ cells in prenatal male and female mice lacking the Cx43 gene (Juneja SC et al.,1999). Postnatal proliferation of spermatogonia is also impaired in Cx43 null mutants (Roscoe WA et al.,2001). Insertion of Cx32 or Cx40 coding regions into the Cx43 coding region of Cx43–/– mice restored oogenesis and other deficiencies caused by Cx43 deletion, but spermatogonial amplification and spermatogenesis remained defective (Plum A et al.,2000). The importance of Cx43 gap junctions to the regulation of spermatogenesis in adults is supported by the fact that Cx43 immunoreactivity is reduced in spermatogenesis-deficient mutants (Batias C et al.,2000; Batias C et al.,1999). Thus, Cx43 is an essential component of GJIC pathways, which support the early phases of spermatogenesis.

Previous studies have shown that nonylphenol can affect spermatogenesis (Nagao T et al.,2000; Hossaini A et al.,2001; Chitra KC et al.,2002; Adeoya-Osiguwa SA et al.,1990).

The effects of nonylphenol on intercellular communication and Cx43 expression were both dose- and time-dependant. Time-response studies in which cells were exposed to 10 μM of nonylphenol indicated that there was a decrease in Cx43 over time, which peaked 24 h after the start of the exposure. These results suggest that nonylphenol does not appear to act at the level of the gap junction itself, but rather exerts a progressive effect either on the level of renewal of the connexins, or on their synthesis, or both. Further studies will be necessary to clearly establish whether or not this effect is at the transcriptional or post-transcriptional level.

Cx43, like most connexins, is a phosphoprotein with multiple electrophoretic isoforms when analyzed by SDS-PAGE. Phosphorylation has been implicated in the regulation of a broad variety of connexin processes, such as trafficking, assembly/disassembly, degradation, and gating of gap junction channels. Alterations in the phosphorylation status of Cx43 appear to influence gap junctional communication, whether it is hyperphosphorylated or hypophosphorylated (Musil LS et al.,1990; Crow DS et al.,1990; Laird DW et al.,1991; Brissette JL et al.,1991; Kadle R et al.,1991; Berthoud VM et al.,1992). Exposure of Sertoli cells to nonylphenol resulted not only in a decrease in the levels of Cx43, but also in its phosphorylation. Our results suggest that nonylphenol inhibited GJIC between Sertoli cells and that aberrant phosphorylation may have contributed to this effect. These effects were not observed when the cells were treated with 17ß-estradiol. These observations are consistent with other studies that show that Cx43 dephosphorylation is correlated with a reduction in communication via gap

junctions (Matesic DF et al.,1994; Kenne K et al.,1994; de Feijter AW et al.,1996; Nomata K et al.,1996; Sato H et al.,2003; Matesic DF et al.,2001).

The protein kinases responsible for phosphorylating Cx43 or the site or sites targeted in unstimulated cells are unknown. Moreover, the exact number of phosphorylated sites per Cx43 is not known; however, at least five different Cx43 phosphopeptides have been observed, suggesting that Cx43 is phosphorylated at multiple sites (Cooper CD et al., 2000), on serine and threonine residues (Crow DS et al.,1990; Kanemitsu MY, et al.,1997). Cx43 has been shown to be phosphorylated by PKC (Saez JC et al.,1997; Lampe PD et al.,2000) and MAPK (Warn-Cramer BJ et al.,1996).

To understand the mechanism by which nonylphenol decreased the phosphorylation of Cx43, we studied whether or not the p38-MAPK and PKC pathways were altered in nonylphenol-treated Sertoli cells. When cells were treated with nonylphenol, p38-MAPK activity was significantly reduced in a dose-dependent manner. Of interest, there was no change in the PKC activity. The decrease in p38-MAPK suggests that nonylphenol may be selectively inhibiting this pathway, thereby resulting in a decrease in Cx43 phosphorylation. This is in contrast to another environmental contaminant, lindane, which increases Cx43 phosphorylation in Sertoli cells by stimulating the extracellular signal-regulated kinases (ERK) without altering either the JNK or p38-MAPK pathways (Mograbi B et al.,2003). The hyperphosphorylation of Cx43 by lindane also resulted in an increase in the cytoplasmic localization of Cx43, which is absent in nonylphenol-treated Sertoli cells in which Cx43 is hypophosphorylated. This suggests

that hyperphosphorylation and altered ERK pathways are implicated in the localization of Cx43 in Sertoli cells (Mograbi B et al.,2003).

Clearly, the effects observed in the present study indicate that nonylphenol alters intercellular communication via an estrogen-independent pathway, despite the fact that nonylphenol is considered an estrogenic chemical (Soto AM et al.,1991; Shelby MD et al.,1996). Studies have reported that nonylphenol can alter the production of reactive species (ROS) in neuronal cells (Okai Y et al.,2004). Furthermore, it has been shown that Cx32 expression is sensitive to increased ROS production in cultured hepatocytes. Therefore, it is tempting to speculate that nonylphenol action on intercellular communication in Sertoli cells may be mediated by an increased production of ROS. Furthermore, the possibility that nonylphenol is acting on other aspects of cell-cell interactions, such as cell adhesion, and that these affect GJIC cannot be discounted.

In rats exposed to nonylphenol, there is a decrease in circulating levels of serum testosterone and altered spermatogenesis (Tan BL et al.,2003; Han XD et al.,2004). Our results indicate that nonylphenol can decrease intercellular communication and Cx43 levels, which have been shown to be important regulators of both Leydig cell function and spermatogenesis. These results suggest that the in vivo effects of nonylphenol on the testis may be, in part, the result of a decrease in intercellular communication. Furthermore, it has been reported that the activation of p38-MAPK by transforming growth factor-ß is essential for the formation and maintenance of the blood-testis barrier, which is formed by tight junctions between adjacent Sertoli cells (Lui WY et al.,2003; Lui WY et al.,2003). The results from this study would therefore suggest that as

a result of decreased p38-MAPK activity, nonylphenol may also be targeting the blood-testis barrier. It has been reported that Cx43 colocalizes with tight junction proteins in the blood-testis barrier (Cyr DG et al.,1999; Segretain D et al.,2004); hence, the complex association of both tight and gap junctions in the blood-testis barrier may be altered by nonylphenol. Further studies will be necessary to establish whether or not this is the case, and whether these effects all contribute to nonylphenol-induced testicular dysfunction.

In conclusion, the present study provides the first evidence that nonylphenol impairs gap junctional intercellular communication between Sertoli cells. This effect is, in part, the result of a decrease in the expression and phosphorylation of Cx43. The effects of nopnylphenol on testicular GJIC are mediated via an estrogen receptor-independent mechanism and an inhibition of the p38-MAPK pathway.

Compound	Nonylphenol concentration	Number of viable cells†	
		Per plate × 10^4	% of control
Control (untreated)		209 ± 4	
Vehicle (0.05% ethanol)		209 ± 6	100
	0.1 nM	209 ± 3	100
	10 nM	209 ± 7	100
	1 μM	207 ± 15	99
	10 μM	206 ± 21	98
	25 μM	196 ± 21	93
	50 μM	152 ± 32	72

* Results are expressed as the mean + SEM of three separate experiments.
† Viable cells were counted with a hemocytometer.

TABLE 5.1. TM4 cell viability after 24 h treatment with nonylphenol

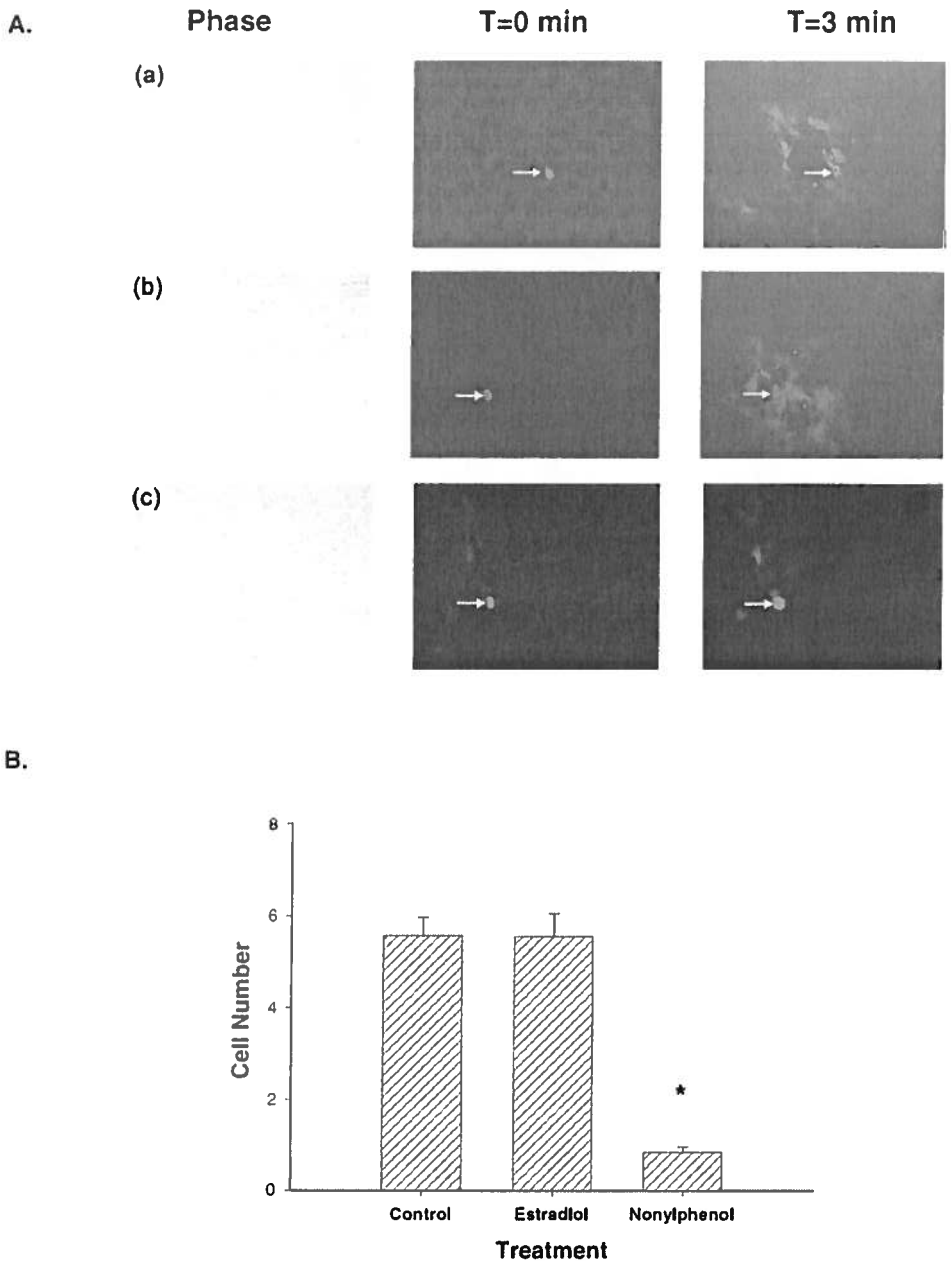

FIG. 5.1. Functional analysis of gap junctions in TM4 Sertoli cells. Confluent cell cultures of TM4 Sertoli cells were treated for 24 h with either vehicle (0.05% ethanol), 1 μM 17ß-estradiol, or 10 μM nonylphenol. Cells were microinjected with Lucifer yellow dye and examined 3 min postinjection for fluorescent dye transfer. Several cells from different cultures were microinjected to establish the effect of treatment. A) Each longitudinal series of panels show the cells before microinjection, the single cell microinjection with Lucifer yellow under fluorescent light, and the transfer of Lucifer yellow between cells after 3 min. Vertical panels indicate (a) control, (b) 1 μM 17ß-estradiol treated cells, and (c) 10 μM nonylphenol treated cells. Arrows indicate the cell injected with Lucifer yellow. Magnification x400. B) Quantitative analysis of GJIC in TM4 cells treated with either vehicle (control), 17ß-estradiol, or nonylphenol (10 μM). Asterisk indicates a significant difference from both control and estradiol-treated groups (ANOVA, $P < 0.05$)

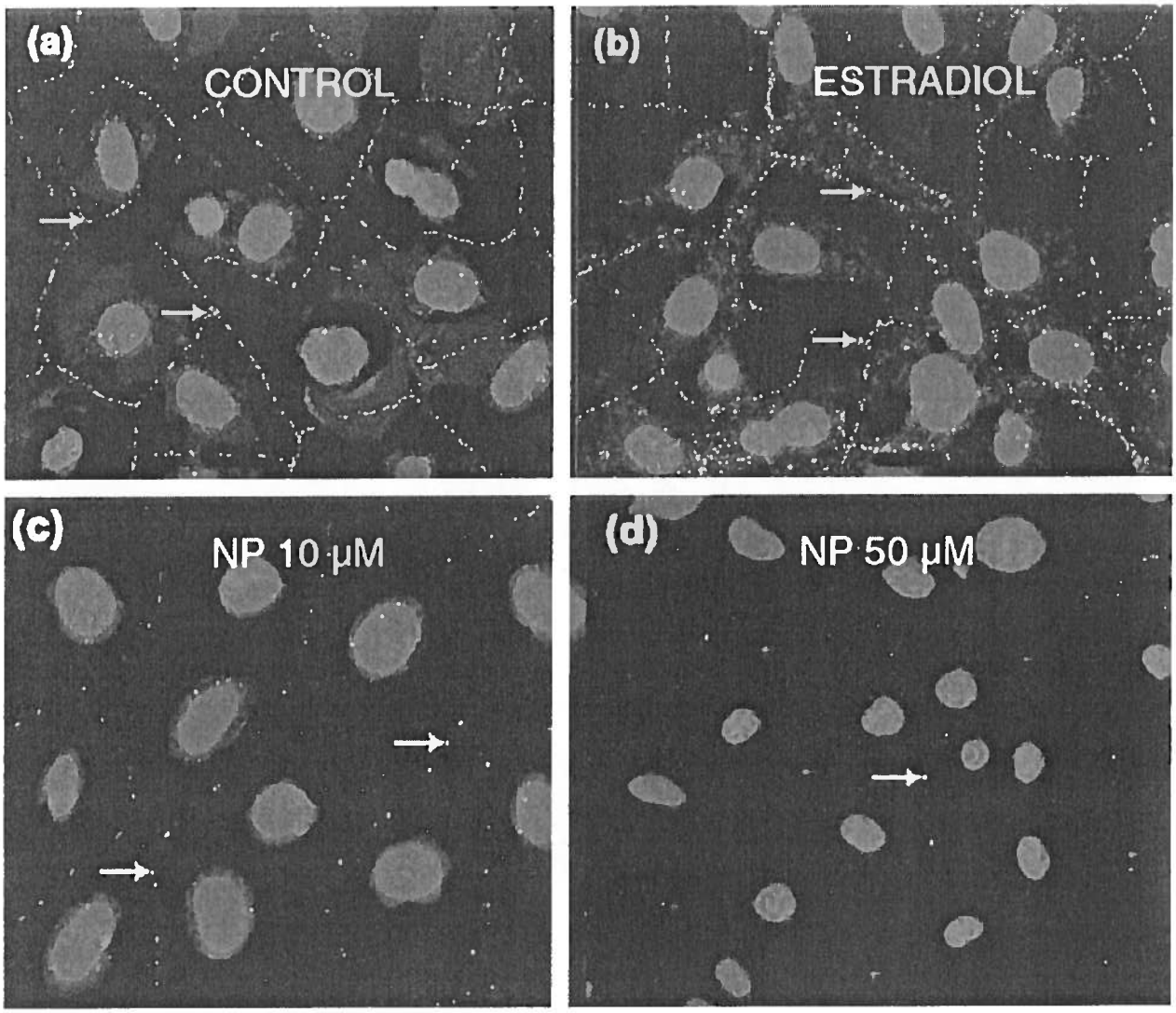

FIG. 5.2. Photomicrographs of TM4 Sertoli cells showing the immunolocalization of Cx43. a) In the untreated TM4 Sertoli cells, punctate Cx43 immunofluorescence (arrows) is prominent in the appositional plasma membranes between adjacent cells. b) 17ß-Estradiol treated cells did not show significant changes in Cx43 staining. In cells incubated with either 10 μM (c) or 50 μM (d) of nonylphenol for 24 h, Cx43 immunostaining (green) is dramatically decreased (arrows). Nuclei are stained with propidium iodide (red). Magnification x600

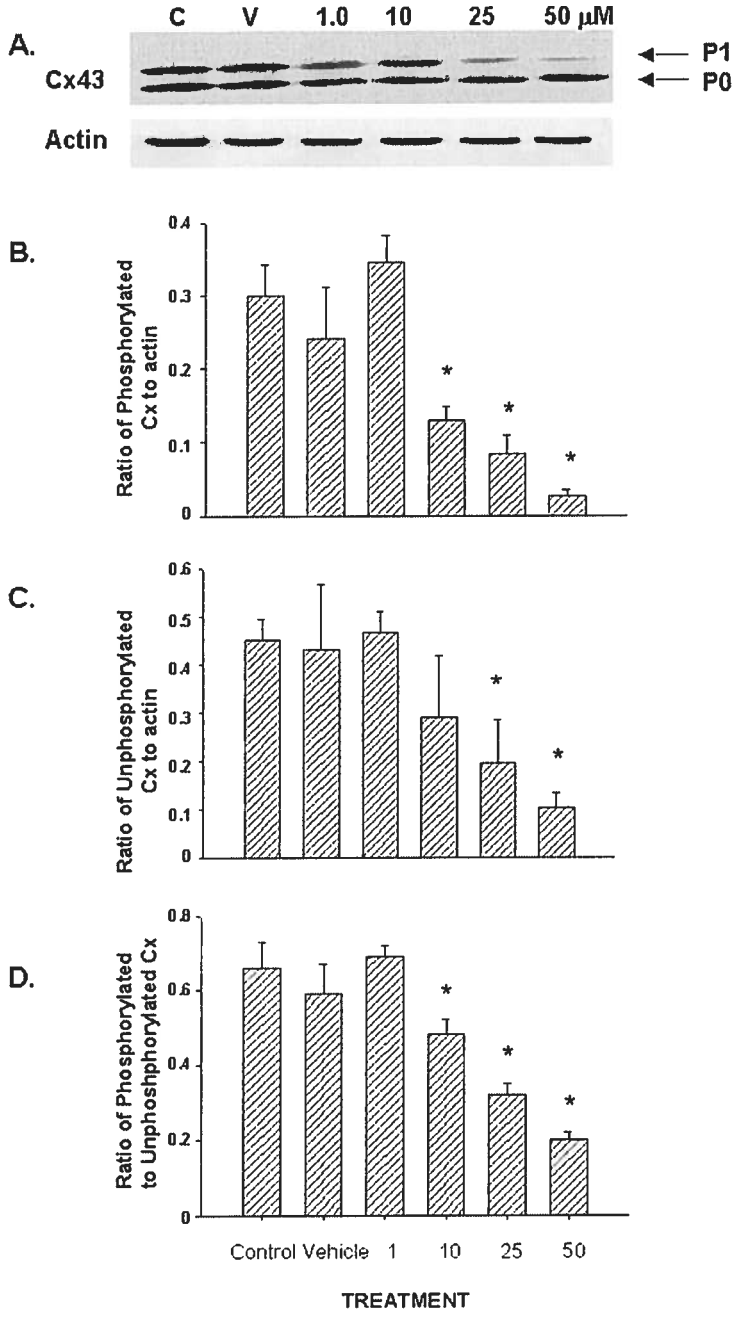

FIG. 5.3. Western blot analysis of Cx43 levels in nonylphenol treated TM4 Sertoli cells. Cells were incubated with either medium alone (C), vehicle (0.05% ethanol), or varying concentrations of nonylphenol (1, 10, 25, or 50 μM) for 24 h. Total proteins (40 μg) were extracted from TM4 cell monolayers and proteins were separated by SDS-PAGE. Cx43 was detected using an alkaline phosphatase-conjugated secondary antibody and revealed by chemiluminescence (A). Protein loading was standardized using actin as an internal control (A). Unphosphorylated (B) and phosphorylated (C) Cx43 levels were determined by densitometry and corrected for protein loading using actin levels. The ratio of phosphorylated to nonphosphorylated Cx43 was also assessed (C). Data are expressed as the mean + SEM of four different experiments. Asterisks indicate significant differences from control and vehicle group (ANOVA; $P < 0.05$)

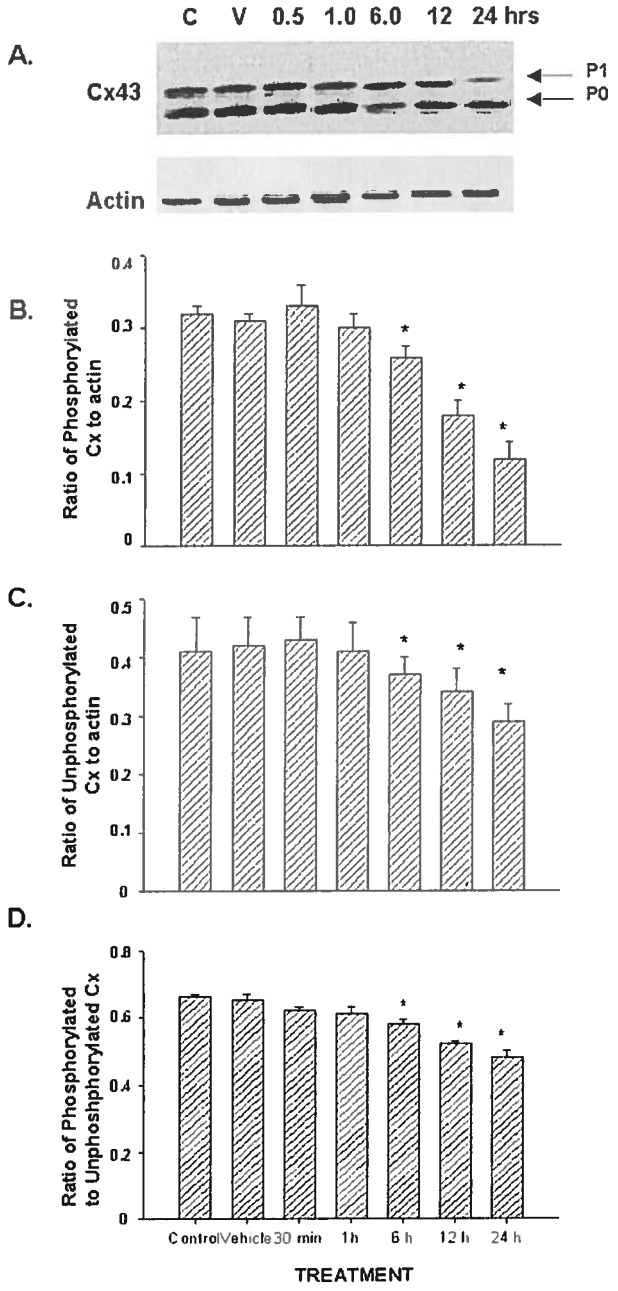

FIG. 5.4 Western blot analysis of Cx43 levels in nonylphenol treated TM4 Sertoli cells at different times following the start of treatment. Cells were incubated with either medium alone (C), vehicle (0.05% ethanol), or nonylphenol (10 μM). Total proteins (40 μg) were extracted from TM4 cell monolayers and proteins were separated by SDS-PAGE at 0.5, 1, 6, 12, and 24 h following the addition of nonylphenol. Cx43 was detected using an alkaline phosphatase conjugated secondary antibody and revealed by chemiluminescence (A). Protein loading was standardized using actin as an internal control (A). Unphosphorylated (B) and phosphorylated (C) Cx43 levels were determined by densitometry and corrected for protein loading using actin levels. The ratio of phosphorylated to nonphosphorylated Cx43 was also assessed (D). Data are expressed as the mean + SEM of four different experiments. Asterisks indicate significant differences from control and vehicle group (ANOVA; $P < 0.05$)

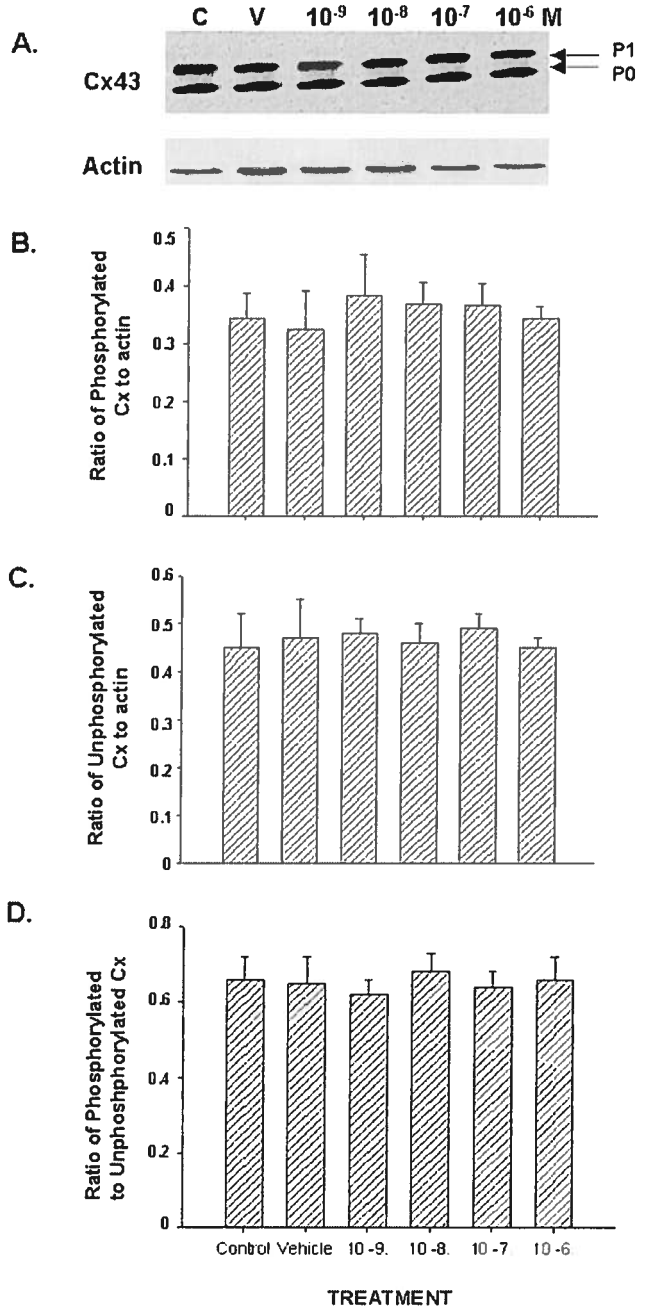

FIG. 5.5 Western blot analysis of Cx43 levels in 17ß-estradiol treated TM4 Sertoli cells. Cells were incubated with either medium alone (C), vehicle (0.05% ethanol), or varying concentrations of 17ß-estradiol (10–9 to 10–6 M) for 24 h. Total proteins (40 μg) were extracted from TM4 cell monolayers and proteins were separated by SDS-PAGE. Cx43 was detected using an alkaline phosphatase-conjugated secondary antibody and revealed by chemiluminescence (A). Protein loading was standardized using actin as an internal control (A). Unphosphorylated (B) and phosphorylated (C) Cx43 levels were determined by densitometry and corrected for protein loading using actin levels. The ratio of phosphorylated to nonphosphorylated Cx43 was also assessed (D). Data are expressed as the mean + SEM of four different experiments. There were no significant differences between the control and vehicle exposed groups.

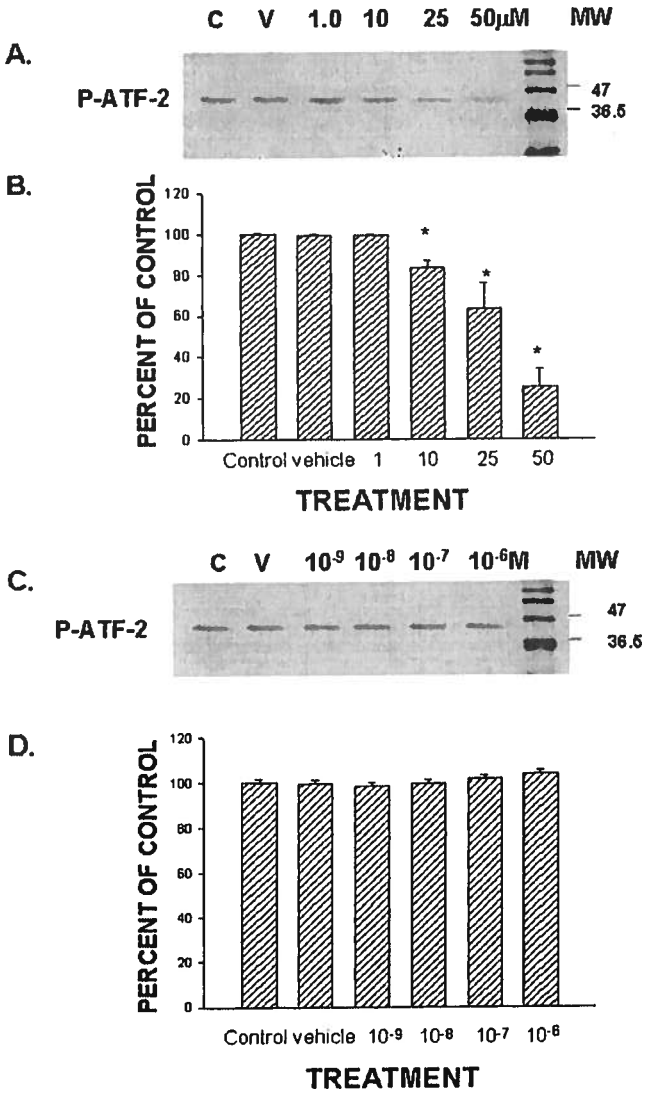

FIG. 5.6 Effects of nonylphenol on p38-MAPK activity in TM4 Sertoli cells. Using a commercial assay kit, p38-MAPK activity was determined by measuring the phosphorylation levels of the ATF-2 peptide. Protein extracts from TM4 cells were analyzed by immunoprecipitation followed by kinase activity assay. Cell extracts were immunoprecipitated with immobilized phospho-p38 MAPK (Thr180/Try182) monoclonal antibody. In vitro p389-MAPK assays were performed using the ATF-2 fusion protein as a substrate. Phosphorylation of ATF-2 at Thr71 was measured by Western blotting using a phospho-ATF-2 (Thr71) antibody and quantified by densitometry. A) Cells were incubated with either medium alone (C), vehicle (V, 0.05% ethanol), or varying concentrations of nonylphenol (1, 10, 25, or 50 μM) for 24 h. Data are expressed as the mean + SEM from four separate experiments (B). To assess whether p38-MAK was altered by estradiol, cells were incubated with either medium alone (C), vehicle (0.05% ethanol), or varying concentrations of 17ß-estradiol (10–9 to 10–6 M) for 24 h (C). Data from these experiments are expressed as the mean + SEM from four separate experiments (D). Asterisks indicate a significant difference from either control or vehicle exposed cells (ANOVA; $P < 0.05$)

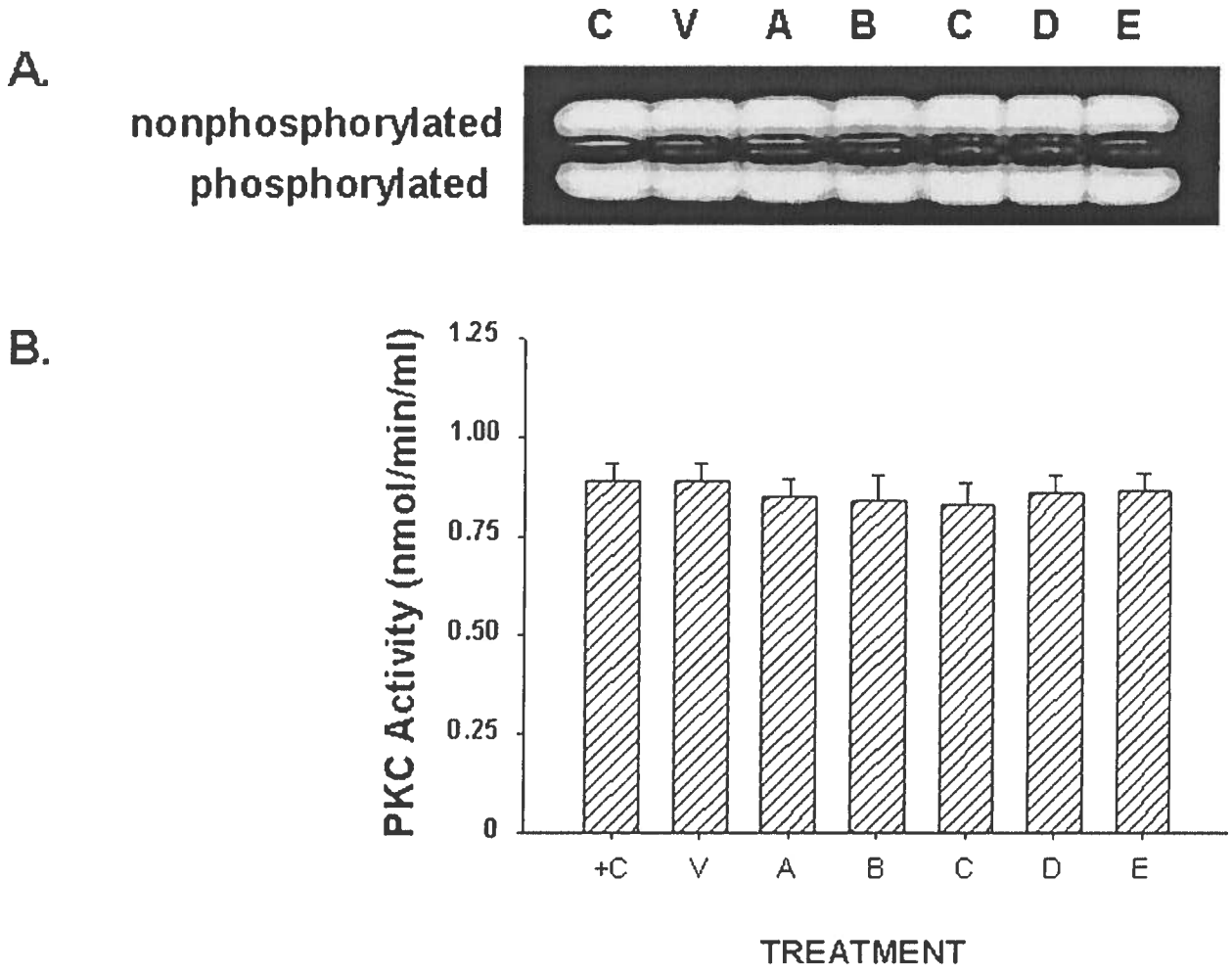

FIG. 5.7. Activation of PKC in TM4 Sertoli cells treated with either nonylphenol or 17ß-estradiol for 24 h. Total cell lysates were prepared and subjected to the PepTag assay. The phosphorylated peptide bands (A) were excised, and PKC activity was estimated by spectrophotometry (B). Cells were incubated with either medium alone (C), vehicle (V, 0.05% ethanol), 17ß-estradiol (A; 1 μM), or varying concentrations of nonylphenol (1 μM, B; 10 μM, C; 25 μM, D; 50 μM, E) for 24 h (A). Data are expressed as the mean + SEM from four separate experiments (B). There were no significant differences between any experimental group (ANOVA)

6.0 DISCUSSION AND CONCLUSIONS

The objectives of my first study was to determine the presence of estrogenic chemicals in the St. Lawrence River using the biomarker vitellogenin and their effects on male reproduction in the spottail shiner (*Notropis hudsonius*). Hepatic vitellogenin (VTG) mRNA levels in immature shiners was measured by PCR using gene specific primers. Results indicated extensive estrogenic contamination spanning almost 50 km both upstream and downstream from the island of Montreal. To determine effects on spermatogensis, stages of spermatogenesis were assessed in fish captured at sites having varying levels of estrogenic contamination. In control fish, 95% had testis of either stage IV (50%) or stage V (45%) of spermatogenesis, which indicate normal spermatogensis. At Ile Dorval, where VTG mRNA levels are moderate, fish had testes of stage III (38%) and IV (45%) and only 15% of fish were at spermatogenic stage V. In contrast, at Ilet Vert and Ile Beauregard, located in the sewage effluent plume from the City of Montreal and where hepatic VTG mRNA levels are high in fish, none of the fish were at stage V and 8% of fish at Ilet Vert were at stage II of development. This indicates a dramatic effect on spermatogenesis, when fish are exposed to xenoestrogens. Sperm concentration and various motility parameters were significantly lower in shiners from Ilet Vert as compared with those from Iles de la Paix (reference). Histological analyses of testes revealed that more than one-third of the fish captured at sites with the highest estrogenic contamination displayed intersex, a condition in which ovarian follicles were developing within the testis. From this study we conclude that there is significant estrogenic contamination in specific sites we looked in the St. Lawrence River. This contamination resulted in impaired reproductive function in male fish.

As a continuation of the first study, we were intersted in determining if transfer of endocrine-disrupting effects occurs through the food chain. The transfer of chemicals, particularly from the aquatic ecosystem, on fish-eating predators must be established. Therefore in the second part of my study we wanted to determine if lactational exposure to contaminated fish could alter the development of the male reproductive system in rats. It should be emphasised here that such experiments cannot be done on humans due to obvious ethical and practical reasons. Three experimental groups were used: rats (dams) gavaged with (a) distilled water (control), or (b) homogenized fish from a reference site (Iles de la Paix) or (c) homogenized fish from a xenoestrogen-contaminated site (Ilet Vert). Pups were exposed via lactation and sampled on either day 21 or day 91. Day 21 was chosen because it was at this age, the pups were weaned. In rats day 91 is considered as adulthood. There was no effect on the body weights or in the male reproductive organ weights between groups except for adult epididymal weight, which was significantly decreased in the xenoestrogen group. Adult sperm concentrations and sperm motility parameters were all significantly decreased in the xeonestrogen group as compared to the reference and control groups. Interestingly, the distribution of stages of spermatogenesis was altered in the xenoestrogen group, indicating an effect on the kinetics of spermatogenesis. Immunoreactivity of Cx43, a gap-junctional protein, was markedly decreased in the seminiferous epithelium of the xenoestrogen group, suggesting that the intercellular coordination of testicular function may be affected. From this study we conclude that contaminants from xenoestrogen environments may pass through the food chain and exert effects on male reproductive functions.

After having shown that exposure of fish to xenoestrogens, can result in significant effects on spermatogenesis in fish. Furthermore, rat pups exposed to xenoestrogen conatminated

fish via lactation have altered spermatogenesis and decreased sperm count. After having shown that consumption of xenoestrogen contaminated fish results in adverse effects in Cx43, a gap-junctional protein, in the seminiferous epithelium of rats, we were interested in looking at the mechanism responsible for this effect. The major contaminant in the sites that we looked at was nonylphenol. Nonylphenol, an environmental contaminant distributed along the St. Lawrence River, has been reported to act as a weak estrogen. Differences in doses of nonylphenol and their effects at different times on sertoli cells need to be clearly addressed, in order to develop a better understanding of the risk of these chemicals to spermatogenesis. Also, it is important to establish whether or not the effects of nonylphenol on spermatogenesis and other testicular functions are mediated by endocrine disruption, or if their effects are the result of other non-endocrine medaited signalling pathways. Gap junctional intercellular communication (GJIC) in the testis is critical for coordinating spermatogenesis. The objectives of this study were to determine the effects of nonylphenol on GJIC and Cx43 in a murine Sertoli cell line, TM4. Cells were exposed for 24 h to different concentrations (1 to 50 μM) of either nonylphenol or 17β-estradiol. GJIC was determined using a microinjection approach in which Lucifer yellow was injected directly into a single cell, and GJIC was assessed 3 min postinjection. Nonylphenol exposure decreased GJIC between adjacent cells by almost 80% relative to controls. A significant concentration-dependent reduction in GJIC was observed at nonylphenol concentrations between 1 and 50 microM. Cx43 immunofluorescent staining was reduced at both 10 and 50 microM doses of nonylphenol. Cx43 phosphorylation, as determined by Western blot analysis, was reduced at both 10 and 50 microM concentrations, which may explain, at least in part, the inhibition of GJIC. In contrast, no effect on GJIC or Cx43 protein was observed in cells exposed to 17beta-estradiol at these concentrations. Cx43 has been reported to be phosphorylated via the p38-

mitogen-activated protein kinase (MAPK) pathway. P38-MAPK activity was assessed in both control and nonylphenol-exposed cells. A dose-dependent decrease in p38-MAPK activity was observed in nonylphenol-exposed Sertoli cells. Protein kinase C activity was also measured and was not influenced by nonylphenol. These results suggest that nonylphenol inhibits GJIC between Sertoli cells and that this is modulated via nonestrogenic pathways.

In conclusion, this study has shown that exposure of immature and male spottail shiners to xenoestrogens in the St. Lawrence River is widespread, as indicated by the induced levels of VTG mRNA. Furthermore, this exposure was shown to have marked effects on their reproductive function in males. Exposure to xenoestrogens was linked to delayed spermatogenesis, reduced spermatozoal production, decreased sperm motility, and high incidence of intersexuality. This is among the first studies which demonstrate that exposure of wild fish to xentoestrogens is correlated with a reduction in male reproductive function, and suggests that fish populations may be affected in the St. Lawrence River as a result of altered reproductive functions.

Further we have shown that exposure of immature rats to fish from a xenoestrogen-contaminated environment during the early postnatal period exerts long-lasting effects on epididymal sperm concentration and motility. This effect appears to result in part from alterations in spermatogenesis that are associated with a decrease in the gap- junctional protein Cx43. Together, these results suggest that the consumption of fish from xenoestrogen-contaminated ecosystems may alter the postnatal development of the male reproductive tract, resulting in permanent effects on male reproductive parameters. These results may be particularly important for both fish-eating humans and other riverine mammalian species and

they raise serious concerns regarding the transmission of endocrine-disrupting effects through the food chain.

In the third part of our study, we provide the first evidence that nonylphenol impairs gap junctional intercellular communication between Sertoli cells. This effect is, in part, the result of a decrease in the expression and phosphorylation of Cx43. The effects of nopnylphenol on testicular GJIC are mediated via an estrogen receptor-independent mechanism and an inhibition of the p38-MAPK pathway.

7.0 SYNTHÈSE DE LA MÉMOIRE REDIGÉ EN FRANÇAIS

L'EXAMEN HISTOLOGIQUE DES CONSÉQUENCES DE L'EXPOSITION AUX OESTROGÈNES SUR LE SYSTÈME REPRODUCTEUR MÂLE CHEZ LE (*NOTROPIS HUDSONIUS*)

L'impact négatif de l'activité humaine sur l'environnement a été bien documenté ces dix dernières années. Il a été découvert que plusieurs substances toxiques présentes dans l'environnement ont une action potentielle sur les hormones en agissant de manière agoniste ou antagoniste. Plusieurs études ont été menée afin d'évaluer l'impact de ces substances sur la vie aquatique et les populations humaines. L'écosystème aquatique représente un important milieu d'accumulation de plusieurs substances chimiques qui finissent par interagir avec la physiologie des poissons, affectant ainsi différents systèmes tels que la reproduction, le développement, croissance et la réponse immunitaire.

Ces effets semblent être le résultat des hormones déversées dans les milieux aquatiques par les eaux usées et de ruissellement provenant des zones agricoles. L'interaction entre ces différentes substances chimiques et la vie aquatique a conduit à la féminisation de certains mollusques et poissons.

Depuis ces dernières vingt années, de nombreuses études ont signalé la présence de perturbateurs endocriniens dans le milieu aquatique. La majorité de ces études ont insisté sur la présence de composés oestrogéniques ou des xénoestrogènes, et de l'induction de la protéine hépatique vitellogénine (VTG) soit chez les poissons immatures ou chez les mâles qui ne devraient pas en produire. L'utilisation de la VTG comme biomarqueur d'exposition aux

xénoestrogènes a permis aux scientifiques de déterminer la manière de se répandre de ces produits chimiques, leur mode d'action sur la vie aquatique puisqu'ils pourraient se lier aux récepteurs d'œstrogène et agir ensuite comme des composés oestrogéniques. Beaucoup de questions se posent sur dispersion de ces produits dans l'environnement et les effets à long terme associés à l'exposition à ces xénoestrogènes.

Cependant, on dispose de très peu d'informations des effets néfastes sur les spermatozoïdes chez les populations de poissons sauvages de la rivière Saint-Laurent le long de l'île de Montréal. Des expériences à long termes sont utiles pour une meilleure compréhension des conséquences physiologiques associées à une exposition chronique aux xénoestrogènes. Donc, mon but premier était de déterminer la présence ou non des oestrogènes dans la rivière Saint –Laurent dans les environs de l'île de Montréal et d'évaluer les effets de ces contaminants sur les fonctions reproductives du poisson nommé « queue à tâche noire » (*Notropis hudsonius*). Ces espèces ont été choisies afin d'évaluer l'impact des effluents municipaux sur les poissons du fleuve Saint-Laurent parce qu'on les retrouvent à des sites spécifiques, ce qui facilite les études pour des effets liées aux sites. La durée de vie de ces poissons est de 5 ans, ils atteignent leur maturité entre 1 an et 2 ans.

Nos études ont montré que les eaux provenant des effluents municipaux de la ville de Montréal contiennent des composés oestrogéniques pouvant être détectés par le niveau de l'ARNm dans la vitéllogénine des poissons « queue à tâche noire » immatures. De manière inattendue, les résultats ont montré que même les poissons en amont du point de décharge des effluents municipaux de Montréal ont aussi présenté un niveau élevé d'ARNm dans la

vitéllogénine, par exemple à l'île de Dorval. Ces résultats seraient attribuables aux rejets d'oestrogènes par d'autres usines situées en amont le long de la rivière Saint-Laurent loin du point de décharge principal.

En outre, la rivière des Outaouais semble aussi contribuer au niveau d'oestrogènes dans la rivière Saint-Laurent comme on le constate par la quantité élevée d'ARNm dans la vitéllogénine des poissons échantillonnés à l'embouchure de la rivière des Outaouais.

Il existe des preuves que les oestrogènes environnementaux peuvent altérer les fonctions testiculaires chez les poissons. Nos résultats montrent un délai marqué dans les stades de spermatogenèse chez les poissons « queue à tâche noire » des sites très contaminés par les xénoestrogènes. En fait, aucun des poissons des sites contaminés et qui présentaient un niveau élevé d'ARNm dans la vitéllogénine n'ont été trouvés avec des testicules au stade V de la spermatogenèse comparativement à ceux du site de référence qui l'étaient à 45%. Ces résultats corroborent avec ceux de l'étude sur les effets des oestrogènes sur la spermatogenèse chez la truite mâle. L'exposition aux oestrogènes par la diète a montré une baisse marquée de la spermatogenèse chez la truite arc-en-ciel (Billard et al., 1981) comparativement aux truites exposées aux oestrogènes naturelles. D'autre part, une étude des effets des alkylphénol sur la spermatogenèse chez les truites matures n'a pas présenté d'effets sur la spermatogenèse (Jobling et al., 1996). Ceci peut s'expliquer par le fait que la période de spermatogenèse durant laquelle ces cellules se développent est plus sensible aux effets des oestrogènes et peut être associée à l'arrêt du développement des cellules de Sertoli (Billard et al., 1982). Une des différences entre les études de laboratoire et celles menées sur le terrain est que, dans le deuxième cas, les

poissons sont exposés aux oestrogènes durant toute leur cycle de reproduction, ce qui peut être difficilement reproductible en laboratoire.

Les récepteurs d'oestradiol ont été identifiés dans les testicules des poissons. Wu et al. (2001) ont reporté que les isoformes a et b des récepteurs d'estradiol sont localisés sur les spermatocytes secondaires et spermatides du poisson-chat. Entre autre, Bouma and Nagler (2001) ont mentionné que les récepteurs d'oestrogène sont localisé dans les cellules précurseurs de Leydig dans les testicules. Nous n'avons pas la certitude si oui ou non les effets des xénoestrogènes présents dans la rivière Saint – Laurent sur la spermatogenèse chez le poisson « queue à tâche noire » sont le résultat d'un effet direct sur le développement des cellules germinales, d'inhibition de la synthèse d'androgène ou une action via l'axe gonado-hypothalamo-pituitaire (Christiansen et al., 1998).

Tandisque d'autres facteurs environnementaux tels que la température est reconnue pour altérer la spermatogenèse, la température de l'eau dans la présente étude varie en dessous de 2^{o} C entre les sites d'échantillonnage des poissons qui s'effectue en espace de 4 jours pour chaque zone. D'autre part, des facteurs tels que le niveau de dissolution de l'oxygène ne varie pas entre les sites dans le fleuve Saint-Laurent qui est bien oxygéné.

Dans la présente étude, il est difficile de limiter toutes les variables; le poids de l'évidence suggère fortement que les effets observés sur la spermatogenèse sont les résultats des effets liés à la présence de xénoestrogènes.

Les données sur la motilité des spermatozoïdes chez les poissons de ce site exposés aux oestrogènes ont été comparé aux résultats des sites de référence sans œstrogène utilisant une méthode informatisée (CASA). Cet approche a été utilisée pour mesurer les effets des métaux lourds sur la qualité du sperme (Rurangwa et al., 1998) afin de tester l'efficacité de la cryoconservation et du stockage (McNiven et al., 1993; Rurangwa et al., 2001) ainsi que l'optimisation du des conditions de fertilisation (Duplinsky, 1982; McMaster et al., 1992; Morisawa et al., 1983; Toth et al., 1995). Les concentrations du sperme chez les poissons de l'île vert étaient significativement basses comparé à ceux des poissons de l'île de la paix (site de référence).

Une étude récente sur le poisson gougon au Royaume-Uni a montré que la densité des concentrations du sperme varie parmi les poissons; la concentration du sperme la plus élevée a été observée chez le poisson « queue à tâche noire » au attribuée aux différentes méthodes d'échantillonnage. Les différences entre les périodes d'échantillonnage ou l'âge des poissons pourraient contribuer à la différence interespèces.

La baisse de concentration des gamètes chez les queues à tâche noire de l'îlet vert était corrélée au niveau élevé de l'ARNm de la vitellogénine, à la grande incidence d'intersexualité des poissons. Et le délai observé dans la spermatogenèse. Des études ont montré que l'exposition aux oestrogènes et xénoestrogènes qui induisent la VTG chez les poissons (Christiansen et al., 1998; Hill and Janz, 2003) a des effets inhibiteurs sur leur spermatogenèse de ces poissons (Hassanin et al., 2002; Jobling et al., 2002) sur la production (Haubruge et al., 2000; Jobling et al., 2002), du sperme et leur motilité (Jobling et al., 2002; McMaster et al., 1992).

Dans cette étude, les queue à tâche noire présentent un faible pourcentage de la mobilité progressive des spermatozoïdes comparé à ceux de notre site de référence. Dans le but d'une évaluation plus approfondie de la motilité des spermatozoïdes, ils ont été répartis en quatre classes selon leur vitesse (rapide, moyen, lent et stationnaire).

Nos observations suggèrent que les différentes populations de spermatozoïdes basées sur la vélocité du sperme coexistent avec sa laitance. La distribution du sperme pour les différentes catégories de vélocités varie entre les poissons de l'île de la paix et de l'îlet vert. Il a eu une augmentation significative du nombre des spermatozoïdes statiques en même temps une baisse du nombre des spermatozoïdes rapides des poissons « queue à tâche noire » de l'îlet vert. Les résultats de la présente étude montrent que la concentration et le pourcentage des spermatozoïdes motiles ne peuvent pas relater toute l'histoire, particulièrement si les effets causent des anomalies à la mobilité des spermatozoïdes.

Les spermatozoïdes des queue à tâche noire présentent un modèle linéaire de motilité. Les paramètres de vélocité (VAP, VSL, VCL) s'avéraient basse pour les spermatozoïdes des queue à tâche noire de l'Îlet vert. Ces paramètres expriment direct le mouvement des spermatozoïdes à atteindre les œufs pour les féconder. Les investigations ont révélé que les spermatozoïdes doivent nager activement dans le canal micropylaire pour féconder (Hart, 1990; Iwamatsu et al., 1993).

La motilité des spermatozoïdes s'active une fois en contact avec l'eau et cette motilité ne dure que quelques minutes. Une relation entre la motilité et la fertilité des spermatozoïdes a été établie par plusieurs auteurs (Billard and Cosson, 1992; Ohta et al., 1995).

L'examen histologique des testicules des poissons « queue à tâche noire » a révélé que les poissons qui ont induit un niveau élevé d'ARNm de VTG présentaient une grande incidence d'intersexualité .Cet incidence se situe entre 2.6% pour le site de référence et 31% pour l'île verte où le niveau de d'ARNm de la vitellogénine était élevé. Cette découverte a été confirmée dans la récente étude plubliée sur l'intersexualité chez le Gardon blanc (Jobling et al., 2002). Dans ces expériences, les échantillonnages ont été effectué en amont et en aval des usines de traitement d'eaux usées au Royaume-Uni. Ils ont rapporté que l'intersexualité a été aussi retrouvée sur toutes les sites. En conclusion, cette étude a montré que l'exposition des mâles immatures de queue à tâche noire aux xénoestrogènes dans le fleuve Saint-laurent était répandue. Ces expositions ont montré un effets marqué sur les fonctions reproductives des mâles.

EFFETS DE LA CONSOMMATION DE POISSONS CONTAMINÉS DE XÉNOESTROGÈNE À TRAVERS L'ALLAITEMENT CHEZ LES RATS.

L'usine de traitement des eaux usées de Montréal déverse ses éfluents municipaux à un seul endroit du fleuve Saint-laurent. La première partie de notre étude a montré que les poissons « queues à tâche noire » exposés à ces effluents exhibaient des niveaux élevés d'induction de vittellogénine, un retard de spermatogénèse, une réduction de la production de sperme et de la motilité des spermatozoïdes, et une incidemce élevée d'intersexualité.

La pêche sportive est souvent pratiquée dans le Saint-Laurent. Kosatsky et collaborateurs ont rapporté que dans la région de Montréal, les pêcheurs sportifs consomment en moyenne trois fois par semaine les poissons issuent de leur pêche. Annuellement, on a estimé cette consommation à plus de 18 kg de poissons du Saint-Laurent. On a observé chez ces plus grands consommateurs de ces poissons des niveaux élevé mercure dans les cheveux et notemment de de PCB et dichlorodiphenyl dichloréthylène dans le sang circulant. Quelques études suggèrent aussi que les habitants de Montréal d'origine Asiatique consomment plus de poissons du Saint-Laurent que d'autres pêcheurs sportifs (Kosatsky et al., 1999b). Ceci entraîne des niveaux plus élevés de contaminants chez cette population asiatique que les niveaux moyenne retrouvés chez d'autres pêcheurs sportifs. Le fait que les poissons du Saint_Laurent sont exposés à des composés oestrogèniques suggère que les personnes, ainsi que les mammifères piscivores, peuvent être exposés eux aussi aux perturbateurs endocriniens.

L'exposition aux contaminants environnementaux pendant les périodes critiques du développement représente un risque majeur pour les animaux et les êtres humains qu'une exposition à l'âge adultes. Les perturbateurs endocriniens sont particulièrement problématiques pour le développement des animaux, parce qu'ils peuvent provoquer des dérèglements endocriniens, pouvant engendrer des défauts physiologiques permanents pour le système immunologique, nerveux ou reproductif (Arukwe 2001; Jobling and Tyler, 2003; Rothcell and Ostrander, 2003). Chez les rats, le tractus reproducteur masculin est soumis à un développement important durant les trois premières semaines de vie. Pendant cette période, les cellules des testicules et de l'épididyme se différencient afin de devenir des cellules similaires à celles des adultes (Pelletier, 2001; Rodriguez et al., 2002).

Les barrières hémato-testiculaire et hémato-épididymaire sont formées, et la première vague de spermatogenèse commence (Cyr, 2001; Cyr et al., 2002; Pelletier, 2001). La période d'allaitement représente l'étape critique pour le développement des rattons mâles, du fait que la mère peut transmettre des contaminants à ses descendants.

La deuxième parti de notre étude a pour but de vérifier si les perturbateurs endocriniens peuvent passer dans la chaîne alimentaire et occasionner des désordres chez les vertébrés supérieures. Par conséquent l'objectif de cette recherche est de déterminer chez les mères, si la consommation de poissons pêchés dans un environnement contaminé aux xénoestrogènes aurait des conséquences néfastes pour le système reproducteur des rattons mâles en période de sevrage. Nous avons voulu aussi constater, si de tels effets se manifestent seulement lorsque les rattons atteingnent l'âge adulte. Cette information nous permettra de développer une meilleure compréhension de l'exposition et risques liés à la consommation de poisson provenant d'environnements contaminés par des composés oestrogèniques.

Le poids corporel des mères et des rattons de notre étude ne présentaient aucune dufférence significatif entre tous les groupes. Cela suggère que la consommation de poisson n'a pas d'effet sur les taux de croissance des rats. Nous déduisons qu'il n'y a pas de différence dans le contenu nutritionnel des poissons entre le site de référence et celui contaminé aux xenoestrogènes.

Chez les rats âgés de 21 jours, on n'a pas trouvé d'effets sur les poids des organes reproducteurs des mâles et dans leur apparence histologique. Mais, toutefois, les rats à l'âge adulte, présentaient une diminution significative du poids de l'épididyme. Les poids d'autres

tissus n'étaient pas affectés. La production de sperme à lieu dans les testicules. Le sperme acquiert l'habilité de nager et féconder dans l'épididyme. Ils y sont aussi stockés jusqu'au moment de l'éjaculation (Yeung and Cooper, 2002). La baisse du poids de l'épididyme qu'on a observé dans cette étude peut résulter d'une diminution dans la concentration de spermatozoïde dans la région caudal de l'épididyme. Des études antérieures effectuées avec des composés oestrogèniques ont montré que le temps requis pour le transit des spermatozoïdes à travers l'épididyme peut être altéré (Hess, 1998; Klinefelter and Suarez, 1997). Une augmentation dans le temps de transit du sperme à travers la région caput/corpus de l'épididyme pouraient expliquer une diminution de son poids.

La concentration, la motilité progressive, et la linéarité de sperme ont été significativement réduites chez les rats exposés aux poissons contaminés par xénoestrogène. On a reporté que chez les humains, il y a une corrélation significative entre les taux de fertilisation et la linéarité (Hirano et al., 2001). La réduction dans le mouvement de sperme et la vitesse linéaire peuvent être des facteurs significatifs dans le début de l'infécondité. L'incidence élevée des cellules lentes et statiques, la baisse des paramètres de vélocité et de linéarité observée chez les rats exposés aux xénoestrogènes suggèrent que leur sperme pourraient présenter un potentiel réduit de fertilisation. Goyal a reporté que l'administration de 10 μg diethylstilbestrol (DES), aux rats à tous les deux jours durant les 12 premiers jours de la période néonatale a occasionné une diminution de la motilité et linéarité du sperme. Le fait que nous ayons observé des effets sur les paramètres de la motilité du sperme sans dommage extensif aux testicules suggère que les produits chimiques oestrogéniques peuvent provoquer des effets plus subtils à des doses environnementales.

La spermatogenèse est un processus linéaire avecdes associations cellulaires adjacentes (14 étapes chez le rat; Leblond and Clermont, 1952) progressant d'une étape à l'autre en structure ondulatoire (Parvinen and Vanha Perttula, 1973). On dispose d'informations très limitées concernant les 14 étapes de la spermatogenèse du rat suite au traitement à des doses environnementales de xénoestrogènes. Dans la présente étude, la fréquence des étapes de spermatogenèse à été altérée, un signe de perturbation dans la cinétique de la spermatogenèse. Ces observations soulèvent la question de savoir si cette altération est-elle du soit à certains stades de maturation plus rapides ou plutôt à un arrêt de maturation chez certaines cellules germinales. Alternativement, l'effet primaire peut avoir été produit sur les cellules germinales à l'extérieur de la barrière hématotesticulaire pendant la période postnatale précoce. On considère que les contaminants l'environnementaux tels que le diethylcarbamyl methyl-2-4- dinitropyrrole (Patanelli and Nelson, 1964), 2,5- hexanedione (Chapin et al.; 1983), et éthylène glycol monométhyle éther (Chapin et al., 1984; Creasy et al., 1985) modifient la fréquence des étapes de la spermatogenèse. Les changements dans la cinétique et l'absence de grands effets histopathologiques sur les cellules germinales observées dans cette étude suggèrent que les xénoestrogènes peuvent provoquer des effets subtils sur la fonction reproductive masculine.

La spermatogenèse est un processus synchronisé et un processus à modélisation spatiale de la prolifération et différentiation cellulaire dont le couplage intercellulaire des jonctions communicantes joue un rôle important dans la coordination de ces fonctions. Le nombre relativement grand de connexions et leur localisation dans les testicules temoignent de l'importance de la communication et jonctions intercellulaires dans les fonctions testiculaires

(Batias et al., 2000; Risley et al., 1992; Tan et al., 1996). Chez les rongeurs, l'interaction entre les cellules de Sertoli et la communication entre les cellules germinales (spermatogonome et spermatocyte) et celles de Sertoli sont facilités par les jonctions lacunaires contenant le Cx43 (Batias et al., 2000). On croit que la communication intercellulaire testiculaire facilité par les jonctions communicantes de Cx43 représente un processus essentiel pour la spermatogènèse, car la spermatogènese est arrêté dans des testicules dépourvus de Cx43 (Roscoe et al., 2001). La localisation immunocytochimique du Cx43 montre que dans chaque groupe expérimental, certains tubules affichent une immunoréaction de Cx43 plus intense que d'autres. Des études antérieures ont montré que l'immuno-coloration de Cx43 dépends du stade du tubule (Batias et al., 2000; Risley et al., 1992; St-Pierre et al., 2003; Tan et al., 1996). Nos résultats indiquent que l'expression de Cx43 testiculaire est réduite chez les rats exposés à des composés xénoestrogèniques pendant l'allaitement. Cette réduction de Cx43 peut être responsable, en partie, des changements observés dans la cinétique de la spermatogènèse. Des études récents ont montré que le lindane peut modifier l'expression de Cx43 dans les cellules de Sertoli (Mograbi et al., 2003). En outre, Defamie a reporté des modifications dans l'expression de Cx43 chez des patients souffrant d'azoospermie et des cellules de Sertoli non différenciées, une découverte qui suggère des conséquences pathologiques liés à la communication intercellulaire.

Bien que la nature des produits chimiques responsables de ces effets est encore inconnue à ce jour. Des analyses chimiques des sédiments autour de l'Île de Montréal ont montré des niveaux de contaminants généralement peu élevés, sauf pour le zinc, dans les eaux des effluents, et des alkylphénoles présents en aval du point de déchargement des eaux usés de Montréal dans le fleuve Saint-Laurent (Gagnon and Saulnier 2003; Sabik et al., 2003). Soit que ces produits

chimiques ou des produits chimiques non identifiés sont responsables des effets de toxicité reproductive observés dans cette étude. Cependant, il est claire que certains produits chimiques peuvent être transférés à la progéniture en développement et exercent des effets permanents sur le développement du système reproducteur mâle.

En résumé, cette étude demontre que l'exposition de rats immatures à des xénoestrogènes pendant la période postnatale produit des effets prolongés sur la concentration et motilité des spermatozoides épididymaires. Cet effet semble être en partie le résultat de modifications dans la spermatogènèse associées à la baisse de l'expression de la proteine des jonctions lacunaire Cx43. Ensemble, ces résultats suggèrent que la consommation de poissons qui proviennent des écosystèmes contaminés aux xénoestrogènes peut modifier le développement postnatal de la région reproductive du mâle, ayant pour résultat des effets permanents sur les paramètres de reproduction mâle. Ces résultats peuvent être particulièrement importants pour les humains qui mangent du poisson et d'autres espèces mammifères riveraines et soulève de sérieux problèmes concernant la transmission des effets perturbateurs endocriniens à travers la chaîne alimentaire.

MÉCANISME D'ACTION DU NONYLPHÉNOL:

Le nonylphénol est le produit final de la biodégradation du nonylphénol polyethoxylates, un important contaminant du Saint-Laurent (Ekeland E et al.,1993; Giger W et al.,1984). Les rats dont les mères ont mangé du poisson de ces endroits durant l'allaitement ont une expression réduit de Cx43 testiculaire. Dans cet étude nous avons demontré que les cellules de Sertoli TM4 forment de jonctions intercellulaires fonctionnelles entre les cellules adjacentes en permettant le passage de la microinjection du colorant « jaune Lucifer » micro-injecté dans les cellules. En

utilisant une analyse d'immuno-cytochimie et de Western blot, nous avons démontré que les jonctions lacunaires des cellules de Sertoli TM4 sont constituées de Cx43. Ces connexions apparaissent commis des structures ponctuées localisées aux contacts cellule-cellule entre les cellules adjacentes. Les jonctions lacunaires sont présentes entre les cellules de Sertoli adjacentes dans les testicules (Gilula NB et al.,1976). McGinley et al., à reporté que les jonctions lacunaires étaient présentes entre les cellules de Sertoli et les cellules germinales devant le développement (McGinley DM et al.,1979). Tan et al. ont d'abord reporté que la Cx43 était localisé entre les cellules de Sertoli adjacentes, ainsi qu'entre les cellules germinales en développement et les cellules de Sertoli chez le rat adulte (Tan IP et al.,1996) . D'autres études ont reporté la présence de Cx 26, 31, 32, 33, 37, 40, et 45 dans les tubule séminifères du rat (Risley MS et al.,2000).

Tandis que l'exposition des cellules à l'estradiol n'altère pas la communication intercellulaire, l'exposition au nonylphénol réduit dramatiquement la communication intercellulaire à toutes les doses testées. Cet effet suggère que, tandis que le nonylphénol est considéré comme substance oestrogénique (Tapiero H et al.,2002), ses effets sur la communication intercellulaire ne sont pasmédiés par le récepteur d'estradiol. Cela se passe malgré le fait que les récepteurs d'œstrogène sont exprimés dans les cellules de Sertoli TM4 (Nakhla AM et al.,1984; Nakhla AM et al.,1989).

Même s'il n'existe pas d'études concernant les effets du nonylphénol sur la communication intercellulaire, il y a une liste croissante de toxiques de l'environnement qui semblent modifier la communication intercellulaire. En employant la technique de micro-injection, nous avons été capables de démontrer que le nonylphénol inhibait presque

complètement la communication intercellulaire via les jonctions communicantes (CIJC) à une forte dose d'exposition. Des études sur des tissus non reproductifs ont montré leur potential pour réduire la CIJC. L'hexachlorobenzène, un organochloré, peut favoriser la promotion de tumeurs spécifiques en bloquant la communication intercellulaire entre les hépatocytes chez le rat (Plante I et al.,2002). D'autres toxiques tels que les dioxines, hydrocarbures aromatiques polychlorés, et cadmium, peuvent aussi diminuer la communication des jonctions communicantes dans les hepatocytes en culture (Jeong SH et al.,2000; Krutovskikh VA et al.,1995). Des études de Defamie et al., 2001 ont montré que le lindane peut abolir la CIJC entre les cellules de Sertoli des rats à une dose de 50 μM. L'importance des jonctions communicantes de Cx43 pour la spermatogènèse est indiquée par une sévère perte de cellules germinales chez les souris mâles et femelles prénatales déficientes en expression du gène Cx43 (Juneja SC et al.,1999). La prolifération postnatal des spermatogonoies est aussi altéré dans les souris 'Knock-out' depouvu de Cx43 (Roscoe WA et al.,2001). L'insertion de régions codants pour la Cx32 ou Cx40 dans la région codante Cx43 -/- l'oogenèse restaurée du souri et d'autres déficiences provoqués par l'inhibition de la Cx43, mais l'amplification spermatogonial restent défectueuse (Plum A et al.,2000). L'importance des jonctions communicantes Cx43 pour la régulation de la spermatogènèse chez les adultes est supporté par le fait que l'immunoreactivité Cx43 est réduite chez les mutants avec déficience de spermatogènèse (Batias C et al.,2000; Batias C et al.,1999). De cette façon, Cx43 est un composé essentiel de la voie CIJC, qui supporte les phases précoces de la spermatogènèse. Des études antérieures ont montré que le nonylphénol peut affecter la spermatogènèse (Nagao T et al.,2000; Hossaini A et al.,2001; Chitra KC et al.,2002; Adeoya-Osiguwa SA et al.,1990).

Les effets du nonylphénol sur la communication intercellulaire et l'expression de Cx43 dépendent de la dose et du temps. Des études à réponse temporelle dans lesquels les cellules ont été exposées à 10 μM de nonylphénol ont montré une baisse de Cx43 en fontion du temps, qui a attendu le sommet 24h après le commencement de l'exposition. Ces résultats suggèrent que le nonylphénol ne semble pas agir au niveau de la jonction communicante, mais plutôt exerce un effet progressif soit sur le niveau de renouvellement des connexions, ou bien sur leur synthèse, ou sur les deux. Des études supplémentaires seront nécessairement pour établir clairement si oui ou non cet effet est au niveau de la transcription ou de la post-transcription.

La Cx43, comme la plupart de Cx's, est une phosphoprotéine avec plusieures isoformes. La phosphorylation a été impliquée dans la régulation des Cx's, tels que le ciblage intracellulaire, assemblage/désassemblage des connexins. Des altérations dans le statut de phosphorylation de la Cx43 causent des effets sur la communication des jonctions lacunaires, qu'elle soit hyper ou hypophosphorylé (Musil LS et al.,1990; Crow DS et al.,1990; Laird DW et al.,1991; Brissette JL et al.,1991; Kadle R et al.,1991; Berthoud VM et al.,1992). L'exposition des cellules de Sertoli au nonylphénol a eu pour résultat non-seulement de causer une diminution des niveaux de Cx43, mais aussi de sa phosphorylation. Nos résultats suggèrent que le nonylphénol inhibe la CIJC entre les cellules de Sertoli et que la phosphorylation aberrante de la Cx 43 peut avoir contribué à cet effet. Ces effets n'ont pas été observés lorsque les cellules ont été traitées avec 17ß-estradiol. Ces observations sont similaires à d'autres études qui montrent que la déphosphorylation de Cx43 est corrélé à une réduction de communication intercellulaire (Matesic DF et al.,1994; Kenne K et al.,1994; de Feijter AW et al.,1996; Nomata K et al.,1996; Sato H et al.,2003; Matesic DF et al.,2001).

Une ou plusieurs protéines kinase sont responsables pour phosphoryler la Cx43. Le nombre exact de sites phosphorylés sur la Cx43 n'est pas encore connu; cependant, au moins cinq phosphopeptides Cx43 différents ont été observés (Cooper CD et al., 2000), suggérant que le Cx43 est phosphorylé à plusieurs endroits, sur les résidues de sérine et thréonine (Crow DS et al.,1990; Kanemitsu MY, et al.,1997). La Cx43 semble avoir été phosphorylé par la Protein Kinase C (PKC).

Afin de comprendre le mécanisme par lequel le nonylphénol a induit une baisse de la phosphorylation de la Cx43, nous avons étudié, si oui ou non, les voies de la p38-MAPK et PKC ont été altérés dans les cellules de Sertoli traités avec nonylphénol. Lorsque les cellules ont été traitées avec nonylphénol, l'activité de la p38-MAPK était significativement réduite de manière dose-dépendante. Une chose remarquable : il n'y a pas eu de changement dans l'activité de la PKC. La diminution de la p38-MAPK suggère que le nonylphénol inhiberait sélectivement cette voie et de ce fait entrainerait une diminution de la phosphorylation de Cx43. Ceci est en contraste avec un autre contaminant de l'environnement, le lindane, qui augmente la phosphorylation de Cx43 dans les cellules de Sertoli via une stimulation d'une kinase extracellulaire régulée par les voies JNK ou p38-MAPK (Mograbi B et al.,2003). L'hyper-phosphorylation de Cx43 par le lindane a induit une augmentation dans la localisation cytoplasmique de la Cx43. Cela suggère que l'hyper-phosphorylation et les voies de signalisation de l'ERK sont impliqués dans la localisation de Cx43 dans les cellules de Sertoli (Mograbi B et al., 2003).

Clairement, les effets observés dans la présente étude indiquent que le nonylphénol modifie la communication intercellulaire par des voies indépendante du-récepteur aux oestrogènes, malgré le fait que le nonylphénol est considéré comme étant un produit chimique oestrogènique (Soto AM et al.,1991; Shelby MD et al.,1996). Des études ont reporté que le nonylphénol peut modifier la production d'espèces réactives oxygénés (ROS) dans les cellules neuronales (Okai Y et al.,2004). En outre, on a montré que l'expression de Cx32 est sensible aux effets de ROS dans des hépatocytes en culture. Par conséquent, on peut spéculer que l'action du nonylphénol sur la communication intercellulaire dans les cellules de Sertoli peut être facilité par une production augmentée en ROS. En outre, la possibilité que le nonylphénol agisse sur d'autres aspects des interactions cellule-cellule, tels que l'adhésion cellulaire, et que cela affecte la CIJC demeure une possibilité.

Chez les rats exposés au nonylphénol, il y a une diminution dans les niveaux de testostérone sérique et une spermatogenèse altérée (Tan BL et al.,2003; Han XD et al.,2004). Nos résultats indiquent que le nonylphénol peut diminuer la communication intercellulaire et les niveaux de Cx43, qui ont été considérés comme des importants régulateurs de la fonction cellulaire des cellules de Leydig et de la spermatogenèse. Ces résultats suggèrent que les effets in vivo du nonylphénol sur le testicule peuvent provenir, en partie, d'une diminution de la communication intercellulaire. En outre, on a reporté que l'activation de la MAP Kinase p38 par TGF- ß est essentiel pour la formation et le soutien de la barrière hématotesticulaire, qui est formée par des jonctions semées entre les cellules de Sertoli adjacentes (Lui WY et al.,2003; Lui WY et al.,2003). Les résultats de cette étude pourraient suggérer que bien qu'étant le résultat d'une baisse de l'activité p38-MAPK, le nonylphénol pourrait aussi avoir des effets sur la

barrière hématotesticulaire. On a reporté que le Cx43 est co-localisée avec les protéines des jonctions serrées dans la barrière hématotesticulaire (Cyr DG et al.,1999; Segretain D et al.,2004); par conséquence, l'association complexe de toutes les jonctions serrés et lacunaires dans la barrière hématotesticulaire paurait être altérée par le nonylphénol. Des études supplémentaires devront nécessairement établir si cela est le cas ou non, et si tous ces effets contribuent au dysfonctionnement testiculaire provoqué par le nonylphénol.

En conclusion, à cette étude démontre pour la premieére fois que le nonylphénol inhibe la communication intercellulaire via les jonctions lacunaires entre les cellules de Sertoli. Cet effet est, en partie, le résultat d'une diminution dans l'expression et phosphorylation de la Cx43. Les effets du nonylphénol sur la CIJC testiculaire sont le resultat d'une inhibition de la voie de signalisation de la p38 MAPK.

Bref, nous avons montré que l'exposition des queues à tache noire immatures mâles aux xénoestrogènes dans le fleuve Saint_Laurent est étendu, et que cette exposition à des effets marqués sur la fonction reproductive des mâles. L'exposition aux xenoestrogènes est liée à un retard de spermatogènèse, réduction de la production de sperme, diminution de la motilité, et une haute incidence d'intersexualité. En outre l'exposition des rats immatures aux poissons qui proviennent d'un environnement contaminé avec xenoestrogène pendant la période postnatale précoce produit des effets de longue durée sur la concentration et motilité des spermatozoides épididymaire. Cet effet semble résulter en partie des modifications dans la spermatogènèse est la diminution de la protéine Cx43 des jonctions lacunaires. Nous avons montré que le nonylphénol inhibe la communication intercellulaire des jonctions lacunaires entre les cellules de Sertoli. Cet

effet est, en partie, le résultat d'une diminution de l'expression et phosphorylation de la Cx43. Les effets du nonylphénol sur la communication intercellulaire par le biais des jonctions communicantes des testicules sont médié par un mécanisme indépendant du récepteur aux d'œstrogènes et une inhibition de la voie p38 MAPK.

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