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Original Communication

Hepatic and reproductive toxicity of a physico-chemically treated municipal effluent to fathead minnows

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ABSTRACT

Municipal effluents are known to release a variety of contaminants that have the potential to compromise neuroendocrine signaling pathways in aquatic organisms. The purpose of this study was to examine the hepatic and reproductive toxicity of a physically-chemically treated effluent to adult fathead minnows following 21 days of exposure at 25 °C. The following biomarkers were determined after the exposure period: oxidative stress (lipid peroxidation and DNA damage), estrogenicity (vitellogenin and glucose-6-phosphate dehydrogenase) and energy budget (mitochondrial electron transport, total sugars and lipids in the liver). Reproductive function was also determined by observation of secondary sexual characteristics, egg production and hatching. The results revealed that fish exposed to municipal effluent had increased levels of hepatic vitellogenin, higher energy expenditure (electron transport activity in mitochondria) and increased levels of lipids and sugars in the liver. The only observed changes in reproduction were decreased egg production and slight discoloration of males. Egg production was negatively correlated with lipid peroxidation in gills and total lipids in the liver. Vitellogenin also contributed significantly to energy expenditure in the liver, suggesting that energy reserves and oxidative stress were involved in decreased egg production. Analysis of the eggs revealed that the exposed eggs had lower lipid content and higher sugar content compared to the control eggs. The study supports the hypothesis that exposure of fish to high concentrations of effluent discharges (>20%) could have detrimental effects on energy expenditure, egg production and egg energy content which impact their reproduction.

KEYWORDS: fathead minnows, municipal effluents, vitellogenin, mitochondrial electron transport activity, energy reserves, egg production, oxidative stress

INTRODUCTION

Municipal effluents are major sources of various anthropogenic pollutants in the aquatic environment. The ecotoxicological effects of municipal effluents on aquatic organisms include endocrine disruption, feminization, serotonergicity, genotoxicity and oxidative stress, including inflammation [1, 2, 3]. Municipal effluents consist of complex mixtures of metals, polyaromatic and halogenated hydrocarbons, and endocrine disrupting substances, some of which are capable of disrupting reproduction in aquatic organisms [4]. Exposure to municipal effluents is known to activate the estrogen signaling pathways leading to the production of the egg-yolk protein precursor vitellogenin (VTG). In Canada, it is estimated that about one half of treated municipal wastewater retain their estrogenic properties after treatment [5]. The estrogenic properties of municipal effluent were more dependent on the incoming untreated effluent than the treatment process applied, highlighting the relative difficulty in removing estrogenic compounds by municipal treatment plants.

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The synthesis of VTG occurs in the liver of vertebrates and gonad tissues of some invertebrates [4, 6]. The synthesis of VTG is an energy-intensive process, as it contains lipids, sugars, phosphates and calcium, which serve as the main source of energy for the developing embryo and fry. Fathead minnows exposed to municipal effluent were found to have increased plasma VTG levels and evidence of ovipositors in males, properties usually observed in females [7]. The secondary sexual characteristics of male minnows were decreased as shown by loss of male banding coloration, fewer nuptial tubercles, little development of the dorsal pad, and absence of the dorsal fin. Municipal effluents contain various steroids (estradiol-17β, testosterone, progesterone, coprostanol and cholesterol) that are bioavailable in fish with an estimated bioconcentration factor of 32 after 141 days of exposure to the effluents [8]. In another study, hospital effluents contributed to elevated levels of lipid peroxidation (LPO) in the gill, liver and brain of carps, indicating that pharmaceutical-rich wastewaters lead to oxidative stress [9]. This was also shown in rainbow trout exposed to secondary and tertiary-treated municipal effluent [10]. However, the tertiary-treated municipal effluent had a less potent effect on LPO, demonstrating the benefit of additional treatment processes.

Municipal effluents can also produce other effects that are independent of the endocrine system. For example, they are able to induce oxidative stress in fish and mussels [5, 11]. The levels of lipid peroxidation in organisms collected near sewage pollution were found to be significantly elevated, which suggests that the antioxidant capacity of cells was unable to prevent oxidative damage to membranes (polyunsaturated lipids). Sustained exposure to contaminants in urban discharges could increase metabolic energy demand for the elimination of xenobiotics in aquatic organisms. Fathead minnow and rainbow trout hepatocytes exposed to municipal effluent had elevated activity in cytochromes P4501A1 and 3A4, which are involved in the oxidative elimination of polyaromatic and polycyclic aliphatic hydrocarbons [5, 12]. Energy expenditure can be determined by monitoring electron transport activity in mitochondria [13]. Electron transport activity is associated with consumption of O_2 and release of CO_2 in mitochondria, which require glucose as a carbon source [14]. It has been previously shown that mussels exposed to municipal effluents had elevated mitochondrial electron transport (MET) activity in visceral and gill tissues [15], suggesting that organisms spend more metabolic energy and have less energy in the form of lipids and sugars in effluent-polluted environments.

The purpose of this study was to examine reproductive toxicity, oxidative stress and energy allocation in adult fathead minnow exposed to a primary treated municipal effluent. Reproduction was determined by measuring changes in VTG in both males and females, daily egg (production) spawning activity, total egg production and hatchability. Energy allocation was measured by monitoring changes in MET, total sugars and lipids in the liver of exposed fish. Special attention was focused on the relationships among effluent estrogenic action on egg production, energy budget (the balance between energy expenditures and sugar/lipid reserves) and oxidative stress in fathead minnow exposed for 21 days to typical physically-chemically treated municipal effluent.

METHODS

Fathead minnow reproduction assay and exposure to municipal effluent

Fish were cultured and bred at a fathead minnow colony at the wet laboratory of the aquatic toxicology laboratory at the Montreal wastewater treatment plant (Pointe-aux-Trembles, QC, Canada). The study utilized a 21-day continuous exposure regime with a 2:1 female to male ratio in 12.5 L tanks that continuously received diluted municipal effluents (1 L per hour). Two male and four female fish were held in each 12.5 L aquarium for 7 days prior to exposure to the effluent. The aquariums contained spawning tiles composed of 2 x 8 cm polyvinyl pipes with a diameter of 10 cm (cut longitudinally in half) and were monitored in the morning for egg fixation to the tiles. Successfully fertilized eggs were examined under a microscope and groups showing the highest egg fertilization rate were selected for the experiment. In fully active fish, exposure to the effluent was initiated using concentrations of 5, 10 and 20% v/v at 25 °C. These concentrations were selected based on the

dilution of the dispersion plume of the effluent in the Saint-Lawrence River: 5% effluent concentration corresponds to a distance of 4-6 km while the 20% concentration corresponds to 0.5-1 km downstream the dispersion plume. The effluent was continuously renewed (1 L/hr flow rate) and was pre-heated to 25 ^{o}C before being pumped into the aquariums. The exposure experiments were repeated with 2 replicate tanks for each treatment group. The fish were fed daily with commercial feed during the exposure experiments (10 g per aquarium). Control fish were exposed to charcoal-filtered and UV treated tap water from the City of Montreal. The fish were exposed at 25 ^{o}C for 21 days under constant aeration and a 16 h-light/8 h-dark photoperiod. Water pH, dissolved oxygen and temperature were monitored daily, and the spawning tiles were checked for the presence of eggs (tiles were replaced with new ones when the egg density covered a good proportion of the tile). At the end of the exposure period, the fish were anesthetized in MS-222 (50 mg/L, Sigma-Aldrich, Ontario, Canada) in accordance with the recommendations of the animal care committee. Fork length and wet body, gonad, gill, brain and liver weights were recorded. The organs were then mixed in 3 volumes of homogenization buffer before freezing at -85 ^{o}C. The homogenization buffer was composed of 25 mM Hepes-NaOH, pH 7.4, containing 140 mM NaCl, 1 mM dithiothreitol and 10 µg/ml aprotinin (protease inhibitor). The tissues were homogenized using a teflon pestle tissue grinder (5 passes) on ice. A portion of the homogenate was centrifuged first at 2000 x g for 15 min at 4 °C, and the resulting supernatant (S2 fraction) was centrifuged at 10000 x g for 30 min at 4 °C. The supernatant (S10 fraction) pellet (crude mitochondria), S2 fraction and the homogenate were then conserved at -85 °C until analysis. Total proteins in these fractions were determined using the Coomassie Blue dye binding assays [16]. Standard solutions of bovine serum albumin were used for calibration. The male and female secondary sexual characteristics were assessed by visual inspection: ovipositor in females; banding coloration, nuptial tubercles and head-sponge appearance in males.

Oxidative stress and DNA damage

Lipid peroxidation (LPO) was determined in liver homogenates using the thiobarbituric acid method [17]. A volume of 50 µL of the homogenate was mixed with 300 µL of 10% trichloroacetic acid containing 1 mM $FeSO_4$ and 150 µL of 0.7% thiobarbituric acid and heated at 70-80 °C for 10 min. The mixture was cooled at room temperature and centrifuged at 10000 x g for 5 min to remove any precipitated materials. A 200 µl volume was transferred to a 96-well dark microplate and fluorescence readings were taken at 520 nm excitation and 600 nm emission. Standard solutions of tetrametoxy-propane (stabilized form of malonaldehyde) were prepared for calibration in the blank (homogenization buffer). Results were expressed as µmole thiobarbituric acid reactants (TBARS)/mg total proteins in the homogenate. DNA damage was determined using the alkaline precipitation assay [18], which is based on the potassium detergent precipitation of protein-bound DNA. Protein-free DNA strand breaks were measured in the supernatant using fluorescence spectroscopy. A 25-µL volume of the homogenate was mixed with 225 µL detergent solution (2% sodium dodecylsulphate containing 10 mM sodium ethylenediaminetetraacetate, 10 mM TRIS base and 40 mM NaOH) for one min, followed by the addition of 250 µL of 0.12 M KCl. The mixture was mixed by inversion, heated at 60 °C for 10 min, cooled on ice for 15 min and centrifuged at 8000 x g for 10 min. A 50-µL volume of the supernatant was mixed with 150 µL of 100 µg/mL Hoechst dye in 0.1 M Tris-acetate, pH 8.5, containing 4 mM sodium cholate and 0.4 M NaCl. Fluorescence readings were taken at 360 nm excitation and 450 nm emission. Standard solutions of salmon sperm DNA were prepared for calibration. The data were expressed as µg DNA strand/mg total protein in homogenate.

Mitochondrial activity and vitellogenin assessments

The livers were removed from the fish and rinsed in the homogenization buffer at 4 ^{o}C to remove excess blood. They were then homogenized as described above and centrifuged at 2000 x g for 10 min at 4 ^{o}C. The supernatant was centrifuged again at 10000 x g for 20 min at 4 ^{o}C and the resulting pellet (crude mitochondria fraction) was removed from the supernatant (S10 fraction for vitellogenin assessment). Vitellogenin (VTG) levels in fathead minnows were determined using a commercial

immunoassay for VTG (Biosense, Cayman Chemicals, USA). The assay is based on the competitive binding of standard VTG to fixed antibodies in the presence of the S10 fraction. The data were expressed as ng VTG per mg of total protein in the S10 fraction of the liver homogenates. Mitochondrial electron transport activity was determined using a dye reduction method measured by colorimetry [14]. The assay is based on the reduction of a tetrazolium dye in the presence of isolated mitochondria, which was found to be coupled with cellular respiration rates in miscellaneous organisms [14]. Briefly, mitochondria (100 µg/mL) were mixed with one volume of 0.1 M Tris-HCl, pH 8.5, containing 0.1 mM $MgSO_4$, 0.1% Triton X-100 and 5% polyvinylpyrrolidone for 1 min before adding 1 mM NADH (reduced nicotinamide adenine dinucleotide) and 0.2 mM NAPDH (reduced nicotinamide adenine phosphate dinucleotide) as the electron source. The reaction was started by adding 1 mM of p-iodonitrotetrazolium. The reaction was allowed to proceed at 20 ^{o}C for 30 min and absorbance readings were taken at 520 nm at 5-min intervals. The data were expressed as absorbance increase/30 min/mg mitochondrial protein content.

Data analysis

The exposure experiment consisted of 2 males and 4 females per treatment aquarium and the experiment was repeated three times. Tissue biomarkers were analyzed in 4 males and 4 females using two-way factorial analysis of variance (exposure groups and sex) after verifying for homogeneity of variance and normality using Levene and the Shapiro-Wilk tests, respectively. Correlation analysis was also performed using the Pearson product-moment procedure. To determine the physiological changes induced by exposure to increasing effluent concentration, discriminant function analysis and factorial analyses were performed. All statistical tests were performed using Statistica software (version 8). Significance was set at $\alpha = 0.05$.

RESULTS

The basic chemical parameters of the diluted municipal effluent (Table 1) were determined on days 1, 2 and 15 to monitor the changes during the 21-day exposure period. The chemical parameters were found to be constant in time. All chemical parameters changed with effluent concentration, with the exception of nitrate levels, which remained

Table 1. Physical and chemical properties of the effluent.

Effluent concentration % v/v	Water temperature °C	Conductivity us/cm	pH	Redox potential mv	Ionized ammonia (mg/L)	Ammonia (NH_3 mg/L)	Nitrates (mg/L)	Oxygen saturation %
0 day 1	24.9	307	7.9	141	0.2	0.01	0.64	110
0 day 3	24.9	307	8.0	151	0.2	0.01	0.78	110
0 day 15	24.8	308	8.1	141	0.2	0.01	0.66	109
5% day 1	24.1	*312**	8.1	139	0.2	0.02	0.66	108
5% day 3	24.8	*312*	7.8	151	0.2	0.01	0.67	108
5% day 15	24.4	*308*	7.8	145	0.3	0.01	0.65	108
10% day 1	24.7	*314*	7.9	*135*	*0.3*	*0.01*	0.67	108
10% day 3	24.7	*316*	8.0	*140*	*0.3*	*0.01*	0.71	107
10% day 15	24.1	*318*	7.8	*140*	*0.3*	*0.01*	0,73	109
20% day 1	24.8	*366*	*7.8*	*122*	*0.7*	*0.03*	0.78	*97*
20% day 3	24.6	*383*	*7.7*	*134*	*1*	*0.03*	0.74	*92*
20% day 15	24.2	*377*	*7.7*	*125*	*0.8*	*0.02*	0.77	*93*
Mean ± SD	24.7 ± 0.2	323 ± 28	7.9 ± 0.12	140 ± 9	0.34 ± 0.28	0.01 ± 0.007	0.74 ± 0.14	106 ± 7

*Values in italics are statistically different from control tap water (0%): ANOVA and LSD at $p < 0.05$.

constant. Water conductivity and levels of un-ionized and ionized ammonia increased significantly with exposure concentration. Water pH, redox potential and O_2 saturation were significantly lower at the highest effluent concentration (20%). Dissolved ammonia concentrations were below the toxic threshold (approximately 17.6 mg/L total ammonia) at 0.029 mg/L and 0.85 mg/L for un-ionized and total ammonia, respectively. Total dissolved oxygen decreased from 110% saturation in the control tanks to 94% saturation (7.62 mg/L) at 20% effluent, but conditions were not considered hypoxic.

The morphological characteristics of the species were examined and are shown in figure 1. The condition factor (fish weight/fork length) was significantly influenced by sex only (factorial analysis of variance or ANOVA), $p < 0.01$ for sex and $p > 0.1$ for effluent concentration). In the controls, males were significantly larger than females, and increasing the effluent concentration had no significant effect (Figure 1A). No change in hepatic somatic index was observed (Figure 1B). The gonado-somatic index (GSI) was significantly influenced by sex only (Figure 2A), being generally higher in females compared to males. The sexual characteristics were qualitatively observed by monitoring changes in fish appearance based on the presence of ovipositors, nuptial

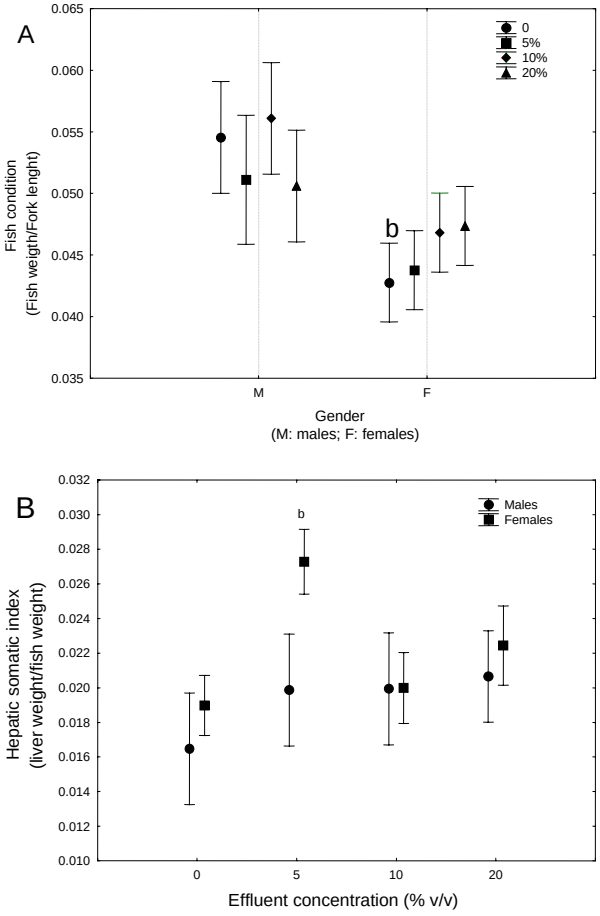

Figure 1. Fish morphological characteristics. Fathead minnows were exposed for 21 days to increasing effluent concentrations. Fish weight and fork length were determined (A) and the hepatic somatic index was calculated (B). In this figure and the following figures, the letter b represents significance between males and females.

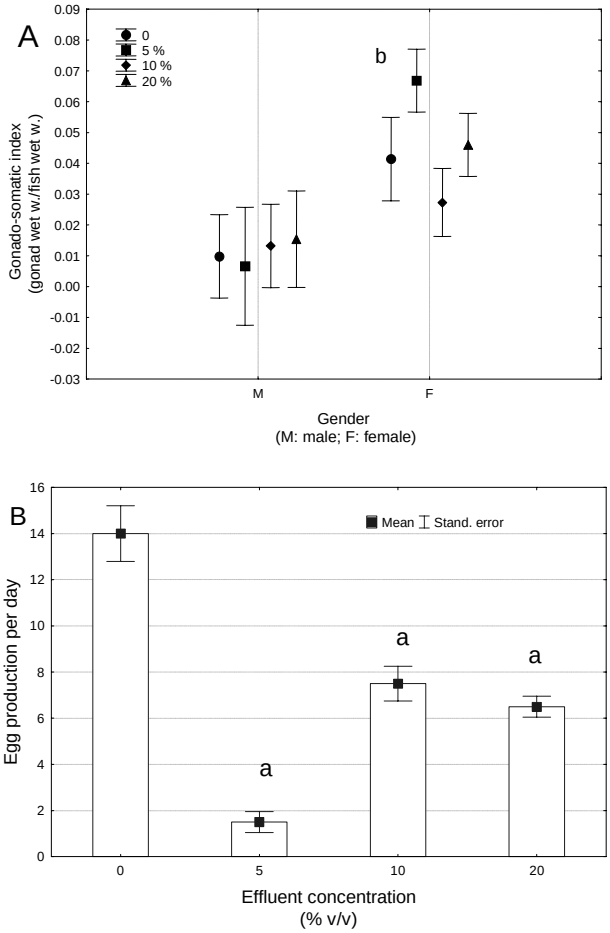

Figure 2. Gonad activity in fathead minnows exposed to effluents. Fathead minnows were collected and analyzed for gonado-somatic index (A) and egg production/ deposition (B). The letters a and b indicate significance in males and females, respectively, relative to controls ($p < 0.05$).

tubes, head sponge and banding colour in males (Table 2). The only change consisted in male discoloration at effluent concentrations of 10 and 20%. VTG levels were determined in male and female livers (Figure 2B). The number of eggs per day significantly dropped for all effluent concentrations. In the control fish, 1190 eggs were laid on the tiles. In fish exposed to effluent concentrations of 5, 10 and 20%, the total number of eggs was 178, 622 and 561, respectively. Based on visual observations, hatching rate, however, did not appear to be influenced by effluent concentration. VTG levels were significantly higher in males, with a median concentration of 1500 ng VTG/mg total protein at 20% effluent concentration (Figure 3A). In females, VTG levels were higher in the controls compared to males, with a median value of 122 ng VTG/mg total protein. VTG levels were significantly increased at 10 and 20% effluent concentrations, reaching values of 1400 ng VTG/mg total protein. The activity of glucose-6-phosphate dehydrogenase, a marker enzyme for lipogenesis, was significantly induced in females at 10 and 20% effluent concentrations (Figure 3B). No significant effects were observed in males. Correlation analysis revealed that VTG was not correlated with the above endpoints (condition factor, GSI and number of deposited eggs per day) (Table 3).

Metabolic energy was determined by monitoring changes in total sugars, lipids and mitochondrial electron transport activity in fish liver (Figures 4A, B and C). For total sugars, two-way analysis of variance revealed that effluent concentration had a significant effect, and that sex had no effect. Total sugars were significantly higher in livers of fish exposed to 5 and 20% effluent, reaching 2.6 times those of the controls. For hepatic lipid content, factorial analysis of variance revealed that only effluent concentration had a significant effect. Lipid contents were significantly higher at 5 and 20% effluent (Figure 4B). Lipid contents were highly variable at 10% effluent, hence not significant. Cellular energy expenditure was studied by monitoring mitochondrial electron transport (MET) activity (Figure 4C). Factorial 2-way ANOVA revealed that sex had no significant effect on MET activity, but effluent concentration had a significant effect. MET activity increased at 10 and 20% effluent, with a two-fold induction. Correlation analysis revealed that total liver sugars were significantly correlated with VTG ($r = 0.45$; $p < 0.05$). Hepatic lipid levels were negatively correlated with eggs produced/day ($r = -0.40$; $p < 0.01$). MET activity was correlated with VTG ($r = 0.58$; $p = 0.001$) and marginally correlated with hepatic sugars ($r = -0.40$; $p = 0.06$) and lipids ($r = 0.30$; $p = 0.07$). The activity of glucose-6-phosphate dehydrogenase was used as marker of lipogenic activity in fish liver (Figure 4). The activity was significantly increased in females exposed to 10% effluent while at 20% the increase was not significant ($p = 0.1$). Tissue damage was also examined in fish exposed to the municipal effluents (Figure 5). The levels of LPO in gills were significantly induced at 5 and 10% and returned to control values at 20% concentration in both male and female fish (Figure 5A). DNA strand breaks, a proxy for DNA repair activity, was significantly induced at 10% effluent concentration in both male and female fish (Figure 5B).

Table 2. Secondary sexual characteristics.

Effluent concentration (%v/v)	Fork length (mm)	Ovipositor	Nuptial tubercles	Head sponge	Male colour
0	M : 61.7 ± 2 F : 59 + 1	8/8	3/4	4/4	3/4
5	M : 62 ± 2 F : 58 ± 1.5	8/8	2/3	4/4	3/4
10	M : 61.5 ± 2 F : 58.5 ± 1	8/8	3 /4	3/4	2/4
20	M : 59.5 ± 2 F : 58.5 ± 1	8/8	4/4	4/4	2/4

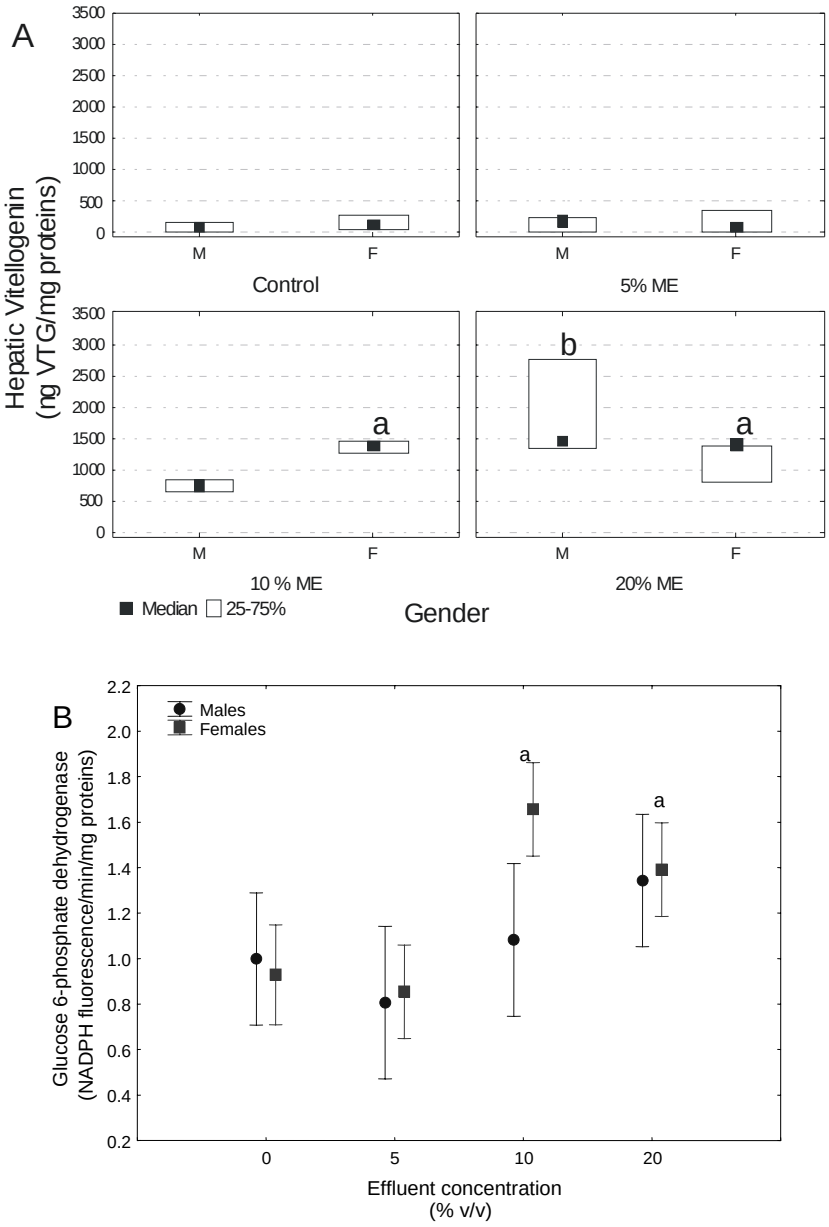

Figure 3. Estrogenic activity of fathead minnows exposed to the effluent. Fathead minnows were collected and analyzed for vitellogenin (A), and glucose-6-phosphate dehydrogenase (B). The letters a and b indicate significance from the controls and in males and females, respectively ($p < 0.05$).

The data was also analyzed using discriminant function analysis to identify biomarkers that best explain the effect of municipal effluent exposure on fish (Figure 6). The analysis revealed that increasing the effluent concentration displaced the square-root transformed data mostly on the y axis (second component). The biomarkers with the highest factorial weights were egg production, MET activity and hepatic VTG. On the x axis, the root function contributed to the discrimination of 5% effluent concentration which was best explained by egg production, MET and VTG levels. Biomarker responses in the controls and the 5% effluent exposure group formed distinct clusters, with classification efficiency of 100%, while the 10 and 20% formed a more confounded cluster, but were separated from the 5% effluent exposure group and controls and were classified at 83% efficiency.

Table 3. Correlation analysis of biomarker data.

	Eggs (d^{-1})	**CF**	**GSI**	**DNA breaks**	**LPO (gills)**	**Sugars (liver)**	**VTG (liver)**	**MET (liver)**	**G6PDH**	**Lipids (liver)**
Eggs (d^{-1})	1	0.06 p > 0.1	-0.24 p > 0.1	0.15 p > 0.1	**-0.48 p < 0.01**	-0.03 p > 0.1	-0.15 p > 0.1	-0.21 p > 0.1	0.07 p > 0.1	**-0.40 p < 0.01**
CF		1	*-0.31 p = 0.06*	0.04 p > 0.1	0.01 p > 0.1	0.11 p > 0.1	-0.005 p > 0.1	*0.30 p = 0.08*	0.03 p > 0.1	-0.13 p > 0.1
GSI			1	0.17 p > 0.1	-0.08 p > 0.1	-0.30 p > 0.1	0.15 p > 0.1	-0.23 p > 0.1	-0.11 p > 0.1	*0.28 p = 0.1*
DNA breaks				1	-0.17 p > 0.1	*-0.35 p = 0.1*	*-0.36 p = 0.08*	-0.30 p > 0.1	*-0.32 p = 0.08*	-0.04 p > 0.1
LPO (gills)					1	-0.01 p > 0.1	0.12 p > 0.1	-0.1 p > 0.1	-0.17 p > 0.1	-0.15 p > 0.1
Sugars (liver)						1	**0.45 p < 0.05**	*-0.40 p = 0.07*	*0.35 p = 0.07*	-0.1 p > 0.1
VTG (liver)							1	**0.59 p = 0.001**	0.10 p > 0.1	-0.002 p > 0.1
MET (liver)								1	0.07 p > 0.1	*0.31 p = 0.07*
G6PDH									1	0.11 p > 0.1

Significant correlations are highlighted in bold and marginally significant correlations ($0.1 < p < 0.05$) are given in italics.

The analysis showed that biomarker responses in the control group and fish exposed to the low effluent concentration group formed distinct clusters from those in the fish exposed to 10 and 20% effluent concentrations, which were more confounded albeit well classified at 83%. Factorial analysis revealed that egg production and VTG endpoints were found on each component (x and y axis), suggesting major effects on reproduction. Egg analysis for alkali-labile phosphates (ALP), total sugar and lipid content were also performed (Table 4). Egg phosphorylation state did not change as a function of effluent concentration, but had reduced lipid content and increased sugar content, which suggests a shift from lipid-rich VTG to VTG with a higher sugar content. No change in labile zinc levels were found in eggs from fish exposed to increasing effluent concentrations. In hatched fry, increased lipid peroxidation was found at 20% effluent (Table 4).

DISCUSSION

Exposure of adult fathead minnows to a physically-chemically treated municipal effluent led to increased VTG expression and readily increased MET activity in the liver of both male and female fish. Increased VTG expression is dependent on the activation of estrogenic signaling pathway [19]. In fathead minnows exposed to treated municipal effluents, increased steroid expression, lipid transport and oxidative stress were also observed, in addition to increased plasma VTG [20]. This is consistent with increased lipid content in the liver and increased oxidative stress in gills of fish exposed to municipal effluent in this study. This indicates that the estrogenicity of municipal effluent is associated with the mobilization of energy in fish and could lead to oxidative stress. Indeed, MET activity, one of the major sources of oxidative stress in cells, was significantly correlated with hepatic VTG levels and marginally related with hepatic sugar content. Furthermore, an analysis of covariance of MET against effluent concentration with VTG as the covariable revealed that MET activity was more strongly related to VTG levels ($F = 26$; $p < 0.001$) than effluent concentration ($F = 8.6$; $p = 0.001$), which suggests that VTG synthesis was a significant

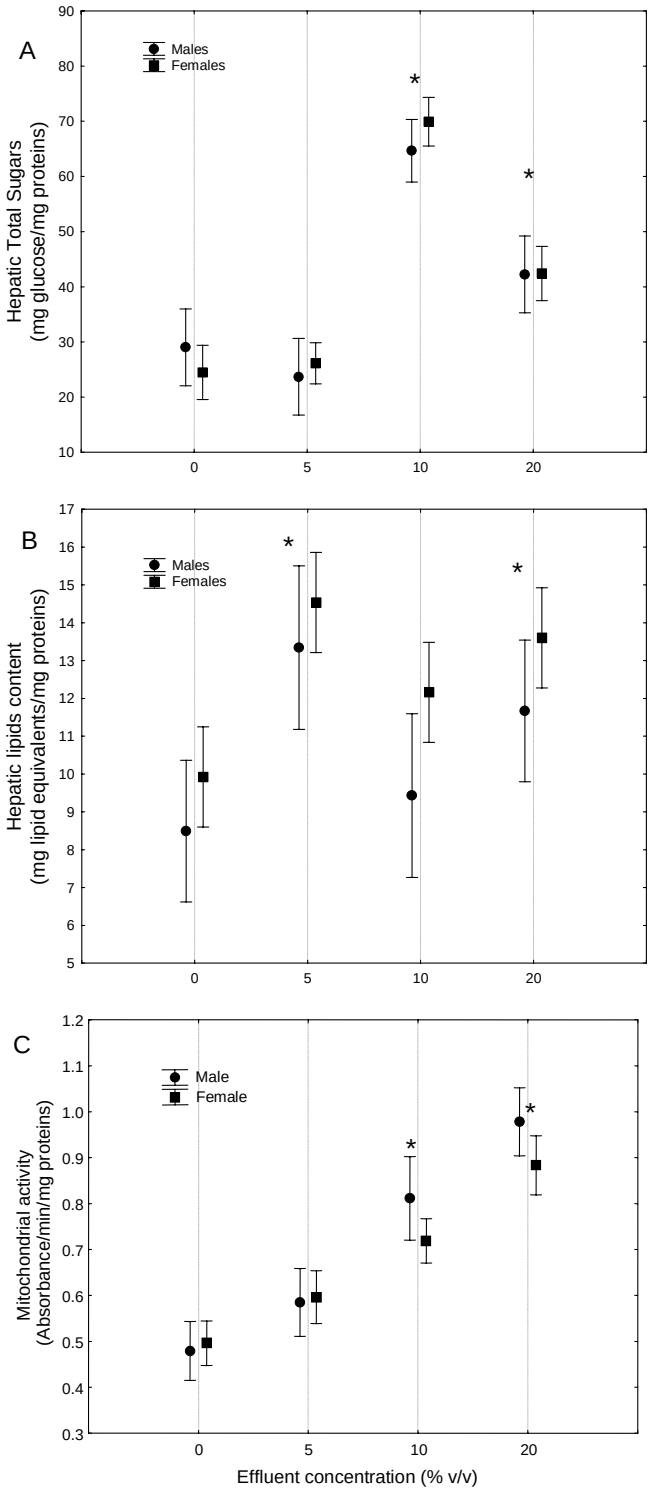

Figure 4. Energy status in fathead minnows exposed to municipal effluents. The levels of hepatic sugars (A), lipids (B) and mitochondrial electron transport activity (C) were determined. The star (*) symbol indicates significance at $p < 0.05$ level.

contributor to energy expenditure. Indeed, VTG is an energy-rich glycolipophosphoprotein that contains lipids, sugars and phosphates as nutrients for the developing embryo. Based on the relative levels of energy components (total sugars and lipids) and energy expenditure (MET), fish undergoing vitellogenesis that are exposed to municipal effluent do not show a negative energy

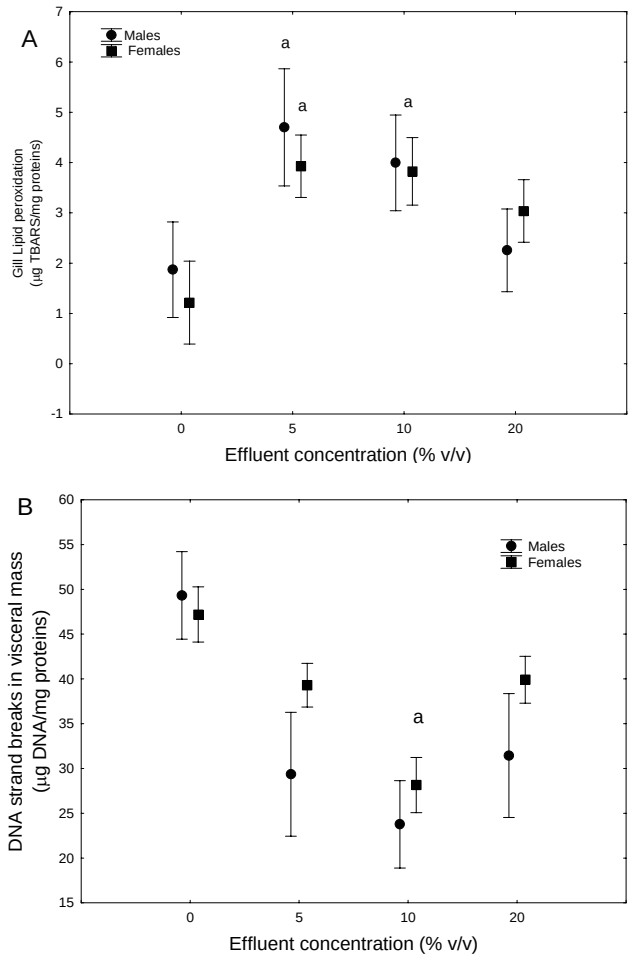

Figure 5. Toxic stress in fathead minnows exposed to a municipal effluent. Toxic stress was determined by monitoring LPO in gills and DNA strand breaks in the visceral mass. The letter a indicates significance at $p < 0.05$ level from the controls.

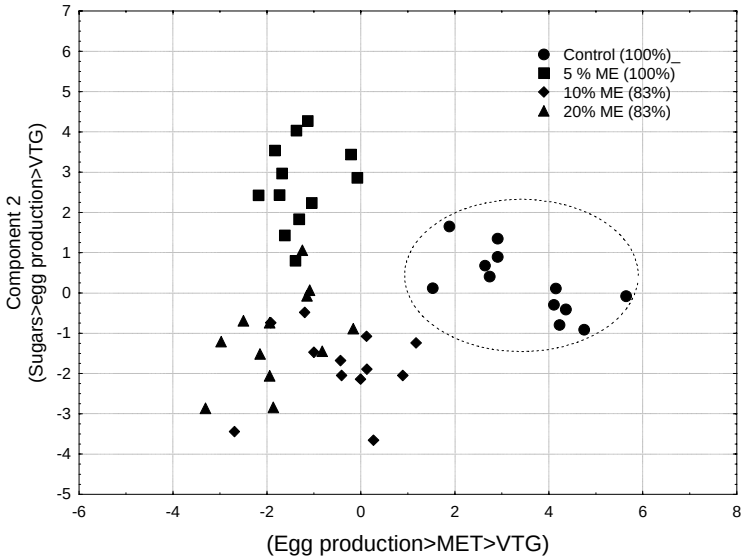

Figure 6. Discriminant function analysis of biomarker data. The biomarker data were analyzed by discriminant function analysis followed by factorial analysis. The biomarkers with the 3 highest factorial weights are shown in the y and x axis. The ellipse regroups the control responses.

Table 4. Egg and larvae analysis.

	Eggs				Larvae	
Effluent concentration (% v/v)	**ALP** µg Pi/mg proteins	**Lipids** mg lipids/mg proteins	**Sugars** µg glucose/mg proteins	**Zn**	**DNA strand breaks** µg DNA/mg proteins	**LPO** µg TBARS/mg proteins
0	64.8 ± 6	6.1 ± 0.6	301 ± 15	1381 ± 200	162 ± 13	5.5 ± 1
5	75 ± 3	**4.4 ± 0.2***	350 ± 14	1121 ± 57	**238 ± 7***	5.3 ± 2
10	80 ± 10	**2.7 ± 0.3***	**400 ± 14***	**862 ± 106***	154 ± 20	**12 ± 5***
20	61 ± 9	**4.1 ± 0.3***	**479 ± 30***	1037 ± 150	149 ± 20	**31 ± 2***

*Significant at $p < 0.05$ (in bold).

budget since energy expenditure is offset by increased levels of sugars and lipids. However, the production of VTG is an energy demanding process and hence its contribution to MET activity in fish. There was only a marginal decrease in total sugar levels with MET activity, suggesting a slight decrease in energy budget in the form of sugars in the liver of fish. However, sugar contents were elevated in eggs from fish exposed to the municipal effluent. In a previous study, fathead minnows collected downstream of an effluent outfall weighed less and were shorter [7]. Male fathead minnows had reduced secondary sexual characteristics (lack of dorsal fin dot, fewer nuptial tubercles and little development of the dorsal pad), thickening of gill lamellae and kidney inflammation. Only a slight discoloration was observed in banding in males exposed to the effluent for 21 days at 10 and 20%, but gill LPO readily increased as a result of exposure to the effluent and was negatively correlated with daily egg production.

One noteworthy effect of exposure to municipal effluents was decreased daily and total egg production. In caged fatheads minnow at urbanized sites, a decrease in egg production was also observed [21]. Globally, there was a negative relationship between egg production and urbanization (human population density) and a positive relationship with wetlands area. The estrogenicity of other municipal effluents was also observed using the fathead minnow bioassay [22]. However, in fish exposed to chlorinated or granular activated charcoal-treated municipal effluents, but not to ozonated effluents, plasma VTG levels remained constant. Interestingly, egg production was reduced by exposure to the effluents, but ethinylestradiol (EE2) at 10 ng/L alone could not reduce egg production. In another study, exposure of fathead minnows to a municipal effluent resulted in decreased egg production, which was proportional to the estrogenic content of the effluents [23]. However, the tested effluents would have had greater effects on egg production based on their capacity to induce VTG levels or when compared with an equivalent estrogenicity potential of EE2 alone. The identification of other contaminants responsible for the observed effects is difficult. Reduced egg production could also be the result of other forms of toxicity (i.e., non-specific toxicity) of various contaminants present in the effluent. For example, oxidative stress and inflammation could reduce the capacity of the fish to produce healthy eggs [21]. For instance, the pesticide atrazine was shown to reduce egg production in Japanese medaka [24] by affecting/blocking oocyte maturation processes and blocking egg ovulation in females. On the other hand, a 28-day exposure to benzo(a)pyrene (1 and 10 µg/L), a common polyaromatic hydrocarbon found in municipal effluent, had no effect on VTG levels or egg production in *Fundulus heteroclitus*, although it reduced estradiol-17β levels [25]. The reproductive effects of six pharmaceuticals and trichlosan revealed no effects on egg production or other reproductive endpoints in fathead minnows, suggesting that pharmaceuticals are not major contributors, at least when tested individually [26]. The tested pharmaceuticals were naproxen, diclofenac, gemfibrozil, ibuprofen, salicylic acid,

acetaminophen from the analgesic (non-steroidal anti-inflammatory drugs), triclosan, triglyceride reducer (clofibrates) and bacteriostatic therapeutic classes. Estrogens are a key class of compounds which, when present in municipal effluents, are capable of reducing egg production in fathead minnows. In an earlier study, egg production in fathead minnows exposed to EE2, a component of oral contraceptives, exhibited a biphasic (hormetic) response, i.e., it was induced at low EE2 concentration (0.1 and 1 ng/L) and decreased at higher concentrations (3-100 ng/L) after 3 weeks at 25 ^{o}C [27]. Exposure to 29 ng/L of estradiol resulted in the rapid production of VTG in male fathead minnows after 2 days and remained elevated for up to 70 days after cessation of exposure [28]. However, in females expressing high VTG levels, exposure to estrone (307 ng/L) and an aromatase inhibitor (fenarimol at 500 ng/L) was also associated with reduced egg production.

The hatched eggs were shown to have a lower lipid content but a higher sugar content (Table 3). Hatched larvae (post 24 hr) exhibited increased LPO as well. The long-term consequences of these effects are unknown at this time. Current studies reveal that exposure of fathead minnows to estrogenic compounds has little impact on F1 generations at environmentally relevant concentrations [29, 30]. For example, in a study in which adult fathead minnows were exposed to bisphenol A (BPA) the no observed effect concentration (NOEC) for this compound was found to be well above the median and upper 95th percentile concentrations of BPA in fresh waters in North America and Europe. However, mixture effects were not thoroughly examined and concerns exist about the cumulative or joint effects of mixtures on aquatic life. In the second study, exposure of breeding pairs of fathead minnows to the weak estrogen 4-tert-pentylphenol for 21 days had no discernible effects on the F1 generation (sex ratio, sexual differentiation and development) whereas exposed parents exhibited decreased egg production/spawning and hatching rate with increased plasma VTG in males exposed to 560 µg/L tert-pentylphenol. However, F1 males in the high concentration (> 180 µg/L) group contained higher VTG levels. These studies indicate that the long-term consequence of increased sugars and decreased lipids in eggs with signs of oxidative damage in the larvae are unknown at present.

CONCLUSION

In conclusion, exposure of fathead minnows to a physically-chemically treated effluent for 21 days resulted in increased VTG expression, oxidative stress, lipids, sugars and energy expenditure (MET). Hepatic VTG levels were strongly correlated with higher energy expenditure, which suggests that the estrogenic activity of municipal effluent contributes to energy expenditure in fish. However, as in previous studies, a decrease in egg production was observed, indicating that estrogenic effluents do not lead to increased egg production and fertilization. On the basis of hatching success, fertilization did not appear to be affected. The eggs had lower lipid content and higher sugar content with no changes in the phosphorylation state. However, the hatched larvae exposed to municipal effluent had increased LPO levels, suggesting a transfer of oxidative stress, which have potential impacts on the next generation of fathead minnows exposed to municipal effluent.

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CONFLICT OF INTEREST STATEMENT

There are no financial or commercial conflicts of interest in relation to this article.

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