

## LONG TERM EXPOSURE OF FRESHWATER MUSSELS TO A MUNICIPAL EFFLUENT PLUME LEADS TO FEMINISATION.

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### SUMMARY

The purpose of this study was to verify the hypothesis of feminisation of the freshwater mussel *Elliptio complanata* exposed for one complete reproductive cycle (1 year) to a municipal effluent plume. Mussels were harvested from a reference lake and placed in specially-designed cages adapted for long-term exposure to a suspended matter-rich river environment. After exposure, mussels were collected to measure growth-related indices, vitellogenin-like proteins and sex ratio. Results showed that mussels from the downstream sites had increased condition factor and gonado-somatic index. They also displayed significantly more vitellogenin related proteins in their gonads and the proportion of females was increased from 41 % (upstream site) to a high of 66 % (farthest downstream site). This high proportion of females is not usually observed in natural populations of *Elliptio complanata* and indicates a feminisation effect on freshwater bivalves by estrogenic compounds continually discharged into the receiving environment.

KEY WORDS : Estrogens, municipal effluent, vitellogenesis, feminisation, gonado-somatic index.

### INTRODUCTION

Municipal effluents are notorious for their release of miscellaneous contaminants and microorganisms into the aquatic environment. For example, pharmaceutical products [1], heavy metals, polyaromatic hydrocarbons, pesticides and endocrine disrupting compounds such as estrogens are released by these sources [2,3]. In municipal wastewaters, the major compounds responsible for estrogenicity are estradiol-17 $\beta$  (and 17 $\alpha$ -ethinylestradiol), nonylphenol and bisphenol A. Estrogens, in terms of their endocrine disrupting potential on the environment, are by far the most studied. They are generally known to activate estrogen receptors leading to several changes normally associated with female sexual characteristics for reproduction.

The bivalve, *Elliptio complanata*, is ubiquitous in the freshwater portion of the St.-Lawrence River and nearby rivers and lakes. It is dioecious with distinct male or female characteristics and a relatively low incidence of hermaphrodites [4]. In this oviparous species, sex ratio was found to

be fairly skewed towards males with a natural proportion ranging from 55 to 60 % of males according to their size [5]. Moreover, this species is somewhat protandrous because younger and smaller mussel populations have more males in respect to older and larger ones [5]. Thus, younger mussels begin their life as males and some of them will gradually change into females by passing through an hermaphrodite stage as they increase in size. In oviparous organisms, vitellogenin (Vtg), a high energy content egg-yolk protein precursor, is regulated by the estrogen receptor and hence induced by estradiol-17 $\beta$ . In natural conditions, oocytes secrete estradiol-17 $\beta$  to activate the target organ responsible for vitellogenesis (i.e., liver in the case of fish or gonad follicular cells in bivalves), whose function it is to produce large amounts of Vtg for developing embryos. However, other estrogen mimics such as p-nonylphenol (NP) may also activate vitellogenesis in both fish [6], as well as in freshwater [7] and marine [8] bivalves. A remarkable biological effect of estrogens is that they have the ability to increase the proportion of females in fish and bivalves [9-11]. Although sexual differentiation mechanisms remain to be fully understood in invertebrates, the process seems to start in nerve tissues where estradiol, serotonin and the neuropeptide (Ala-Pro-Gly-Trp-NH<sub>2</sub>) in neural ganglia are involved [12-13]. The latter controls the male reproductive organs in gastropods whose increased expression is associated with the imposition of male structure in female sexual organs, and hence imposex conditions, in gastropods. In fish brain, females have increased estradiol-17 $\beta$  and lower serotonin levels while the inverse is found in males. In mussels, lower serotonin and catecholamine levels were also reported in the female portion of the gonad in the hermaphrodite bivalve *Argopecten purpuratus* [14]. In oysters, injection of estradiol-3-benzoate during the resting phase of the gonad, led to a 15 % increase in the proportion of females with respect to control oysters [11]. In general, studies indicate that estradiol-17 $\beta$  is able to feminize oysters when administered during the immature stage of gametes and stimulates female gametogenesis including vitellogenesis [15].

The purpose of this study was therefore to observe changes in the sex ratio of mussels after long-term exposure to wastewater of a major urban effluent discharge. Mussels were exposed for one year in cages placed upstream and downstream of a primary-treated municipal effluent. Retrieved animals were analyzed for condition factor, gonado-somatic index, visceral sex and Vtg levels. An attempt was also made to relate the vitellogenin biomarker as a causative factor for feminisation.

## METHODS

### **Mussel collection**

Mussels were collected at Lake de L'Achigan in the Laurentian mountains, Quebec, Canada, in an area known to abound in *Elliptio complanata* [16] during the third week of May 2001 (water temperature 8-10°C). They were hand-picked and kept in coolers at 4°C for their immediate transport to the laboratory. They were maintained in 300 L tanks in dechlorinated, UV-treated and aerated tap water at 12-15°C for several weeks, and fed three times a week with *Selenastrum capricornutum* micro-algae or cultures of commercial coral reef solution.

### ***In situ* exposure of mussels**

Initially, mussels were sorted by size (weight and shell length) and placed in a special benthic cage designed to accumulate and hold suspended particles. In the inner portion of the benthic pen, consisting of a 40 cm diameter cylindrical net (Vexar™ mesh, Aquipro, Aquipro@avantage.com, Quebec, Canada) containing 10 cm of clean sand in its bottom, were placed 27 mussels (ranging from 62.3 to 88.9 mm in length). The cylindrical net was then inserted into a 60 L rigid plastic container with the bottom removed. Three benthic pen cages were deployed at each of three sites located 1.5 km upstream, 8 km and 10 km downstream of a municipal effluent outfall (Figure 1). Temperature monitor probes were also fixed to cages at each site. They were submerged in the river for close to one full year (12<sup>th</sup> of June 2001 until May 25<sup>th</sup> 2002). After the exposure period, cages were collected and mussels were analyzed for survival, shell length, total and soft tissue weights. The gonado-somatic index was also determined.

### **Sex determination**

Sex was determined by microscopic examination of a gonad smear. After a 30-60 min drying time, microscope slides containing each smear were stained by adding 20 drops of hematoxylin-eosin stain (Sigma Chemical Co) for 30 min, washed with water and dried. The slides were then examined at a magnification of 400 x. Smears indicative of large oocytes were classified as females and those showing the presence of small and elongated cells were classified as males. In some cases, when either female or male characteristics were unclear or absent, sex was classified as undetermined.

### **Determination of vitellogenin-like proteins**

Relative levels of vitellogenin-like proteins were determined in gonad tissue by an organic alkali-labile phosphate assay [7] with the following modification. Gonad tissues were dissected out of the visceral mass and homogenized at 4°C with a teflon pestle in 125 mM NaCl, 10 mM Hepes-NaOH, pH 7.4, 1 mM EDTA and 1 mM dithiothreitol. The homogenate was then centrifuged at 12 000 x g for 20 min at 2°C. High molecular weight proteins were precipitated in 35 % acetone. The protein pellet was obtained by centrifuging at 12 000 x g for 5 min at 20°C. The resulting pellet was resuspended in 200 µL of NaOH 1M and heated at 60°C for 60 min. The released phosphates were determined as described elsewhere [17]. Calibration was achieved with rainbow trout vitellogenin and inorganic phosphate. The data are expressed as µg of alkali-labile phosphates/ mussel wet weight (g). The levels of vitellogenin-like proteins were also determined by gel electrophoresis using precast 4-12 % high resolution mini-gels (Invitrogen, USA). One volume of S<sub>12</sub> extract was mixed with one volume of loading buffer (50 % glycerol, 0.1 % SDS, 10 mM dithiothreitol, 100 mM NaCl, 0.001 % bromophenol blue and 10 mM Hepes-NaOH, pH 7.4). After loading 25 uL to the wells, gels were resolved at 90 volts for 90 min and stained with Coomassie blue Biosafe staining kit (Biorad, Canada). Gels were scanned with an horizontal scanner and band density determined by the Unscanit software (USA). Molecular weight markers and rainbow trout Vtg were also used for calibration of the gels. The data are expressed as densitometric units/g wet weight of mussels.

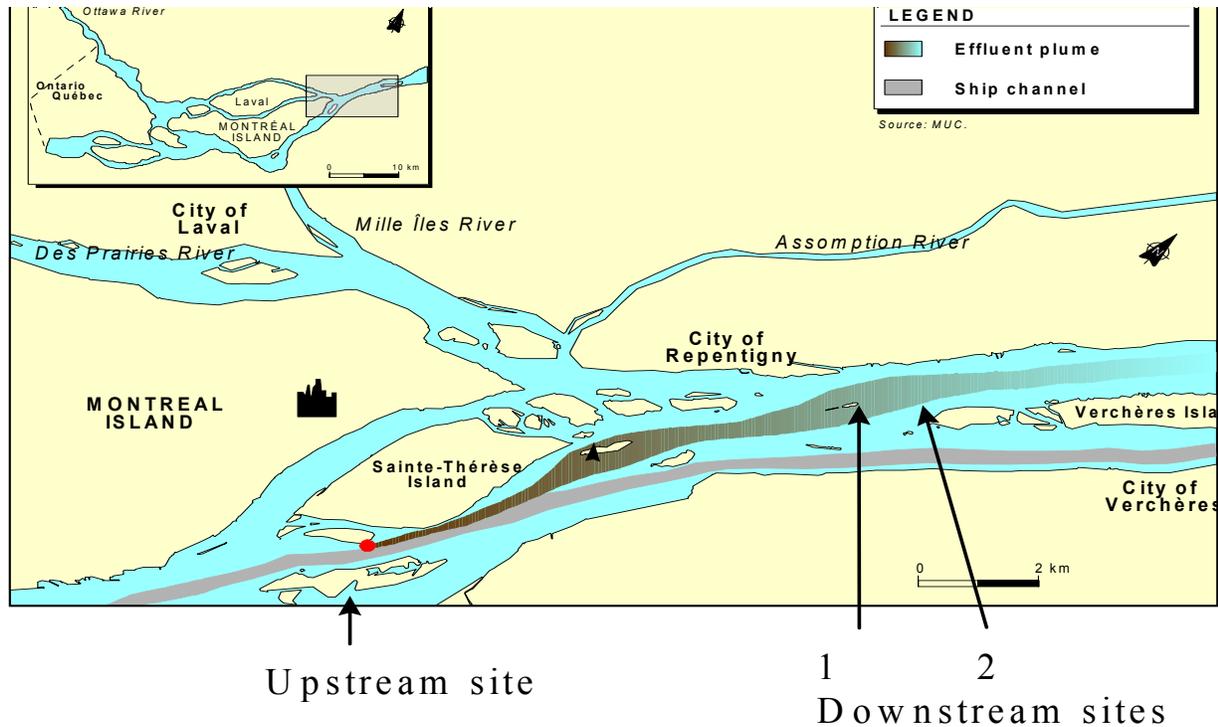


FIGURE 1 Site locations for the one-year exposure of caged mussels.

Freshwater mussels were placed in special benthic cages and submerged at three locations in the St. Lawrence River for one year ( $n = 3$  cages per site each containing 27 mussels). The upstream site is located 1.5 km above the effluent plume; the downstream sites are located at 8 km and 10 km below the plume.

## RESULT AND DISCUSSION

The shell length range of exposed animals in this study was between 62.3 to 88.9 mm with a mean and standard deviation of  $76.36 \pm 0.6$  mm for both upstream and downstream sites. No significant change in shell length was found after the one-year exposure period. It has been reported that natural Eastern *Elliptio* populations in the 50-90 mm range have about 55 to 60 % of males [5]. This is similar to the sex ratio range found in mussels at the upstream site (Table 1). Hence, there is no indication of sex changes at the upstream site with respect to the reference lake. During cage retrieval, significant amounts of particulate matter were observed to have accumulated in the cages very likely resulting from the plume and the water column. In fact, cages were found to contain 8-16 cm of suspended matter which had been deposited over the sand layer. Water temperature did not vary significantly between sites over the year. Overall, mussel survival averaged about 82 % with no clear trend with distance in the plume, albeit

highest mortality occurred at the most downstream site (30 % mortality). Condition factor (or condition index), as defined by total weight to shell length ratio, was significantly higher at the downstream sites in females ( $p < 0.05$ ). The gonado-somatic index was also found to be higher at both downstream sites in both males and females.

Soft tissue weights were higher at the 8 km downstream site in both sexes while no significant changes were obtained at the farthest site (10 km downstream). Shell weight was only found to be significantly increased in females at the first downstream site from the outfall (not shown). In an earlier study in caged mussels, a 60-day exposure of *Elliptio complanata* mussels to the same effluent, but at 4 km downstream of the plume, also had increased total and soft tissue weights [7]. At this particular site, it was also shown that slightly longer exposure periods (70 and 90 days) led to important mortality in caged mussels (> 90 %). Burying of mussels by particles which abound in the plume or toxicity (e.g., ammonia) are suspected factors. In these 60 to 90-day shorter-term studies, the netted cages used, designed more for water column exposure, differed markedly from the benthic cages which were found to be more appropriate for long term exposures in the present study.

Table 1. Morphometric characteristics and sex ratio in mussels.

Sites	% survival in benthic pens	Condition Index (g/mm) <sup>1</sup>	Soft tissue weight ratio (mg/g) <sup>2</sup>	Gonado-somatic index (g/g tissue) <sup>3</sup>	Proportion of females (%)	Undetermined sex condition (%)
Upstream	79	0.486±0.002	0.62±0.3	10.9±1	41	3.5
8 km Downstream	95	0.526±0.008*	0.88±0.09*	15.2±1.4*	62*	4
11 km downstream	70	0.503±0.001*	0.58±0.04	15.7±1.1*	66*	5

1. soft tissue wet weight/mm shell length

2. weighth of soft tissue / total weight (tissue + shells)

3. gonadal tissue wet weight/wet weight of soft tissue

\* indicates statistical difference at  $p < 0.05$  level.

Mussel gonad tissues from both sexes were enriched by high molecular weight protein(s) in the range of 250 kDa which are characteristic of Vtg. The levels of alkali-labile phosphates (ALP) and Vtg-like proteins in the gonad were significantly increased at downstream sites (Figure 2). These responses were observed in both males and females but the effects were more pronounced in females than in males. The increased susceptibility of females to respond to estrogens discharged by a municipal effluent were also observed in an earlier study [7]. Vtg protein was increased to about 4-fold while ALP increased only 2-fold. Vtg production is known to be regulated by the female sex hormone estradiol-17 $\beta$  and other hormone mimics [7,15]. However, the levels of ALP at the farthest site (10 km) did not show a significant increase (1.4-fold increase) with respect to the upstream site. In contrast, protein levels still remained high indicating that phosphorylation of the protein complex was decreased. This discrepancy at the farthest site remains unclear but it has been reported that the chemical composition of Vtg in bivalves (i.e., carbohydrate, lipid, phosphate content) can be altered in contaminated areas as can be the maturation stage of gonads [18]. Another explanation may be linked to the possibility that gonad protein levels masked the increase in ALP at this site or that phosphorylation was less pronounced at this distance. Differing sexual maturation rates between sites may also have had an influence on ALP levels, although we can only assume that these were likely similar based on temperature measurements which did not show variation among sites during the one-year exposure.

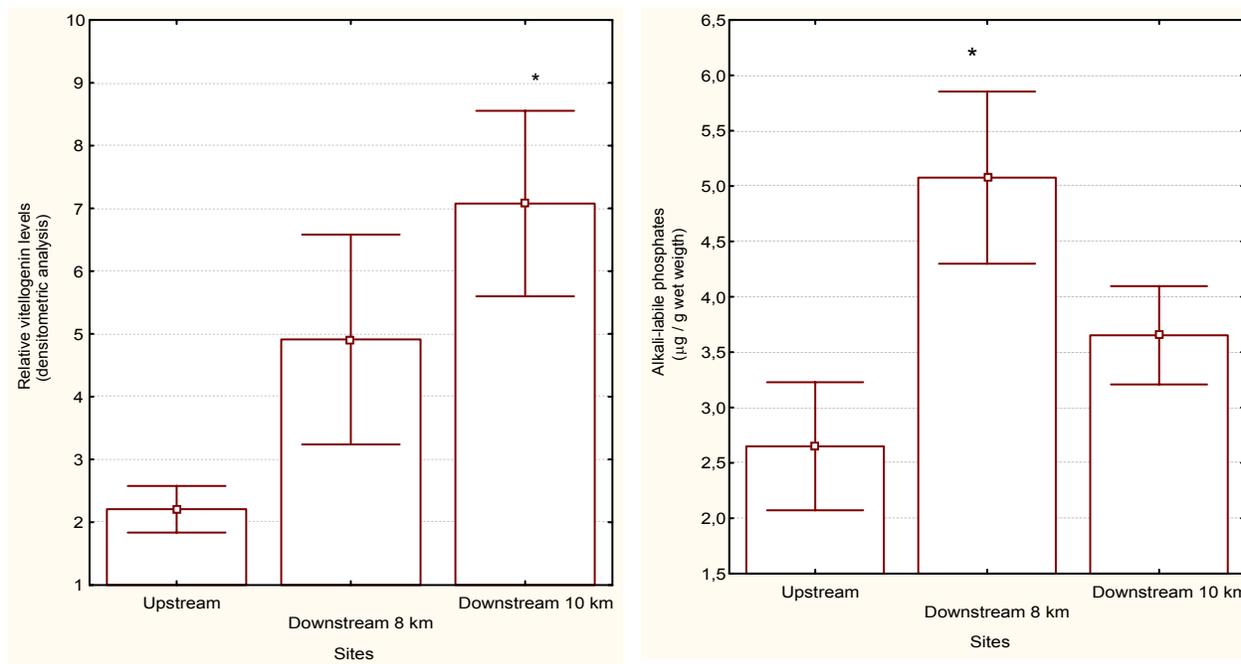


FIGURE 2. Relative vitellogenin-like proteins in mussel gonad.

Relative Vtg levels were determined in mussel gonad by gel electrophoresis. Alkali-labile phosphates were also measured in gonad extracts in the insert. The data represent the mean with the standard error. \* indicates significance at  $p < 0.05$ .

The sex ratio was found to be significantly skewed towards females at both downstream sites with respect to the upstream site (Table 1). This indicates that the proportion of females could be experimentally induced by long-term exposure to a primary-treated municipal effluent plume. Over a one-year period of exposure, feminisation therefore appeared with at least a two-fold induction of ALP or Vtg related proteins. Moreover, both Lake-collected *E. complanata* mussels and those exposed at the upstream site, equivalent in terms of their shell length range, also displayed a similar sex ratio (40-45% females), which corresponds to that found in natural conditions [5]. Hence, an increased proportion of females at the downstream sites does not reflect that of natural settings. Exposure of mussels to an estrogen (estradiol-3-benzoate) during the undifferentiated stage of the gonad was shown to significantly increase the number of females in oysters [11] and to accelerate their gonad maturation rate [15]. Our results also indicate that the gonado-somatic index is readily increased in both sexes at downstream sites. Municipal effluents contain many steroids such as cholesterol, coprostanol and estradiol-related compounds [19] and mussels are thus in a position to accumulate these chemicals which can act as estrogen mimics

such as has recently been demonstrated for coprostanol [20]. In addition, other compounds typically found in sewage waters (e.g., nonylphenol, ethinyl-estradiol and some organochlorine pesticides) which are capable of interacting with estradiol binding sites in mussels can also contribute to increased Vtg-like proteins and mussel feminisation.

This is the first study, to our knowledge, demonstrating that mussels chronically exposed to an urban effluent have increased proportion of females and Vtg. Mussels are particularly at risk to contaminants because they are sessile, thrive in benthic environments and display good longevity (10-year life span or greater). Increases in the proportion of females not normally found in nature could have severe consequences for the survival of the population. In conclusion, mussels were successfully exposed for one complete cycle of reproduction to a municipal effluent plume. Exposed animals displayed increased condition factor, gonado-somatic index and expression of Vtg. The proportion of females was augmented at levels that do not normally occur in natural populations of *Elliptio complanata*.

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