EVIDENCE OF ENDOCRINE DISRUPTION IN MYA ARENARIA LOCATED NEAR THE MOUTH OF THE SAGUENAY FJORD LINKED TO TBT SEDIMENT CONTAMINATION

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ABSTRACT
Previous studies in our laboratories have shown that *Mya arenaria* in the Saguenay Fjord have delayed sexual maturation and decreased immune competence. To verify if TBT in sediments could be harmful to *Mya arenaria*, a bivalve of economic importance, TBT, sex steroid hormones (testosterone, estradiol, progesterone) were measured in gonads. Clams were collected in a site subjected to heavy naval traffic at the mouth of the Saguenay Fjord (Baie Ste-Catherine (BSC), and measured parameters were compared with those of clams sampled at control sites nearby and along the Fjord. Our data show that in the contaminated site, TBT levels were high (400 ng g-1 gonads d.w.) while the gonado-somatic index was significantly decreased. Testosterone levels were increased in both female and male *M. arenaria* and sexual maturation arrived earlier than expected in females. We can therefore conclude that endocrine disruption of sexual maturation of *Mya arenaria* is present in the Saguenay Fjord and that ecological consequences are likely to occur.

RÉSUMÉ
Des études récentes dans nos laboratoires ont démontré un délai de la maturation sexuelle et une compétence immunitaire diminuée chez *Mya arenaria* dans le Fjord du Saguenay. L'objectif du travail présenté ici était de vérifier si le TBT présent dans les sédiments pouvait modifier la maturation sexuelle de la mye. Pour réaliser notre objectif, nous avons mesuré dans les gonades de la mye les hormones stéroïdes sexuelles (testostérone, estradiol et progestérone). Nous avons échantillonné des myes à partir d'un site soumis à un trafic naval intense (Baie Ste-Catherine (BSC), et comparé les résultats à ceux de sites témoins à proximité et au long du Fjord. À BSC, les niveaux de TBT sont très élevés, avec des teneurs près de 400 ng g-1 gonade p.s. tandis que l'indice gonado-somatique est inférieur à celui retrouvé dans les myes témoins. Les teneurs en testostérone sont plus élevées au site contaminé et la maturation sexuelle y est plus précoce chez les femelles. Ces résultats démontrent une perturbation endocrine à l'embouchure du Fjord, ceci constituant une alarme pour d'éventuelles conséquences écologiques.

1. INTRODUCTION

It is now well known since the beginning of the 90's that many contaminants, such as organochlorines, PAHs and organotins, can act as endocrine disruptors in wildlife (Colborn and Clement 1992; Crain and Guillette 1997). There is also a growing awareness that sediments act as a sink to these contaminants (Loring 1978) and that their effects on reproduction could last longer than expected and then lead to a threat for populations exposed. Among the aquatic ecosystems threatened by such substances, the Upper St. Lawrence River has been shown to be highly contaminated with mercury (Gobeil et al. 1997). In addition, the St. Lawrence River is the main entrance to Canadian Great Lakes and is a major shipping route for cargo traffic (tankers, container ships and fishing boats) (Pelletier and Normandeau 1997). Despite the ban of organotin-based paints on boats shorter than 25m (since 1989), a persistent tributyltin (TBT) contamination of marine waters has been shown in Canada (Chau et al. 1997). TBT contamination has been recorded in sediments, mussels, clams, sea stars and beluga whales of the St. Lawrence River (Pelletier and Normandeau 1997; Saint-Louis et al., 1997; Saint-Louis et al., 2000) and the Saguenay Fjord (Gagné et al. 2002). Recent studies have also demonstrated chronic effects of contaminated sediments, like a delay in sexual maturation and decreased progesterone levels (Gauthier-Clerc et al. 2002; Siah et al. 2002). Moreover, a direct link was made (Gagné et al. 2002), at the mouth of the Saguenay Fjord, between tributyltin contamination and effects on reproduction. It has been shown that the sex ratio was significantly skewed toward males in 2001, and that the condition and gonad-somatic indices, vitellin-like proteins in female gonads and the capacity of female gonads to produce estradiol-17β were significantly reduced at the harbor site (Baie-Sainte-Catherine) with respect to the reference sites. In order to further investigate the masculinizing effects of TBT contaminated sediments in Baie Sainte-Catherine, we carried out in 2002 a survey of sex ratio, sexual maturation and steroid hormone levels in both male and female *Mya arenaria* in comparison with results from a reference site (Moulin-à-Baude) and from two sites within the Fjord.

2. METHODS

2.1. Clam collection and tissue preparation

Clams were collected at four sites which include two sites in the Saguenay Fjord: Anse-aux-Érables (AE), upstream and in front of Ste-Rose-du-Nord. This site is considered as directly exposed to upstream contaminants of industrial and domestic origin (Pelletier and Canuel 1988). The other site in the Fjord is Anse-Saint-Étienne (ASE), a reference site with no known or apparent source of contamination. In the St.Lawrence Estuary , we have chosen one harbor site supporting commercial and whale-watching boats (Baie St.Catherine or BSC) and a reference site, located about 10 km north-east of BSC (Baie-du-Moulin à Baude or MAB).

Animals were collected in June 2002 which corresponds to the spawning time for these organisms (Gauthier-Clerc et al. 2002) thus allowing good estimates of sex ratio and gonado-somatic index. Clams were collected from intertidal mud flats at low tide with a shovel (and/or pitchfork) at 15-20 cm depth and transported in a cooled icebox to the laboratory within 3 h. They were aged by counting the number of major grooves (annual rings) on the shell. The shell length, total weight, whole soft and gonad tissue weights were determined. Sex was determined by microscopic observation of a smear of gonadal tissue on a glass slide (400 x magnification). Gonad tissues were then stored on dry ice for subsequent biochemical analyses as described below. Hormonal levels were only carried out on BSC and MAB samples.

2.2. Histological study

Maturation stage of the gonad was determined for sixty organisms collected at each sampling site. A sample of gonad was kept at -80 ^{o}C for histological examination. Sections of gonads were cut at 3 µm using a motorized Cryotome from Thermo Shandon and treated with a Lee's Methylene blue-basic fuchsin staining solution for microscopic examination. The gonad organisation and the cytological characteristics of the germinal and somatic cells for both sexes were determined using a light microscope (Olympus BX50). Five maturation stages were determined according to Coe and Turner (1938): indifferent, development, ripe, spawning and spent.

2.3. Steroid extraction and Enzyme ImmunoSorbent Assay

Testosterone, progesterone and estradiol titers were estimated using a commercial ELISA kit (Cayman chemical). Gonad was homogenized in H_2O (1:5 w:w) and sonicated twice for 30 sec. 400 µL HCl 0.025M were added to 500 µL homogenate for a hydrolysis of 15 min. at 40^{o}C and then 1.25 mL Na_2HPO_4 were added before organic extraction (Siah et al., 2002). Homogenates were extracted twice with 14 ml dichloromethane and organic extracts were evaporated to dryness under nitrogen at room temperature, and redissolved in 250 µl EIA buffer. Testosterone, progesterone and estradiol standards were prepared and determinations carried out in duplicate.

2.4 Data Analysis

A total of 60 clams were collected for the determination of condition factor (clam weight/shell length), gonado-somatic index (gonad wet weight/whole soft tissues wet weight), sexing, sex ratios and hormone levels. Comparisons for statistical significance were made using a one-way analysis of variance to examine differences between the means of each biochemical component. Prior to the analysis of variance, data were subjected to a Levene's test to detect non-parametric distributions of residuals. A t-test was performed for single comparisons in the presence of a parametric distribution of the data and a Kruskall-Wallis ANOVA was used in the case of non-parametric distribution. Data were then subjected to a Tuckey's test (or Dunn's test in the case of unequal number of data from one group to the other) or a Student-Newman-Keuls non-parametric test to confirm critical differences between groups. Statistical and graphical analyses were performed using SPSS package (version 9.0) and Sigma Plot (version 6.0).

3. RESULTS AND DISCUSSION

Clams collected at Baie-Ste-Catherine showed lower gonado-somatic index in 2002 as reported also earlier for samplings in 2001 (Gagné et al. 2002). This decrease could not be due to decreased nutritive conditions in this site since at the mouth of the Saguenay Fjord, there are numerous data reporting the richness of this area due to topographic differences on the floor of the St. Lawrence River (Gauthier-Clerc et al. 2002). This region is well recognised to be a feeding area for belugas, blue and finback whales, with a lot

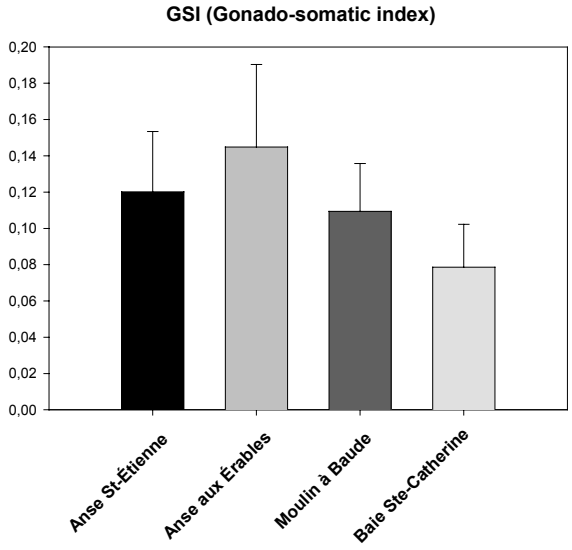

Figure 1. Intersite comparison of the gonado-somatic index. Anse-Saint-Étienne and Anse-aux-Érables are sites located within the Saguenay Fjord, while Moulin-à-Baude and Baie-Ste-Catherine are sites in the St. Lawrence River near the mouth of the Saguenay Fjord. GSI was significantly different between sites (Kruskal-Wallis =88,366; p= 0,000).

of naval activities (whale watching excursions, maritime transport along the St. Lawrence waterway between the Gulf and the Great Lakes).

Despite that we did not observe (Figure 2) a tendency toward an increased male:female sex ratio in 2002 in Baie Ste-Catherine (X^2 =0.62 < X^2 (0.05;4)=7.81), in the St. Lawrence reference site (MAB), 5 % of the individuals analysed were hermaphrodites (see Figure 3 for an example of a structural layout of gonads). This result was quite surprising because of very low levels of contaminants in the reference site and the absence of butyltins (Gagné et al. 2002). According to Coe and Turner (1938), hermaphrodites are very rare in *Mya arenaria* and the frequency naturally observed is about 1 for 1000 individuals.

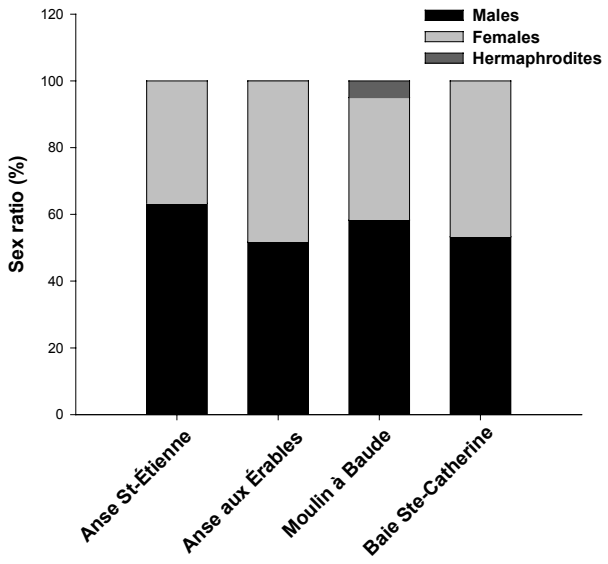

Figure 2. Inter-site comparison of sex ratio.

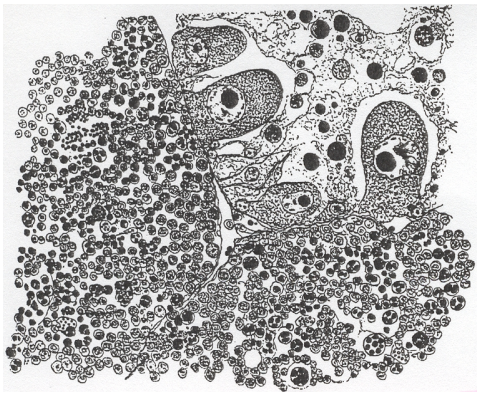

Figure 3. Example of the structural layout of female and male gonad structures in the gonad of hermaphrodites (from Coe and Turner 1938).

In Figures 4 and 5, a clear difference can be seen between males and females. In BSC, males show a delay in their sexual maturation with nearly 20% of clams at the development stage while in MAB, all the male individuals were at the mature stage. Differences are also observed between the two areas, within and outside the Fjord, a difference already reported by Gauthier-Clerc et al. 2002. The contaminated site (AE) within the Fjord shows a marked delay with 100% of male individuals at the development stage. One explanation for this delay could be due to the nutritive conditions of the Fjord, less favourable than in the St. Lawrence (Coté and Lacroix, 1979). However, in ASE, 40 % of clams are at the development stage while 60 % are at the spawning stage, thus demonstrating at least, that some factors influence sexual maturation in the clams of AE, possibly contaminants present in this area. Females show a marked delay in sexual maturation with 100% at the development stage in AE while in the other sites, female maturation is in advance when compared to males, a result similar to those published by Gauthier-Clerc et al. 2002. However, in BSC, females show an advanced sexual maturation with 40% of females at the spent stage. This could be due to the increased testosterone levels shown in Figure 6. It is now well known that TBT-induced imposex is mediated *via* increased androgen levels (Bettin et al. 1996). Since high levels of TBT were measured in gonads of *Mya arenaria* (Gagné et al. 2002) and testosterone seems to play a role in the spawning process (Gauthier-Clerc personal communication), we can therefore suggest that this advanced sexual maturation in female clams could be due to higher testosterone levels induced by butyltins or another toxicant playing a similar role.

In contrast, in 2002, no masculinization pressure was observed in males, reflected by an increased sex ratio, even if levels of testosterone are significantly increased (Figure 6). When compared to maturation in MAB, males from BSC show a delay with near 20% of individuals still in the development stage. We have previously shown (Siah et al. 2002) that in a harbour site, progesterone levels were markedly decreased, this effect being observed concomitantly with a marked delay in sexual maturation. In this study, despite the observation of a delay in gametogenesis, expected high TBT levels, no decreases in progesterone and estradiol levels were observed (data not shown). It is therefore clear that contaminants in BSC may affect steroid hormone metabolism, possibly by inhibiting aromatase or 17 β Hydroxy-steroid-dehydrogenase. TBT has been reported to inhibit cytochrome P450 A1 isoenzyme in fish (Padros et al. 2000) and in mollusks (Morcillo and Porte 1997). A variety of factors were shown to influence sex steroid production, for example exposure to heavy metals that may exert adverse effects via interference with the hormonal control of reproduction by steroids. In Saguenay Fjord, *M. arenaria* exposed to heavy metals exhibited a lower condition factor and lower levels of lipids and proteins in their gonads (Gagné et al. 2002). Moreover, *Mya arenaria* has a low capacity to metabolize organotins (Kure and Depledge 1994; Saint-Hilaire 1997) and negative effects on sexual maturation by TBT were reported in clams (Morcillo and Porte 2000).

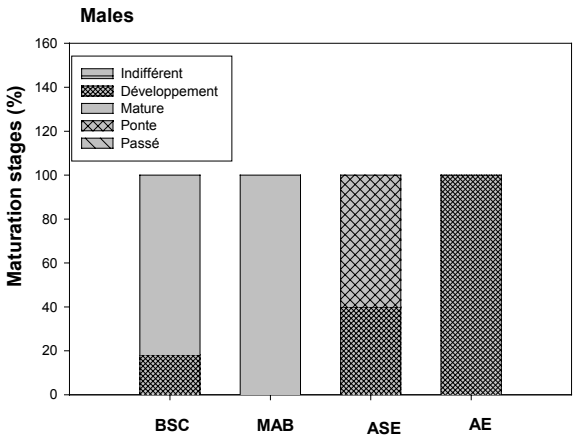

Figure 4. Maturation stages of male *Mya arenaria* in the four sites studied.

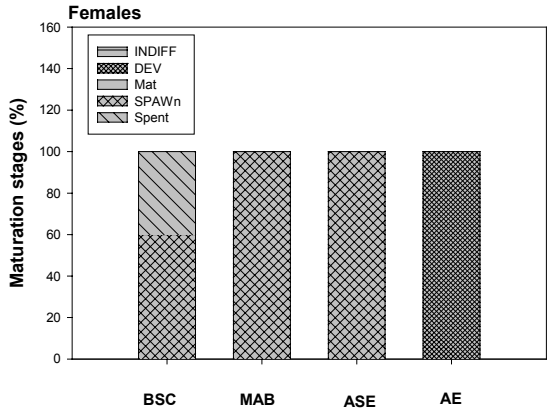

Figure 5. Maturation stages of female *Mya arenaria* in the four sites studied.

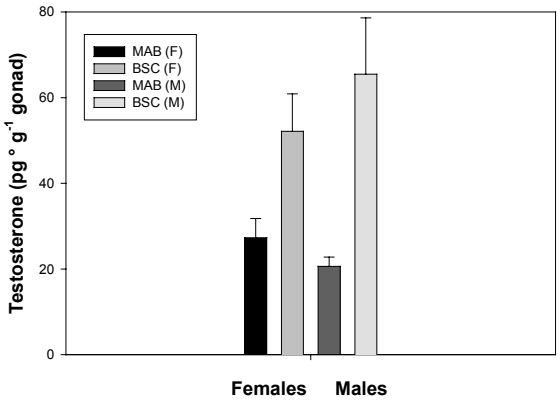

Figure 6. Levels of testosterone in male and female *Mya arenaria*.

4. CONCLUSION

Data shown in this paper demonstrate that endocrine disruptors are present In the Saguenay Fjord, including Baie Ste-Catherine. Additional studies will be necessary to elucidate the effects of TBT and other endocrine disruptors on sex hormone steroids in *M. arenaria*, as well as the role of steroid hormones during gametogenesis. Current studies in our laboratory attempt to clarify these points, particularly using mesocosms and laboratory exposure of marine fauna to contaminated sediments and to TBT.

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