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Neuroendocrine disruption in *Mya arenaria* clams during gametogenesis at sites under pollution stress.

Gagné¹, F., Blaise¹, C., Pellerin², J., André¹, C.

1. River Ecosystem Research, Aquatic Ecosystem Protection Division, Environment Canada, 105 McGill Street, Montréal, Québec, Canada H2Y 2E7. 2. ISMER, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, Québec, Canada G5L 3A1.

Address all correspondence to F. Gagné: francois.gagne@ec.gc.ca.

Abstract

This study examined the neuroendocrine status of clams on intertidal mud flats in the St. Lawrence Estuary and Saguenay Fjord areas during late gametogenesis. The impact of pollution was determined by a test battery of early stress markers (metallothioneins, heme levels, glutathione S-transferase activity), tissue damage (lipid peroxidation and DNA damage) and morphologic characteristics (age, soft-tissue weight ratio and growth index). Neuroendocrinal status was examined by tracking serotonin and dopamine metabolism, monoamine adenylate cyclase activity in synaptosomes, monoamine oxidase and arachidonate cyclooxygenase activities in relation to gametogenetic activity: pyrimidine synthesis, (aspartate transcarbamoylase activity or ATC), vitellogenin-like proteins and gonado-somatic index. The results show that clam soft tissue weights were reduced at sites close to harbours and higher at sites near domestic wastewater outfalls. The age-to-length ratio of clams was generally higher at impacted sites, suggesting reduced growth. The biomarkers of stress or damage confirmed that oxidative stress, DNA damage,

metallothioneins and glutathione S-transferase activity were significantly increased at varying degrees, at the polluted sites. Vitellogenin-like proteins and gametogenic activity were significantly increased at sites influenced by domestic wastewaters. Furthermore, the clams were still in active gametogenesis and not ready for spawning, as indicated by the concordance of the serotonin/dopamine ratio with vitellogenin-like proteins and pyrimidine synthesis. However, gonadal cyclooxygenase activity was increased at polluted sites and significantly correlated with most of the stress biomarkers, suggesting that the clams were in a state of inflammation rather than at the spawning stage. Finally, a multivariate analysis revealed that the sites were readily discriminated with high efficiency (> 71%) and that both neuroendocrine physiological markers and stress responses were identified as the major components, thus explaining the global physiological response of the clams. We conclude that the effects of pollution compromise the clams' health status and that the initiation of gametogenesis in environments contaminated by municipal wastewaters or harbours contributes to the toxic effects of pollution.

Key words: *Gametogenesis, vitellogenin-like proteins, serotonin/dopamine ratio, oxidative stress, inflammation, Mya arenaria.*

Introduction

The St. Lawrence Estuary, along with its unique fjord (Saguenay River), is subject to all the different types of contaminants resulting from urban, industrial and agricultural activities. Indeed, intensive boating activities release chemicals such as polycyclic aromatic hydrocarbons (PAHs) and metals, including organotins, near harbours and marinas. In a previous study, gonadal tissues of *Mya arenaria* clams in the same area of Quebec, Canada, were contaminated with tributyltin (TBT) at concentrations as high as 400 ng/g dry weight (dw) (Pellerin et al., 2004). The proportion of male clams was also higher at a harbour site contaminated by TBT (Gagné et al., 2003). In marine environments, domestic wastewaters are often released into the sea following minimal treatment, if treated at all. These wastewaters are known to contain various contaminants, with clear evidence of endocrine-disrupting effects. Indeed, municipal effluents are recognized to contain both estrogenic and serotonergic compounds, as suggested by increased vitellogenin-like proteins, cyclooxygenase activity and spawning in exposed mussels (Blaise et al., 2003a; Tilton et al., 2003; Gagné et al., 2004). Clam populations at polluted sites adjacent to townships displayed various signs of pollution-related stress, including altered vitellogenin production.

To further investigate the effects of pollution on gametogenesis, specimens of feral clam populations living in intertidal mud flats in the Saguenay Fjord and St. Lawrence Estuary were collected from sites influenced by domestic effluents and harbours (Figure 1). In an attempt to relate reproductive endocrine disruption with anthropogenic pollution, we used

a comprehensive test battery of biomarkers to evaluate exposure to pollutants, defense mechanisms, tissue damage, general health condition and reproductive (gametogenetic) function. Biomarkers selected for monitoring early biological effects and defense mechanisms were metallothioneins (MT) and total heme levels, for heavy metal effects (Viarengo et al., 1997; Simionatto et al., 1984), and glutathione S-transferase, a major enzyme responsible for the conjugation of electrophilic organic molecules such as (hydroxylated) PAHs (Gowland et al., 2002). Next, tissue damage was determined by measuring lipid peroxidation and DNA damage (Aldridge, 1997; Doyotte et al., 1997). Total heme levels were also measured to evaluate hemoprotein breakdown by toxicants such as organotins (Siminiatto et al., 1984). Increased hemoprotein breakdown could result in delayed steroidogenesis owing to decreased cytochrome P450 activity. Neuroendocrine parameters of gametogenesis and spawning were examined by following gonadal serotonin and dopamine metabolism, mono-amine oxidase, cyclooxygenase (COX) activities, vitellogenin-like proteins, aspartate transcarbamoylase (ATC) and the gonado-somatic index. During gametogenesis, dopamine and estradiol-17 β levels are increased to support vitellogenesis (oogenesis) and spermatogenesis (Osada and Nomura, 1989; Li et al., 1998; Gauthier-Clerc et al., 2006). ATC is a step-limiting enzyme complex for pyrimidine biosynthesis to support nucleic acid production in the gametes (Mathieu, 1987). Its activity is also enhanced and closely follows the gonado-somatic index in bivalves. In female gonads, oocytes accumulate vitellogenin-like proteins as the main energy source for the developing embryo (Osada et al., 2003; Li et al., 1998).

Levels of serotonin and prostaglandin synthase or COX are gradually increased during the final maturation of eggs and spermatocytes, reaching maximum values to assist the spawning process (Osada and Nomura, 1990; Osada et al., 1987; Fong et al., 1993).

The objective of this study was to characterize the neuron-endocrine parameters in feral *Mya arenaria* clams at the ripe (late) stage of gamete formation just before spawning. Moreover, the impacts of pollution on the neuron-endocrine status of gametogenesis were also examined in clam populations under the influence of harbors and domestic wastewaters. To this end, biomarkers of health status and tissue damage in clams were examined in relation to those of gametogenetic activity and neuro-endocrine parameters (monoamine metabolism and cyclooxygenase). An attempt was made to examine the interaction of gametogenesis and pollution stress in respect to the health status in feral clam populations.

Methods

Sampling sites and clam handling

Soft-shell clams (*Mya arenaria*) were collected from six sites in the Saguenay Fjord and St. Lawrence Estuary during a field survey conducted in June 2004 (Figure 1): Baie Sainte-Catherine (BSC) where the clam population was located between the mouth of a river draining a small town and a wharf with heavy boat traffic (< 100 m); Anse St-Jean (ASJ), within (< 100 m downstream the source) the municipal effluent dispersal plume of

a population of 2000 residents; Baie-Éternité (BE), close (<10 m) to a wharf for a seasonal commercial sight-seeing boat operation and a marina for small pleasure craft; Anse aux Érables (AE) bay, the farthest upstream site in the Saguenay Fjord (70 km) and influenced by several aluminum and paper mills located in the cities of Alma, Jonquière, Chicoutimi and La Baie, which discharge their effluents into the Saguenay River and Fjord. The wastewaters of 15 seasonal cottages and the upstream waters of Alma also affect site AE. Anse Saint-Étienne (ASE) and Baie du Moulin à Baude (BAU) are not affected by any direct source of anthropogenic activity and therefore considered as the “pristine” sites for the Saguenay Fjord and St. Lawrence Estuary, respectively, in this study (Figure 1). Thirty animals with a shell length greater than 4 cm were collected at each site for biochemical measurements. The clams were immediately placed in coolers chilled with ice ($T = 0-4^{\circ}\text{C}$) for transport to our field laboratory. Twelve animals were randomly selected for each site, frozen in dry ice and brought back to the St. Lawrence Centre laboratories, where they were placed in a -85°C freezer until analysis. The remaining clams were dissected for gill and gonadal tissue weights and sex determinations (Gagné et al., 2003). For the chemical analyses, soft tissues of *Mya arenaria* clams were collected (n=12 individuals per site) and homogenized with a Polytron tissue grinder apparatus. The homogenized sample was then analyzed for heavy metals by ion-coupled plasma mass spectrometry (NLET 1994). Blank values consisted of distilled water that was processed with the Polytron mixer.

Individual morphometrics and tissue processing

Twelve clams of similar size from each sample site were thawed on ice. Each animal was measured (shell length in mm) and weighed (g wet weight of soft tissue) to determine the soft tissue weight ratio (g wet weight of soft tissue/g wet weight of clam). Age was estimated from the number of major grooves on shells. Each specimen's digestive gland (hepato-pancreas), gonadal and gill tissues were removed and homogenized with a Teflon pestle tissue grinder in ice-cold 25 mM Hepes-NaOH buffer, pH 7.4, containing 125 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol at a 1:5 weight/volume ratio. Aliquots of each homogenate were taken for total protein determinations (Bradford, 1976), monoamines, heme levels, DNA strand breaks and lipid peroxidation (LPO). Concentrations of these biomarkers were normalized with the total protein concentrations of each homogenate. A portion of the homogenate was centrifuged at 1500 x g for 10 min for the preparation of synaptosomes and mitochondria. The remaining homogenate was centrifuged at 15 000 x g for 30 min at 2°C. The supernatant was then carefully separated from the pellet for cyclooxygenase (COX), metallothioneins (MT) and glutathione S-transferase (GST) activity determinations. These biomarkers were also normalized with total protein concentrations determined in the supernatant.

Reproductive function

Gametogenesis was estimated by measuring the gonado-somatic index (GSI; weight of gonad/soft tissue weight), relative levels of vitellogenin-like proteins (Vtg) and ATC

activity. Sex was determined by microscopic examination of gonad smears at 400X enlargement. The presence of Vtg in gonad was determined by the organic alkali-labile phosphate (ALP) technique using the acetone fractionation procedure (Gagné et al., 2003). Vtg-like protein levels were expressed as μg of alkali-labile phosphate (ALP)/mg of gonad protein. Gamete activity was determined by measuring the activity of aspartate transcarbamoylase (ATC) in the S15 fraction of gonad homogenate (Mathieu et al., 1982) using the spectrophotometric detection of liberated phosphate in the presence of carbamoylphosphate (Herries, 1967).

General health status and stress biomarkers

Metallothionein (MT) levels were determined in the digestive glands of the clam specimens. Relative levels of MT proteins were determined by the thiol spectrophotometric assay (Viarengo et al., 1997). The homogenate was centrifuged at 15 000 x g for 30 min at 2°C and the supernatant carefully removed from the upper lipid layer. The MT fractionation and assay procedure was performed on the 15 000 x g supernatant. Standards of reduced glutathione (GSH) were used for calibration. The data are expressed as μmoles of GSH equivalents per mg protein. DNA damage (double and single DNA strand breaks) was assessed in gills, digestive glands and gonad by means of an alkaline precipitation assay (Olive, 1988) with fluorescence quantification of DNA strands (Bester et al., 1994). The assay principle is based on K-assisted SDS precipitation of genomic DNA (associated with proteins), which leaves protein-free DNA breaks

(double and single strands) in the supernatant. Salmon sperm DNA standards were added for DNA calibration. The results were expressed as μg of supernatant DNA/mg of proteins. LPO was determined by the thiobarbituric acid method, which measures the production of malonaldehyde (Wills, 1987). Briefly, 20 μL of the homogenate was diluted to 80 μL with distilled water and mixed with 50 μL of 20% trichloroacetic acid solution containing 1 mM FeSO_4 and 100 μL of 0.67% thiobarbituric acid. The mixture then stood in a hot water bath (80°C) for 10 min. Blanks and standards of tetramethoxypropane were prepared in the presence of the homogenization buffer. All assay tubes then stood at room temperature for 15 min to allow denatured proteins to settle. Fluorescence was measured at 520 nm for excitation and 590 nm for emission using a fluorescence microplate reader (Dynatech, Fluorolite-1000). Because the reagent could react with other aldehydes, results were expressed as μg of thiobarbituric acid reactants (TBARS)/mg of homogenate protein. Glutathione S-transferase (GST) was determined by the spectrophotometric method of Boryslawskyj et al. (1988) using 2, 4-dichloronitrobenzene as the substrate. Data are expressed as increase in absorbance/min/mg proteins. Total heme was determined by fluorescence according to the method of Simionatto et al. (1984). Briefly, 20 μL of digestive gland homogenate was mixed with 500 μL of 1 M NaOH in methanol. The mixture was allowed to stand for 5 min at room temperature and centrifuged at $10\,000 \times g$ for 5 min. Total heme was determined in the supernatant at 400 nm excitation and 685 nm emission. Standard solutions of hemin were used for calibration. Total heme levels were expressed as μg

hemin/mg proteins. Cyclooxygenase (COX) activity was measured by the oxidation of 2, 7-dichlorofluorescein in the presence of arachidonate (Fujimoto et al., 2002). Briefly, 50 μ L of gonad or gill $15\ 000 \times g$ supernatant was mixed in 200 μ L of 50 μ M arachidonate, 2 μ M dichlorofluorescein and 0.1 μ g/mL of horseradish peroxidase in 50 mM Tris-HCl buffer, pH 8, containing 0.05% Tween 20. The reaction mixture was incubated for 0, 10, 20 and 30 min at 30°C, and fluorescence was measured at 485 for excitation and 520 for emission (Dynatech, Fluorolite 1000). The data were expressed as the increase in relative fluorescence units/(min x mg proteins).

Neuroendocrine assessment of gametogenesis

Gonad (containing nerve fibers) homogenates were centrifuged at $1500 \times g$ for 15 min at 4°C. The supernatant was overlaid on 0.8 M sucrose containing 10 mM Hepes-NaOH, pH 7.4, and 1 mM EDTA, and centrifuged at $10\ 000 \times g$ for 20 min at 4°C to separate the synaptosomes (in the 0.8 M sucrose phase) from mitochondria (pellet). The synaptosomes were mixed with two volumes of distilled water to prevent osmotic shearing of synaptosomes. Monoamine (serotonin and dopamine) levels were measured in the gonad homogenates using fluorescence-based methodologies (Orsinger et al., 1980; Szabo et al., 1983). For serotonin, 5-hydroxyindolacetate (5-HIAA) and dopamine determinations, a sub-sample of homogenates was mixed with one volume of butanol containing 0.1 N HCl and 0.2 mM reduced glutathione (GSH). The mixture was centrifuged at $2000 \times g$ for 15 min to remove precipitated material and the supernatant was extracted once with one

volume of hexane. For serotonin, the aqueous phase was mixed with one volume of o-phthalaldehyde in 2 N HCl and heated to 70°C for 15 min. Fluorescence was measured at 360 nm excitation and 480 nm emission against standards of serotonin. For 5-HIAA, the deaminated metabolite of serotonin, the organic phase was mixed with one volume of 100 mM sodium bicarbonate pH 9.0, and levels were determined by the o-phthalaldehyde method as described above. For dopamine, the aqueous phase was mixed with a 0.1 volume of alkaline iodide solution (I₂/KI, Lugol, Sigma Chemical Co.), heated at 75°C for 60 min and fluorescence was measured at 325 nm excitation and 385 nm emission. Standards of serotonin, 5-HIAA and dopamine were used for calibration. Data were expressed as ng of monoamine/mg proteins. Serotonin and dopamine transport in the presence of ATP was determined in synaptosomes using the liberation of inorganic phosphate from ATP methodology (Kakko et al., 2003). Synaptosomes (10 µg/mL) were incubated with either 100 µM serotonin or dopamine in the presence of 100 µM ATP in the following assay buffer: 140 mM NaCl, 1 mM KH₂PO₄, 1 mM CaCl₂, 10 µM GTP and 10 mM Hepes-NaOH, pH 7.4. The mixture (200 µL) was allowed to stand at 30°C for 0, 10, 20, 30 and 40 min and the reaction was stopped with 5 µL of 100% trichloroacetic acid. The samples were centrifuged at 10 000 x g for 3 min to reduce turbidity. The levels of inorganic phosphates liberated from ATP hydrolysis were determined using the phosphomolybdate assay (Stanton, 1968). MAO activity was determined using the serotonin analogue tryptamine as the substrate. Mitochondria (0.1 to 0.5 mg/mL proteins) were incubated in the presence of 10 µM dichlorofluorescin, 100 µM

tryptamine in 10 mM Hepes-NaOH, pH 7.4, containing 125 mM NaCl, 0.1 M aminotriazole (a catalase inhibitor) and 1 $\mu\text{g/mL}$ of horseradish peroxidase (Sigma-Aldrich Co., USA). The reaction was allowed to proceed at 30°C for 0, 15, 30 and 60 min. Fluorescence was measured at 485 nm excitation and 520 nm emission. Enzyme activity was expressed as nmole of fluorescein formed/(min x mg proteins). Standard solutions of fluorescein were used for calibration.

Data analysis

Twelve clams ($n = 12$) from each site were held back for biomarker measurements. Data distribution was analysed using Levene's test for homogeneity of variances. When the data proved to be homogeneous, a two-way factorial analysis of variance was performed, with site and gender as the main factors; critical differences between groups were appraised using Dunnett's t test. In the case of nonhomogeneous data distributions, data were \log_{10} -transformed before performing a two-way factorial ANOVA. Correlation analyses between biomarkers were determined using the Pearson-moment correlation and factorial analysis (principal components method). Site classification according to biomarker response was determined by discriminant analysis. Biomarker data (biomarkers of stress or damage) from the spatial survey were also integrated using a ranked-sum approach to yield a biomarker index (Chèvre et al., 2003). Biomarker responses were ranked from 1 to 6 (because there were six sites), where the lowest rank value corresponds to the mean value of the reference site (i.e. 1). The biomarker

responses were corrected to yield values > 1 to rank their relative intensity (e.g. a 0.2-fold decrease was corrected to produce 1.2-fold). Inter-site differences were verified using the Mann-Whitney U test where sites were assigned the same rank value when no difference was obtained between them. The rank values were then summed for each site to yield an index value. All statistical tests were performed using the *Statistica* software package (version 7.1).

Results

Clams were collected at various sites subject to differing types of contamination, as shown in Figure 1. To reiterate, site BE is contaminated by boating activities; site ASJ is contaminated by domestic wastewaters; and sites AE and BSC are contaminated by both these types of contamination. To confirm this, heavy metal loadings were determined in the soft tissues of clams (Table 1). Indeed, these sites were generally more contaminated in cobalt, chromium, copper, iron, mercury, manganese and nickel. The strongest increases were found for iron (14 fold increase), cobalt (2.8 fold increase), chromium (2.7 fold increase) and iron (1.9 fold increase) thus corroborating contamination of *Mya arenaria* clams at these sites. A two-way analysis of variance revealed that site location, but not gender, had a significant effect on soft-tissue weight ratios (Figure 2A). The soft-tissue weight ratio was significantly lower at BSC, but very high at the site contaminated by domestic wastewaters (ASJ; Figure 2A). Age also varied significantly across sites and gender (Figure 2B). Females were somewhat older (mean age of 6.3 y) than males (mean

age value 6 y) with no apparent interaction of gender with site. Clams at BSC were significantly younger than those at BAU (the reference site for the St. Lawrence Estuary) and clams from sites ASJ and BE were significantly younger compared to those at site ASE (the reference site for the Saguenay Fjord). Clams at the most upstream site (AE) were significantly older than those from the reference site (ASE). The age-to-shell-length ratio, a measure of growth, varied with gender and site (Figure 2C). Again, females had somewhat higher growth index than males. The age-to-shell-length ratio was significantly higher at sites BSC, ASJ and AE, suggesting reduced shell growth in clams in the 5- to 8-year-old age groups. A correlation analysis revealed that age was negatively correlated with the soft-tissue weight ratio ($R = -0.3$; $p < 0.01$).

Gametogenesis was appraised by measuring the gonado-somatic index (GSI), vitellogenin-like proteins (Vtg) and gamete activity by aspartate transcarbamoylase (ATC) activity (Figures 3A-C). A two-way analysis of variance revealed that only site had a significant effect on GSI (Figure 3A). GSI was significantly reduced at sites BSC, ASJ and BE, but not at site AE. The levels of Vtg proteins were significantly different across the study sites and gender had a significant effect at $p = 0.05$ (Figure 3B). As expected, VTG was significantly higher in females than in males but no significant interaction between site and gender were observed. Vtg was significantly increased at sites ASJ and AE, but not at site BE, for both female and male clams. Gonad ATC activity varied with site where gender had no significant effects (Figure 3C). ATC activity was readily induced at site ASJ alone, suggesting enhanced pyrimidine

biosynthesis. A correlation analysis of these reproductive endpoints revealed that GSI was positively correlated with age ($R = 0.41$; $p < 0.01$) and negatively correlated with soft-tissue weight ratio ($R = -0.3$; $p < 0.05$). Levels of Vtg were positively correlated with soft-tissue weight ratio ($R = 0.34$; $p < 0.05$), age ($R = 0.27$; $p < 0.05$) and growth index ($R = 0.39$; $p < 0.01$), suggesting that growth is directly associated with the production of egg-yolk proteins. ACT activity was positively correlated with soft-tissue weight index ($R = 0.63$; $p < 0.001$) and slightly so with Vtg ($R = 0.25$; $p = 0.05$).

Several biomarkers of defence and tissue injury were examined to assess the health status of clams (Table 2). A two-way ANOVA indicated that MT levels in digestive gland varied with site but not with gender. MT levels were significantly higher at all polluted sites in respect to their corresponding reference sites. Glutathione S-transferase (GST) activity in the digestive gland varied significantly across all sites, but not with gender. GST activity was readily induced at BSC site alone. Total heme levels varied across all sites, but not with gender. Heme levels were readily increased in gonadal tissues at the ASJ site, while they were unchanged at the other sites relative to the reference sites. A two-way ANOVA for digestive gland DNA strand breaks revealed that only site was significant. DNA strand breaks were readily increased at all impacted sites. LPO in gills and digestive gland varied significantly with site, but not with sex. LPO in digestive gland was significantly induced at all impacted sites with no effect with gender, with the exception of site BE where its activity was not significantly different than the reference

site. Gill LPO revealed significant differences within sites, gender and site-gender interactions (2 way ANOVA $p < 0.01$ for site, gender and site-gender interaction). Female had more LPO in gills than in males. LPO in gills was reduced in females at impacted sites in the Saguenay fjord sector while a significant gill LPO decrease at sites BSC in males only. The biomarker responses were incorporated into an index by which sites were ranked according to toxic stress: BSC > AE = ASJ > BE > BAU > ASE. A correlation analysis revealed that MT was significantly correlated with growth index ($R = 0.4$; $p < 0.01$), GST in digestive gland ($R = 0.76$; $p < 0.01$), LPO ($R = 0.35$; $p < 0.01$), and DNA strand breaks ($R = 0.26$; $p < 0.05$) in digestive gland (Table 2). Digestive gland GST was significantly correlated with GSI ($R = -0.39$; $p < 0.05$), LPO ($R = 0.26$; $p < 0.05$) and DNA strand breaks in digestive gland ($R = 0.39$; $p < 0.01$). Total heme levels in gonad were significantly correlated with soft-tissue weight ratio ($R = 0.45$; $p < 0.01$), Vtg ($R = 0.38$; $p < 0.05$) and ACT ($R = 0.49$; $p < 0.01$). DNA damage in digestive gland was significantly correlated with GSI ($R = -0.31$; $p < 0.05$), Vtg ($R = 0.37$; $p < 0.01$) and LPO in digestive gland ($R = 0.51$; $p < 0.01$). Digestive gland LPO was significantly correlated with GSI ($R = -0.3$; $p < 0.05$) and growth index ($R = 0.4$; $p < 0.01$). LPO in gills was significantly correlated with GSI ($R = 0.41$; $p < 0.01$).

The influence of site quality was examined in various neuroendocrine mediators such as serotonin, 5-HIAA, dopamine, COX and MAO activities in gonadal (and visceral ganglion) tissues (Table 4). Serotonin levels were not influenced by gender, but changed

significantly within sites. Serotonin levels were readily increased at contaminated sites in both areas, with the exception of site BE. Two-way ANOVA revealed that both site and gender were significant factors for 5-HIAA levels. The metabolite of serotonin, 5-HIAA, was somewhat higher in males than in females while its levels were significantly reduced at the BSC and AE sites and elevated at site ASJ. Meanwhile, serotonin transport activity (serotonin activation of adenylate cyclase activity) remained unchanged across all sites and with gender. The serotonin-to-5HIAA ratio varied marginally with gender ($p=0.08$, females having increased serotonin/5HIAA ratio), but did change within sites. The ratio was significantly higher at sites AE and BE, suggesting an accumulation of serotonin in tissues. Dopamine levels were also significantly different within sites, but not with gender. Dopamine levels were significantly elevated at all polluted sites. Moreover, dopamine transport activity (dopamine activation of adenylate cyclase activity in prepared synaptosomes) varied within sites, but gender had no effect. Dopamine transport activity was significantly induced at the most stressed sites (i.e. BSC and ASJ sites) relative to the corresponding control sites (i.e. BAU and ASE, respectively). The serotonin/dopamine ratio varied significantly within sites and with gender. The serotonin/dopamine ratio was higher in females than in males (1.36 fold higher). The serotonin/dopamine ratio was significantly induced at the polluted sites AE and ASJ. A two-way ANOVA for gonad MAO activity showed that both site and sex produced significant changes. MAO activity was 1.15 fold higher in females than in males. MAO activity was significantly elevated at sites in the Saguenay Fjord area (i.e. ASJ, BE and

AE). A two-way ANOVA for gonad COX activity showed that only site was significant. COX activity was significantly elevated at sites BSC and AE. A correlation analysis revealed that serotonin was significantly correlated with growth index ($R = 0.49$; $p < 0.01$), gonad ACT ($R = 0.34$; $p < 0.05$) and gonad COX ($R = 0.32$; $p < 0.05$) activity, and digestive gland MT ($R = 0.49$; $p < 0.01$) and GST ($R = 0.31$; $p < 0.05$) activity. Serotonin transport activity, while remaining unchanged with site, was significantly correlated with growth index ($R = 0.34$; $p < 0.05$), serotonin/5H1AA ratio ($R = 0.42$; $p = 0.01$) and gonad COX activity ($R = 0.37$; $p < 0.05$). The serotonin metabolite 5-H1AA was significantly correlated with total gonad heme level ($R = 0.43$; $p < 0.05$) only. The serotonin/5H1AA ratio was significantly correlated with MAO activity ($R = 0.49$; $p < 0.01$). Dopamine levels were correlated with growth index ($R = 0.48$; $p < 0.01$), gonad ACT levels ($R = 0.34$; $p = 0.01$), Vtg ($R = 0.72$; $p < 0.01$), digestive gland GST ($R = 0.31$; $p < 0.05$) and MT levels ($R = 0.49$; $p < 0.01$) and gonad COX activity ($R = 0.32$; $p < 0.05$). Dopamine transport activity was significantly correlated with gonad ACT level ($R = 0.41$; $p < 0.01$), Vtg ($R = 0.4$; $p < 0.05$), digestive gland GST ($R = 0.54$; $p < 0.01$), DNA strand breaks ($R = 0.32$; $p < 0.05$) and MT levels ($R = 0.35$; $p < 0.05$), gonad heme levels ($R = 0.88$; $p < 0.05$) and dopamine levels ($R = 0.43$; $p < 0.05$). The serotonin/dopamine ratio was significantly correlated with soft-tissue weight ($R = 0.4$; $p = 0.01$), gonad ACT activity ($R = 0.37$; $p < 0.05$) and Vtg ($R = 0.57$; $p < 0.01$), DNA strand breaks in digestive gland ($R = 0.38$; $p = 0.01$) and dopamine transport activity ($R = 0.35$; $p = 0.05$). Gonad MAO activity was significantly correlated with Vtg ($R = 0.55$; $p < 0.01$), digestive gland LPO

($R = 0.48$; $p < 0.01$) and DNA strand breaks ($R = 0.4$; $p < 0.01$), dopamine levels ($R = 0.37$; $p < 0.05$) and serotonin/5-HIAA ratio ($R = 0.49$; $p < 0.01$). Finally, COX activity was significantly correlated with the following biomarkers: GSI ($R = -0.27$; $p < 0.05$), soft-tissue weights ($R = -0.38$; $p < 0.01$), growth index ($R = 0.42$; $p < 0.01$), digestive gland MT ($R = 0.68$; $p < 0.01$), LPO ($R = 0.69$; $p < 0.01$), GST ($R = 0.54$; $p < 0.01$) and DNA strand breaks ($R = 0.37$; $p < 0.01$).

A multivariate data analysis was performed using discriminant analysis (Figure 4) followed by a principal component (factorial) analysis to highlight the most discriminative physiological responses (Table 4). The discriminant analysis revealed that most sites were correctly classified with more than 85% efficiency with the exception of the AE site (71%). For site AE, some clams were misclassified to sites BAU (3) and BSC (3) indicating that some clams were not responding and some displayed a response pattern close to BSC (intense traffic harbor and municipal effluent). In the Saguenay Fjord area, ASJ was the site that differ the most from the pristine site (ASE). A factorial analysis revealed that 63% of the total variance was explained by four factors (Table 5). In descending order of factorial weights (0.79 to 0.61), the biomarkers associated to the factors were: gonad dopamine, soft-tissue weights, heme levels, GST, serotonin, COX, DNA strand breaks and Vtg. These biomarkers were associated with general health status, defense biomarkers and neuroendocrinal aspects of gametogenesis.

Discussion

Clams from the Saguenay Fjord area (sites AE, BE and ASJ) and those from the St. Lawrence Estuary (BSC) were recognized to be suffering anthropogenic stress. Previous studies have shown that clams from BSC site were contaminated by various heavy metals, including organotin products, with clear evidence of reproductive perturbations: decreased GSI, Vtg and ATC and increased male/female ratio and oxidative stress in gonadal tissues (Gagné et al., 2003; 2005a). The male-to-female sex ratio, Vtg and ATC were not statistically different in the present study, but GSI, age and soft-tissue weight ratio still remained significantly reduced. This suggests that pollution inputs are not constant over the years and clams can recover. Nonetheless, clams from site BSC had increased MT, GST, LPO and DNA strand breaks in the digestive gland. Based on the correlative analysis, MT was associated with neither Vtg-like proteins nor GSI but was related with serotonin and COX levels. This suggests that MT could be influenced during the spawning process and the occurrence of inflammation perhaps through infection (Shi et al., 2006) in addition to heavy metal exposure. Although GST is considered a conjugating enzyme of reduced glutathione to less-polar electrophilic organic compounds, its activity was readily increased by organotin contamination in digestive gland acini of the scallop *Pecten maximus* (LePennec and LePennec, 2003). In another study, the population status of *Mya arenaria* clams in the same sector revealed that oxidative stress, in addition to reduced vitellogenesis and GSI, was associated with low clam density and mean age values in the local population (Blaise et al., 2003a). These

results support the observation that this population is under constant anthropogenic stress in this area, but the long-term trends within each biomarker response could vary over the years. Despite these observations, the biomarker index revealed that this site (BSC) was the most impacted one in the area, followed by those affected by domestic wastewaters and industrial inputs (BSC > ASJ = AE > BE). The neuroendocrine biomarker data suggest these clams were experiencing delayed gametogenesis or that they were perhaps in a state related to inflammation. This latter condition was shown by the increased gonadal COX activity accompanied by increased dopamine and dopamine adenylate cyclase activity (dopamine transport in synaptosomes) and decreased serotonin levels. In the scallop *Argopecten purpuratus*, dopamine levels rose during the first phases of gamete development while serotonin was more closely associated with the later steps of gamete maturation and spawning (Martinez and Rivera, 1994). In the scallop, no difference was observed between the sexes (as with the clams in this study) and gonad dopamine was lowest during spawning (Osada and Nomura, 1989). Interestingly, COX activity was elevated but poorly associated with serotonin level and serotonin transport activity. During the spawning process, serotonin and COX are induced for gamete release in the scallop (Osada and Nomura, 1990), and thus significantly correlated with each other. However, a dampening of gametogenesis, as suggested by reduced GSI and increased ATC activity, points to other factors as being responsible for the increased COX activity. The association of COX activity with the condition of inflammation rather than the later stage of gametogenesis was further supported by an analysis of covariance

of COX activity using serotonin and serotonin transport activity as covariates, which showed that COX activity remained significantly elevated at impacted sites. Inflammation might be the results of infections from pathogenic micro-organisms such as viruses, bacteria and macroparasites (Canesi et al., 2002). However, cockles' vulnerability to pollution was enhanced by parasites through the deficit of MT synthesis (Baudrimont et al., 2006). Although MT synthesis was increased during gonad maturation, MT levels from parasitized animals were lower. In the present study, although decreased GSI were observed at impacted sites, we did not observe either the occurrence of major infections of macroparasites in gonad or reduced MT levels at polluted sites. In the Arctic bivalve, the influence of salinity and sexual status was practically negligible in comparison with tissue weight and the copper concentration in tissues (Amiard-Triquet et al., 1998). In a 2 year study with *Mytilus galloprovincialis*, contamination by PAHs were highest during winter but with less tissue lesions in respect to warmer months (Bodin et al., 2004) suggesting that toxicity follows temperature increase and seasons. Changes in salinity were recognized to have moderate effects of some biomarkers such as heat shock proteins and GST activity (Lyon et al., 2003). However, the interaction of temperature and salinity had a significant effect on oxidative stress (Damiens et al., 2004). Indeed, TBARS levels increased as a function of salinity when the temperature was maintained at 20-21°C, whereas at 25.8°C no effect of salinity was obtained in addition to lack of change in GST and catalase activities at 20-21°C. Hence, the effects of salinity, temperature and spawning were considered minimal since

clams were collected during the same week before the spawning period at moderate temperatures (in early June corresponding to 12-20°C).

Municipal effluents were recently recognized to produce an inflammatory syndrome in freshwater mussels, with increased response in oxidative stress and COX activity and reduced phagocytotic activity in hemocytes (Gagné et al., 2005b; 2006). Moreover, COX activity was significantly correlated with MT, GST and LPO in the digestive gland, suggesting the implication of pollutant-related effects with bacterial infection on the state of inflammation in *Mya arenaria* clams. In a recent study, it has been shown that infection by *Vibrio tapetis* lead to modifications in several defence-related factors such as increased hemolymph protein and lysozyme activity (Allam et al., 2006). In addition, COX activity was negatively correlated with soft-tissue weight ratio in clams, perhaps pointing to a harmful effect of this syndrome. Inflammation is a physiological state that precedes various pathologic manifestations where oxidative stress, nitric oxide, prostaglandin production or COX are increased. In general, induced catecholamines (i.e. dopamine and noradrenaline) were shown to stimulate the immune response via the induction of interleukin-1, tumor necrosis factor α and interleukin-8 production usually implicated in pro-inflammation conditions (Esch and Stefano, 2002); cytokines that are also found in bivalves, but the interaction between increased dopaminergic activity and inflammation is not well understood at present in invertebrates (Hughes et al., 1992). MT proteins were induced during infection and inflammation in the striped bass *Morone*

saxitilis (Regala and Rice, 2004), which suggests that the inflammatory state (COX and oxidative stress) is likely to contribute to the MT response. A study on caged freshwater mussels exposed to a municipal effluent dispersion plume found that even though metals were less bioavailable, MT was still induced in the digestive gland (Gagnon et al., 2005). This finding suggests that the inflammatory properties of pollution, and not the presence of metals in municipal effluents, contributed to increased MT synthesis. This hypothesis is supported by an analysis of covariance that revealed that COX activity (covariate) had the greatest effect on inter-site differences in MT levels.

Besides the contamination from commercial and pleasure boating activities, the contribution of domestic or municipal effluents could also have an impact on the health status of clam populations. Although clams from sites ASJ and AE had decreased GSI, their levels of Vtg and ATC activity were significantly increased. Moreover, the growth index was higher in clams from sites ASJ and AE, but the mean age value was significantly lower and higher, respectively, than the pristine site ASE. The increase in Vtg-like proteins at site ASJ and the slight increase at site AE were previously reported elsewhere (Blaise et al., 2002), showing a sustained estrogenic effect of domestic wastewaters on feral clam populations. Although serotonin levels were elevated at polluted sites and weakly correlated with COX activity, the serotonergic signal might have been produced by exposure to domestic wastewaters. Indeed, serotonergic properties were recently identified in freshwater mussels exposed to municipal effluents

(Gagné et al., 2004). It was observed that exposing freshwater mussels to municipal wastewaters increased COX activity in gonadal tissues, with a concomitant increase in monoamine metabolism that favoured serotonergic over dopaminergic effects. Because clams were collected during gametogenesis, the serotonin/dopamine ratio should have been lower. The contrary was true, however, with the serotonin/dopamine ratio being much higher at polluted sites, especially those under the influence of municipal wastewaters (sites ASJ and AE). Interestingly, gonad dopamine levels were more highly correlated with Vtg-like protein and gonad ATC activity, which usually occurs at the beginning of gametogenesis in females, than with COX activity. Thus, exposure to urban wastewaters at the beginning of gametogenesis represents a sensitive period in the manifestation of toxic effects in feral clam populations. Indeed, dopamine levels were also closely associated with oxidative stress (LPO) and DNA strand breaks, suggesting that the increase in catecholamine synthesis during the initiation of gametogenesis might have some DNA-damaging effects, perhaps through oxidative stress. Catecholamines are well known for their sensitivity to oxidation and recognized for enhancing DNA damage by oxidative stress such as nitric oxide production during inflammation conditions (Yoshie and Oshima, 1997). Based on a correlation analysis, DNA damage seems equally linked to the processes of inflammation (COX activity) and gametogenesis, as determined by dopamine metabolism biomarkers (i.e. no significant difference between correlation coefficients was observed). Therefore, the induction of COX activity by urban pollutants

at the time of gametogenesis, where dopamine production is higher, appears to increase oxidative stress and lead to lipid peroxidation and DNA strand breaks in clams.

In conclusion, the neuroendocrine status shows clear signs of disruption in clams influenced by different types of contaminating pollution. The biomarker data revealed delayed gametogenesis and an inflammatory syndrome in these clams, as evidenced by their increased serotonin/dopamine ratio, COX activity and oxidative stress. Furthermore, this inflammatory condition appears more closely related to urban pollution, especially domestic effluents, as suggested by the close relationships with MT, GST and LPO in the digestive gland and the absence of trends with GSI or Vtg. We conclude that pollution not only delays but indeed disrupts the neuroendocrine signals of gametogenesis. Gametogenesis, therefore, appears to increase the susceptibility of organisms to pollution.

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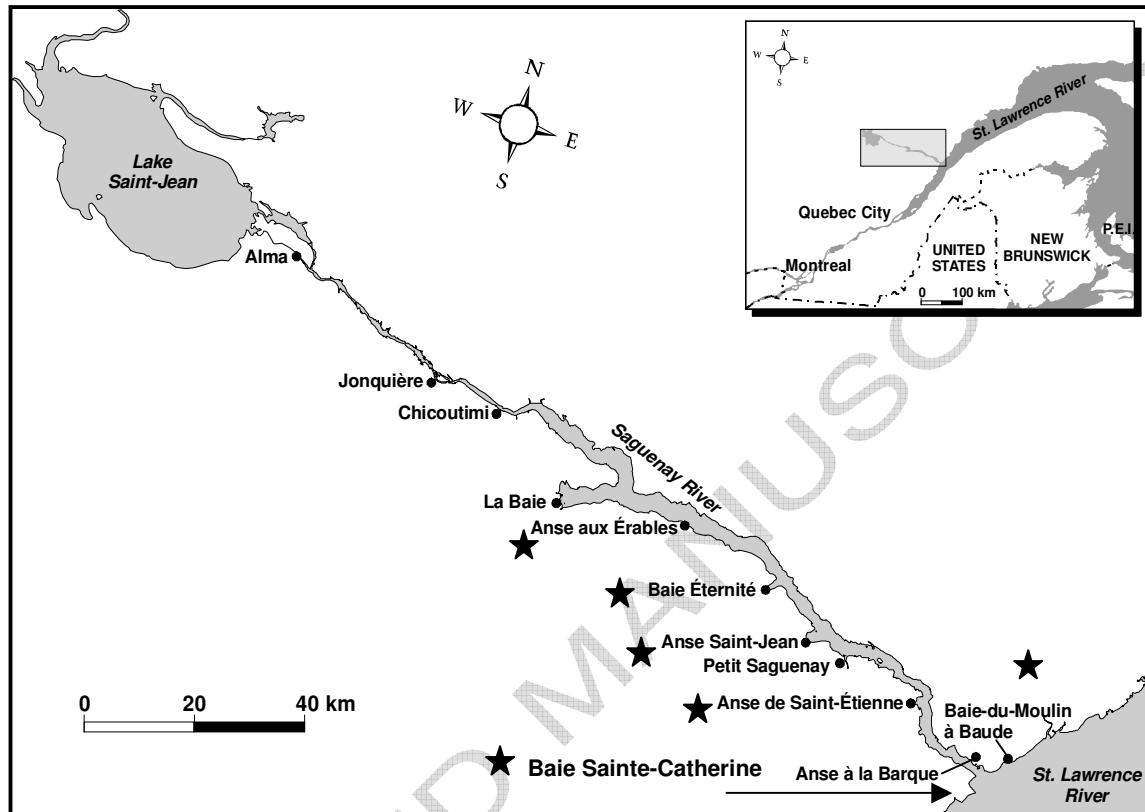


Figure 1. Site location in the Saguenay Fjord area.

Clams were collected at sites (identified by stars) in June 2004. The sites Anse de Saint-Étienne (ASE) and Baie-du-Moulin à Baude (BAU) were considered as the reference (pristine) sites, while Anse aux Érables (AE), Baie-Éternité (BE), Anse Saint-Jean (ASJ) and Baie Sainte-Catherine (BSC) were those affected by pollution.

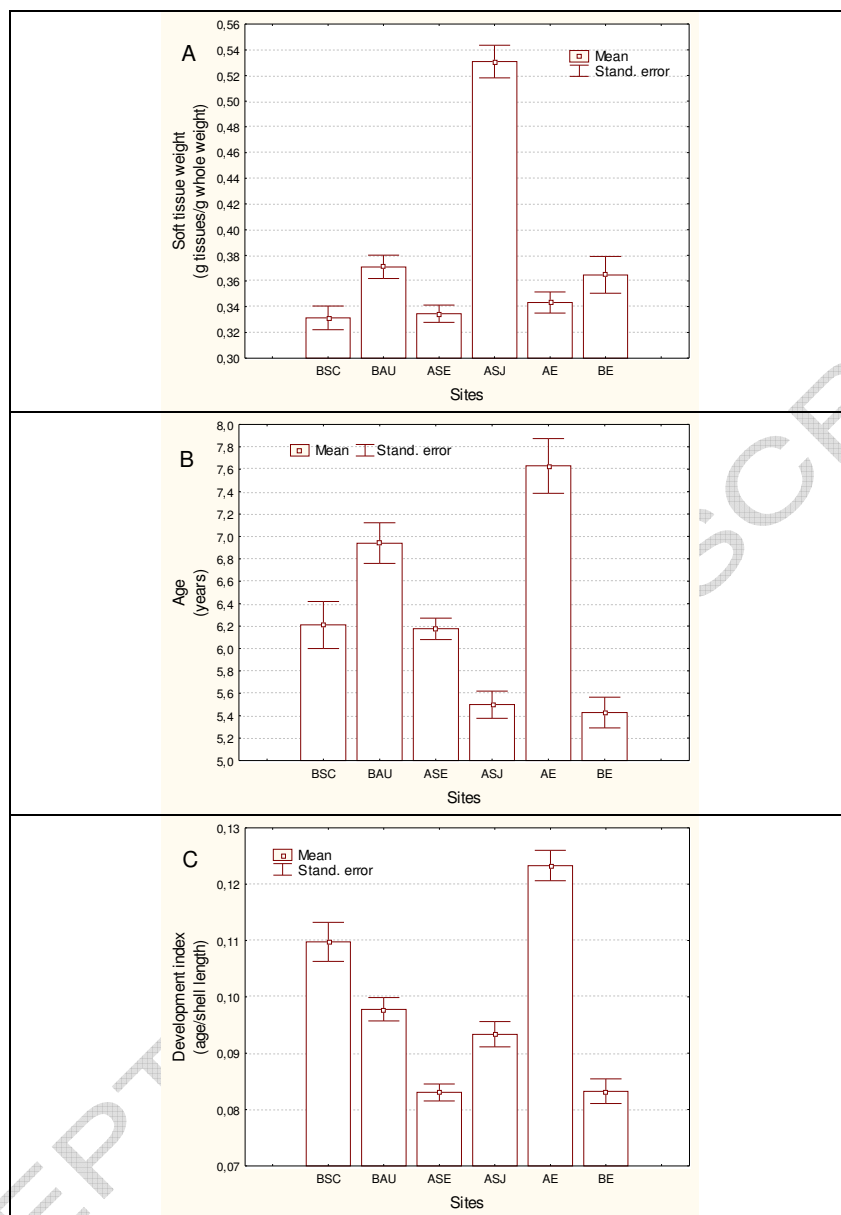


Figure 2. Morphological characteristics of clam populations in the St. Lawrence Estuary and Saguenay Fjord.

Clams were collected at low tide and brought back to the laboratory for analysis of soft tissue weights (A), age (B) and growth index, as determined by the age-to-shell-length relationship (C). Significance was set at $p < 0.05$.

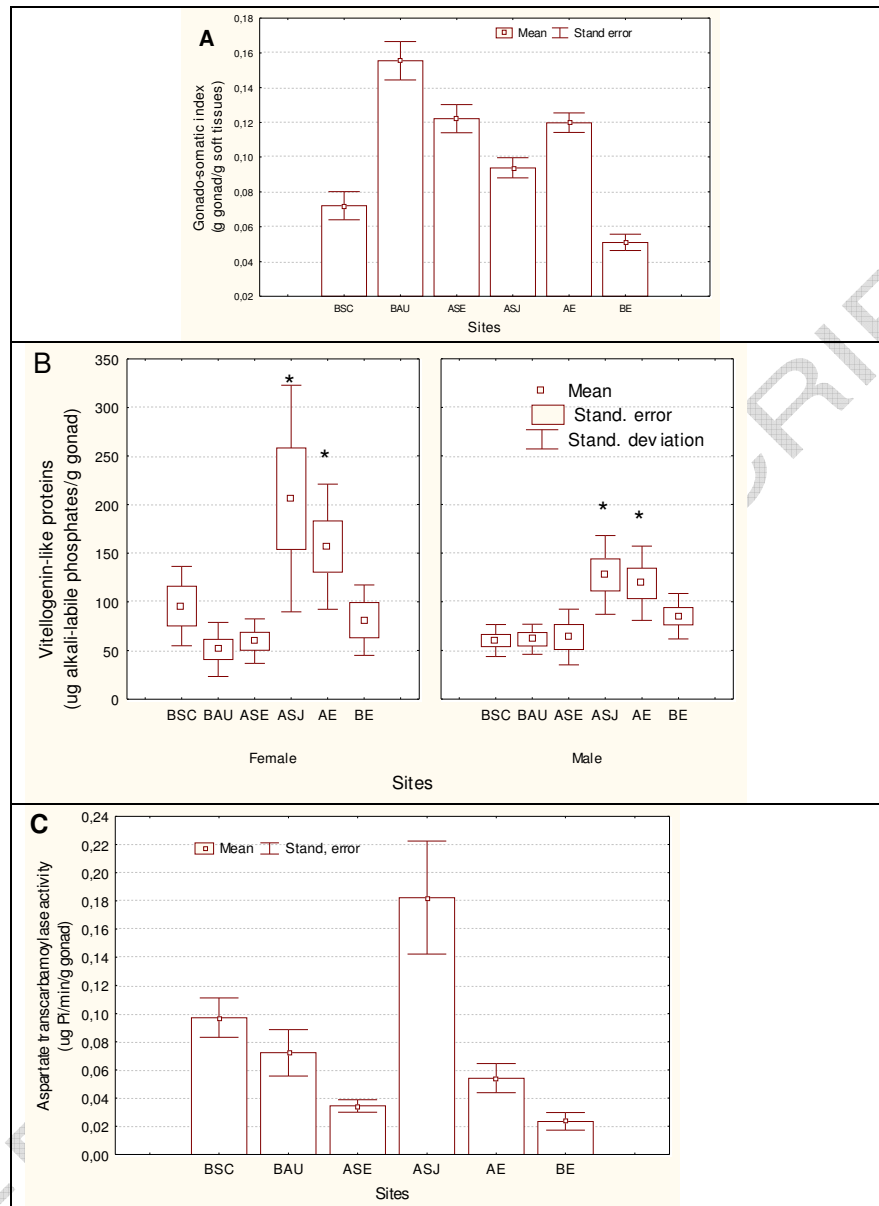


Figure 3. Gametogenetic activity in clams collected from impacted sites.

Clams were collected and the gonad analyzed for the gonado-somatic index (A), for levels of vitellogenin-like proteins (B) and aspartate transcarbamoylase activity (C). Significant at $p < 0.05$.

Table 1. Heavy-metal analysis in whole soft tissues of *Mya arenaria* clams.

SITE	BSC	BAU	ASE	ASJ	BE	AE
Metal						
As	0.91±0.05	0.74±0.07	0.84±0.04	0.48±0.03	0.47±0.025	0.42±0.02
Cd	0.04±0.002	0.06±0.004	0.1±0.01	0.12±0.01	0.11±0.008	0.12±0.007
Co	3.6±0.2	3.8±0.4	2.7±0.2	7.6±0.3	3.3±0.3	6.7±0.5
Cr	11.2±1	7.6±0.5	6.4±0.8	16.3±2	6.4±0.1	15.8±0.9
Cu	1.6±0.15	1.7±0.15	1.3±0.1	1.7±0.2	1.4±0.1	1.45±0.1
Fe	753±60	450±40	580±40	1130±75	744±60	575±45
Hg	0.03±0.005	0.008±0.004	Nd	0.032±0.003	0.046±0.005	0.044±0.005
Mn	21.6±3	9.2±0.5	11.7±1	162±10	50±5	54±4
Ni	6±0.5	1±0.1	1.5±0.1	2.6±0.2	2.2±0.2	1.8±0.15

Data were expressed as the mean with the standard error in $\mu\text{g/g}$ dry weight from N=4 groups of 10 clams per site.

Bold value indicates significance relative to the reference site (either BAU or ASE) at $p<0.05$ level.

Table 2. Biomarkers of defence and tissue injury in *Mya arenaria* clams.

Site	MT (digestive gland)	GST (digestive gland)	Total heme (gonad)	DNA damage (digestive gland)	LPO (digestive gland)	LPO (gill)	Biomarker index	
							Sum	Rank**
BSC	175 ±10*	0.81 ±0.03*	0.29 ± 0.1	63 ± 3*	3.9±0.26*	1.4±0.23*	16	5
BAU	104 ±7	0.36 ±0.02	0.36 ± 0.1	41 ± 4	1.95±0.25	2.5±0.46	8	2
ASE	49 ±5	0.38 ±0.03	0.41 ± 0.07	44 ± 2	2.49±0.26	2.4±0.36	6	1
ASJ	88 ±6*	0.43 ±0.026	0.80 ± 0.03*	63 ± 5*	3.37±0.26*	1.8±0.39	13	4
AE	111 ±8*	0.34 ±0.026	0.34 ± 0 .06	60 ± 3*	4.73±0.38*	1.5±0.17*	13	4
BE	71 ±6*	0.32 ±0.027*	0.39 ± 0.06	58 ± 6*	3.0±0.27	1±0.17*	10	3

* Significance was set at $p < 0.05$ relative to the reference sites (BAU and ASE).

** Sites are ranked in ascending order of toxicological stress.

Table 3. Trend analysis of biomarker data.

Variable 1	Variable 2	Correlation coefficient	P value
Gonado-somatic index	Age	0.34	< 0.01
	GST	0.39	< 0.01
	LPO in digestive gland	-0.28	0.02
	LPO in gill	0.41	< 0.01
	DNA damage in gill	-0.34	0.02
	DNA damage in digestive gland	-0.31	0.018
	COX	-0.27	0.038
Tissue weight ratio (soft-tissue wet weight/total weight)	Age	-0.32	< 0.01
	Development index	-0.21	0.04
	ACT	0.58	< 0.01
	DNA damage in gill	0.61	< 0.01
	Heme	0.45	< 0.01
	Serotonin/dopamine ratio	0.4	0.01
	COX	-0.38	< 0.01
Age	Development index	0.75	< 0.01
	DNA damage in gill	-0.33	0.024
	Serotonin/5H1AA ratio	0.33	0.013
Developmental index (shell length/age)	MT in digestive gland	0.4	< 0.01
	LPO in digestive gland	0.41	< 0.01
	Dopamine	0.34	0.029
	Serotonin	0.48	< 0.01
	Serotonin/5H1AA ratio	0.49	< 0.01
	COX	0.42	< 0.01
ACT	DNA damage in gill	0.47	< 0.01
	Heme	0.49	< 0.01
	Dopamine	0.32	0.039
	Serotonin	0.34	0.012
	Serotonin/dopamine ratio	0.37	0.018

Variable 1	Variable 2	Correlation coefficient	P value
GST	MT in digestive gland	0.76	< 0.01
	LPO in digestive gland	0.26	0.03
	DNA damage in digestive gland	0.4	< 0.01
	Dopamine	0.32	0.032
	Serotonin	0.31	0.018
	COX	0.68	< 0.01
MT	LPO in digestive gland	0.35	< 0.01
	DNA damage in digestive gland	0.27	0.041
	Serotonin	0.49	< 0.01
	COX	0.69	< 0.01
LPO in digestive gland	DNA damage in digestive gland	0.52	< 0.01
	Dopamine	0.4	< 0.01
	Serotonin/5H1AA ratio	0.35	< 0.01
	MAO in in digestive gland	0.48	< 0.01
	COX	0.54	< 0.01
DNA damage in gill	DNA damage in digestive gland	0.47	< 0.01
	Heme	0.55	< 0.01
	Dopamine	0.55	< 0.01
	Serotonin/dopamine ratio	0.54	< 0.01
	MAO	0.4	< 0.01
DNA damage in digestive gland	Dopamine	0.49	< 0.01
	Serotonin/dopamine ratio	0.38	0.01
	MAO	0.4	< 0.01
	COX	0.37	< 0.01
Heme	5H1AA	0.43	0.015
Dopamine	Serotonin/dopamine ratio	0.89	< 0.01
	MAO	0.37	0.016
Serotonin 5H1AA	Serotonin/5H1AA ratio	-0.67	< 0.01
	COX	0.32	0.02
Serotonin/5H1AA ratio	MAO	0.5	< 0.01

N.B.: Only significant correlations ($p < 0.05$) are shown.

Table 4. Neuroendocrine effects of site quality.

Site	Serotonin (ng/ g ww)	Serotonin transport activity	5-HIAA (ng / g ww)	Serotonin/ 5-HIAA	Dopamine (ng/g ww)	Dopamine transport activity	Serotonin/ dopamine	COX activity (x 10 ³)	MAO act (x 10 ³)
BSC	15 ± 2	28 ± 7	7 ± 3.4* ¹	2.5 ± 0.7	16.4 ± 3.9*	143 ± 47*	2.5 ± 0.7	1.7 ± 0.08*	1.8 ± 0.0
BAU	21.7 ± 2*	26 ± 8	16.5 ± 1.*	1.5 ± 0.3	5.04 ± 1	55 ± 12	2 ± 0.5	0.9 ± 0.05	1.6 ± 0.0
ASE	16 ± 2	18 ± 5	7.4 ± 1.9	0.6 ± 0.1	3.97 ± 1	42 ± 13	1 ± 0.27	0.9 ± 0.09	1.5 ± 0.0
ASJ	21 ± 1.8*	27 ± 7	11 ± 1.5*	1.9 ± 0.3	20.5 ± 7*	130 ± 33*	1.9 ± 0.3*	0.74 ± 0.04	2.8 ± 0.0
AE	19 ± 1.7*	51 ± 9	3.4 ± 0.2*	5.8 ± 0.4*	14.6 ± 1*	21 ± 5	5 ± 0.4*	1.3 ± 0.07*	2.9 ± 0.0
BE	13 ± 1.3*	19 ± 3	4.7 ± 1.5	3.1 ± 1.2*	12.4 ± 3.6*	41 ± 5	1.6 ± 0.6	0.9 ± 0.1	2.6 ± 0.0

N.B.: Asterisks (*) indicate significance at $p < 0.05$

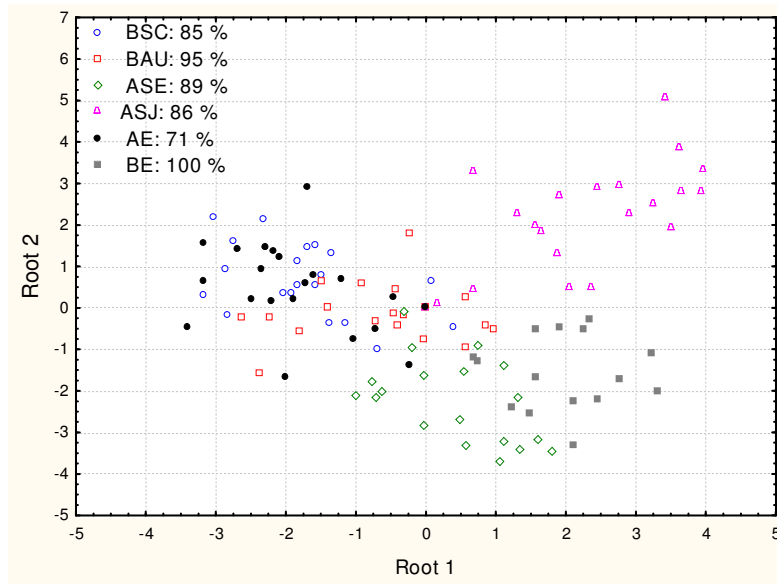


Figure 4. Multivariate analysis of biomarker data.

Biomarker data were analysed by discriminant analysis for site classification. The classificant performance for each sites are expressed as percentages in the legend.

Table 5. Factorial analysis of biomarker data.

Biomarkers	Factor 1	Factor 2	Factor 3	Factor 4
Individual				
Soft tissue weights	-0.17	-0.72	-0.27	-0.024
Gonado-somatic index	0.39	0.19	-0.36	-0.49
Age	-0.06	0.62	-0.52	-0.35
Growth index	-0.51	0.57	-0.33	-0.37
Gonad (Reproduction)				
Vtg	-0.61	-0.22	-0.55	-0.008
ACT	-0.45	-0.57	-0.008	-0.15
Heme levels	-0.24	-0.67	-0.25	-0.27
Dopamine	-0.79	-0.21	-0.24	0.10
Serotonin	-0.48	0.035	-0.054	-0.66
5H1AA	0.29	-0.46	0.045	-0.59
Serotonin/5H1AA	-0.41	0.56	-0.37	0.18
Serotonin/dopamine	-0.56	-0.41	-0.26	0.22
Dopamine transport activity	-0.50	-0.40	0.38	-0.24
Serotonin transport activity	-0.49	0.17	-0.08	-0.11
COX	-0.62	0.48	0.48	-0.08
Digestive gland				
GST	-0.57	-0.015	0.68	-0.20
MT	-0.62	0.25	0.46	-0.32
LPO	-0.67	0.20	-0.02	0.20
DNA strand breaks	-0.63	-0.044	0.19	0.34
MAO	-0.56	-0.0002	-0.44	0.29
Gills				
LPO	0.24	-0.03	-0.20	-0.26
Explained Variance (cumulative)	25%	41%	53%	63%

1. Factorial weights > 0.6 are shown in boldface.