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ScienceDirect
Environment International 34 (2008) 30–43
ENVIRONMENT INTERNATIONAL
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Relationships between intertidal clam population and health status of the soft-shell clam *Mya arenaria* in the St. Lawrence Estuary and Saguenay Fjord (Québec, Canada)

F. Gagné [a,*], C. Blaise [a], J. Pellerin [b], M. Fournier [c], M.J. Durand [d], A. Talbot [a]

[a] *Fluvial Ecosystems Research, Aquatic Ecosystems Research Protection Branch, Environment Canada, 105 McGill Street, Montréal, Québec, Canada H2Y 2E7*
[b] *ISMER-Université du Québec à Rimouski, Rimouski, 310 Grande Allée, Rimouski, Québec, Canada G5L 3A1*
[c] *INRS-Institut Armand-Frappier, Université du Québec, 245 Hymus Blvd., Pointe-Claire, Quebec, Canada H9R 1G6*
[d] *Université de Nantes, CNRS UMR 6144-GEPEA, Département Génie Biologique, De, 18 Bd G.Defferre, 85035 la Roche sur Yon, France*

Received 21 March 2007; accepted 21 June 2007
Available online 6 September 2007

Abstract

The purpose of this study was to examine the impacts of anthropogenic activity on the health status of intertidal clam populations of the Saguenay Fjord and the St. Lawrence Estuary (Québec, Canada). Clams were collected during low tide at sites subject to direct contamination and at sites far from human activity. Clams were analyzed for tributyltin and dibutyltin total levels and toxic stress (glutathione *S*-transferase, gonadal lipid peroxidation and DNA strand breaks), immunocompetence (phagocytic activity, hemocyte count and viability), reproduction (gonado-somatic index, gamete maturation, and vitellogenin-like proteins), energy status (temperature-dependent mitochondrial electron transport, and gonad lipids), and individual status (age, condition factor, and growth index). These responses were compared against population characteristics such as live clam density, number of empty shells, and sex ratio. The results show that clam density decreased with distance from the estuary (high salinity level) to upstream of the fjord (low salinity). There was no clear relationship between the number of empty shells and distance or site quality. Clam density values corrected against distance were significantly correlated with hemocyte viability, phagocytic activity, mitochondrial electron transport (MET), DNA damage in gonad, and temperature-dependent mitochondrial electron transport activity. A canonical analysis of the various groups of biomarkers revealed that population metrics were more strongly related with immunocompetence, followed by energy status and temperature-dependent mitochondrial electron transport activity. However, toxic stress biomarkers were strongly associated with energy status and reproduction. This was further confirmed by non-linear modeling using adaptive artificial neural networks (genetic selection and back propagation learning paradigms), where the following parameters were able to predict population parameters with <20% error: gonad maturation and somatic index, MET (at 4 °C), gonad LPO, DNA damage, and phagocytic capacity. Intertidal clam populations were influenced by a distance gradient effect (salinity), where immunocompetence, in addition to energy status, was the strongest physiological parameter related to clam population metrics.

Keywords: *Mya arenaria* clams; Biomarkers; Population; Toxic stress

1. Introduction

The St. Lawrence Estuary, which receives water inputs from the Saguenay Fjord, is influenced by pollution from local harbors (marinas) and municipal and industrial wastewater discharges. Chemical contamination of this intertidal area is relatively well documented and includes the usual contaminants (polyaromatic hydrocarbons, heavy metals including organotin compounds), as well as endocrine disruptors contained in urban wastewater (Fortin and Pelletier, 1995; Gagné et al., 2005). *Mya arenaria* clams were recently shown to accumulate organotin compounds such as tributyltin, making them a species at risk to this type of contamination (Yang et al., 2006). In an attempt to understand the possible ecotoxicological effects of environmental contamination on intertidal *Mya arenaria* clams, an extensive spatial biomarker survey was undertaken in areas of the St. Lawrence and Saguenay Fjord suspected of being contaminated by organotins and other compounds (Gagné et al., 2005, 2002; Blaise et al., 2003). These studies revealed that

* Corresponding author.
E-mail address: francois.gagne@ec.gc.ca (F. Gagné).

0160-4120/$ - see front matter.
doi:10.1016/j.envint.2007.06.010

clams living near sources of anthropogenic pollution were generally adversely affected. Indeed, the clams' health status was compromised, as evidenced by changes in metallothionein, DNA damage and vitellogenin-like proteins (Vtg) levels. The ecotoxicological effects observed in intertidal clams have raised concerns about the possibility that the effects of anthropogenic pollution might reach the population level.

To examine the impact of pollution on feral clam populations, a spatial survey was undertaken in the intertidal zones of the Saguenay Fjord and St. Lawrence Estuary. Population characteristics were studied in terms of clam density (per surface area), number of empty shells, age structure, and sex ratio. Clams in the 5–8 year age group were amassed for individual biomarker evaluation (shell length, clam/soft tissue weight and growth index), reproductive function, immunocompetence, energy status, and toxic stress. Immunocompetence in peripheral clam hemolymph was examined by phagocytic activity, hemocyte count and viability using flow cytometry technology at the site of clam collection (Gauthier-Clerc et al., 2006). Toxic stress was investigated by measuring oxidative stress through lipid peroxidation of polyunsaturated fatty acids and glutathione *S*-transferase, which is also implicated in the conjugation of polar xenobiotics, leading to DNA damage when these responses are sustained (Gravato et al., 2005; Cheung et al., 2004). Reproduction was studied at the gametogenic level as determined by the gonado-somatic index, male and female gonad maturation index, and levels of Vtg in females. Poor reproduction is expected to impact population status, which can be altered, in turn, by contaminants (Gagné et al., 2002). Vtg is a major, energy-rich egg-yolk protein found in developing embryos of both vertebrates and invertebrates, including bivalve molluscs (Byrne et al., 1989). Vtg synthesis is controlled by the estrogen receptor pathway, in part at least, which may be negatively or positively modulated by environmental chemicals (Osada et al., 2003). Energy status was examined in the mitochondria by determining the rate of electron transport activity and lipid stores in the gonad (Smolders et al., 2004). These biomarkers of cellular energy allocation proved predictive of changes at the individual and population levels in aquatic invertebrates (De Coen and Janssen, 2003). The interaction of temperature with pollution in the context of global warming was also investigated here. The temperature/pollution interaction was examined in isolated mitochondria of gonadal tissues of feral clam populations from sites of differing quality (Gagné et al., 2007). Indeed, pollution heightened the susceptibility of feral clam populations to temperature increases by significantly increasing electron transport activity in mitochondria (energy expenditure), which represents, at the present time, the first means by which to measure the interaction between global warming and pollution in benthic ecosystems.

The main objective of this study was to examine the relationships between clam population metrics at sites previously recognized as impacted by pollution and changes in the abovementioned physiological parameters. In this respect, biomarker responses were examined at different levels based on homeostatic and reproductive functions such as morphological status, reproduction, immunocompetence, toxic stress, and energy status, with its temperature-dependent characteristics. An attempt was made to examine the contribution of various environmental stressors to population maintenance in the context of global warming, water/sediment quality, predation pressures, and habitat quality in feral clam populations.

2. Methods

2.1. Site characteristics, clam density, and morphological metrics

Mya arenaria soft-shell clams were collected by hand on mud flats at morning low tide from four sites in the St. Lawrence Estuary and six sites in the Saguenay Fjord (Fig. 1). Among the St. Lawrence Estuary sites, Baie Saint-Catherine (BSC) is influenced by heavy traffic from sightseeing and whale-watching boats; Pointe aux Alouettes (AL), a few kilometers upstream, is not directly affected by any anthropogenic activity; Tadoussac harbour (TAD), about 5 km downstream on the north shore of the mouth of the Saguenay Fjord, supports intensive commercial boating activities; Baie-du-Moulin à Baude (BAU) was considered the reference site because of the absence of any direct source of pollution. As for the Saguenay Fjord sites, Anse à la Barque (BAR) is close (1–2 km) to TAD on the north shore of the fjord close to ferry traffic; Anse Étienne site (ASE), located on the south shore 20 km upstream of the estuary, is under no direct source of anthropogenic activity and was thus considered as the reference site for the fjord; the site of Petit Saguenay (PS) is located 32 km upstream on the south shore of the estuary and has a small marina for seasonal and pleasure-boating activities; the Anse Saint-Jean (ASJ) site is exposed to the minimally treated (screening) urban wastewater of a population of about 2000 residents and is located 40 km upstream of the estuary; Baie Éternité (BE) is located 50 km upstream of the estuary and supports a moderate (capacity of 10–20 ships <10 m in length) marina for pleasure and tourist boating activities; finally Anse-aux-Érables (AE), the farthest upstream site in the fjord (70 km upstream of the estuary), is located downstream of aluminum smelters and pulp and paper mills, as well as four townships that discharge their wastewaters into the fjord.

Clam density estimates were determined by the number of live animals present in four 0.25-m^2 replicate quadrant at each site. The number of dead animals was estimated by the number of empty shells found at the sites in the same four replicate quadrant. The number of major grooves (annual rings) on shells determined age. Afterwards, about 50 clams were collected for biomarker analyses. A 1-mL portion of the hemolymph was collected with the aid of a syringe from the anterior adductor muscle for immunocompetence evaluation, as described below. The weights of clams, soft tissues and shell length were also determined in the laboratory for gonado-somatic index (wet weight of gonad/wet weight of soft tissues), condition factor (clam weight/shell length), and growth index (shell length/age ratio). Tissues were stored on dry ice for subsequent biomarker determinations. Before freezing, a gonadal smear was examined microscopically for sex characterization and maturation index estimates. After thawing on ice, the digestive gland and gonadal tissues were collected for homogenization. Tissues were homogenized using a Teflon pestle tissue grinder in ice-cold 25 mM Hepes-NaOH buffer, pH 7.4, containing 125 mM NaCl, 0.1 mM EDTA and 0.1 dithiothreitol at a 1:5 v/v ratio. Aliquots of each homogenate were collected for total protein determinations (Bradford, 1976), lipid peroxidation (LPO) and DNA damage. The remaining homogenates were centrifuged at 3000 ×*g* (20 min at 2 °C) for mitochondrial electron transport (MET) activity in mitochondrial-enriched supernatant and the remaining supernatant centrifuged at 15,000 ×*g* (20 min at 2 °C) for vitellogenin (Vtg)-like protein determinations and glutathione *S*-transferase (GST) activity in the supernatant, as described below. All biomarkers were normalized with total protein levels in the corresponding homogenate S3 and S15 supernatant fractions.

2.2. Microbial assay for organotin compounds

Relative levels of tributyltin and dibutyltin compounds were determined using a microbiossay procedure with a recombinant *E. coli*-sensitive strain TBT3 (Durand et al., 2003). Digestive glands were dissected out from $n=3$ clams per site and homogenized in one volume of distilled water with a Polytron tissue grinder. The homogenate was then mixed with two volumes of ethylacetate for 30 min at room temperature (with repeated vortex mixing at 5-min intervals). The mixture was centrifuged at 1000 ×*g* for 5 min to separate

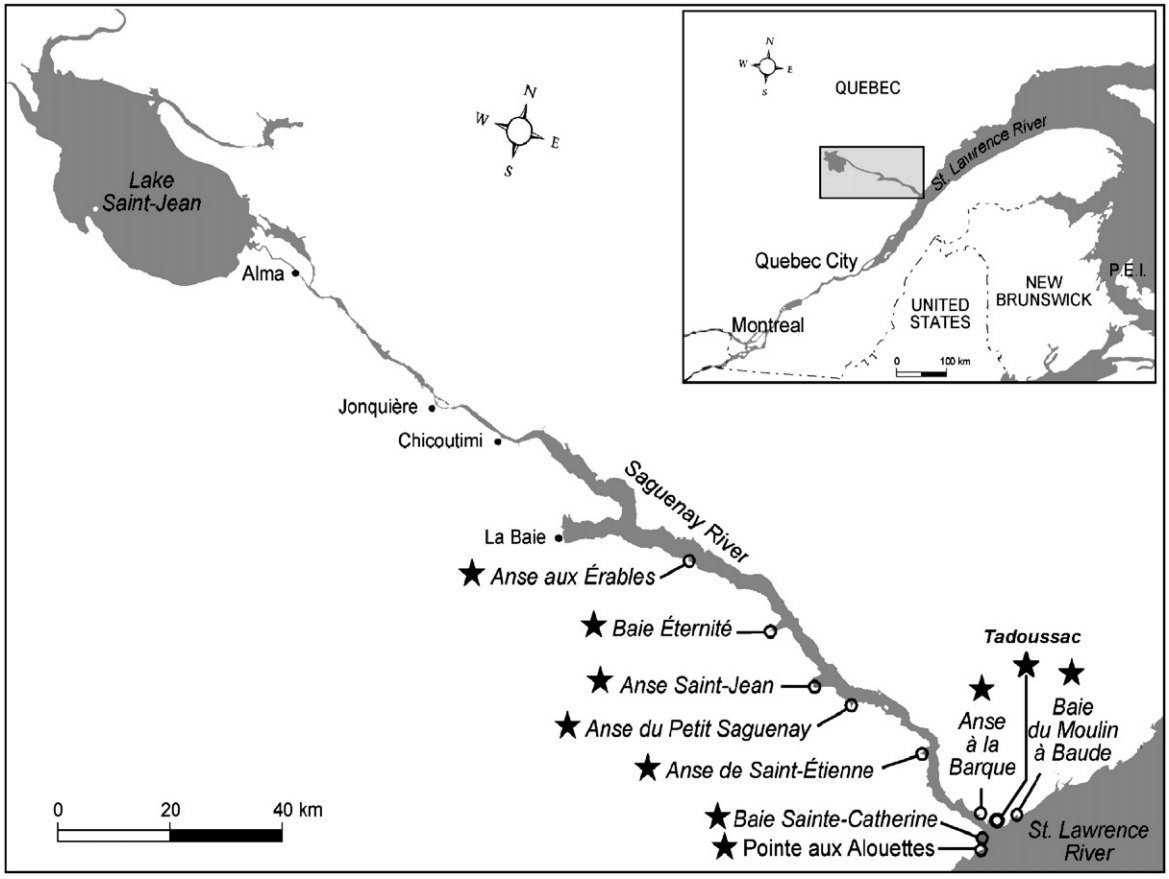

Fig. 1. Location map of Saguenay Fjord sites. Clams were collected at nine sites (identified by stars) in June 2005. The sites Anse de Saint-Étienne (ASE) and Baie du Moulin à Baude (BAU) were considered as the reference ("pristine") sites, while Anse aux Érables (AE), Pointe aux Alouettes (PAL), Baie Éternité (BE), Anse Saint-Jean (ASJ), Anse du Petit Saguenay (APS), Anse à la Barque (BAR) and Baie Sainte-Catherine (BSC) and the city of Tadoussac (TAD) were the pollution-impacted sites.

the phases. The organic phase was carefully collected, passed through calcium sulfate anhydride and evaporated to dryness under a nitrogen stream. The dried material was weighted for normalization. Just prior to performing the microbial assay, the extract precipitate was resuspended in 400 μL of isopropanol and 1/10 and 1/20 dilutions were added to bacterial culture containing glucose medium in 96-well micro plates (optical density of cells $OD_{620\ nm}$ 0.15 with an Hitachi U-1800 spectrophotometer). The micro plate was incubated 1 h at 30 °C, after which luminescence was recorded with a micro plate luminescence reader (Microlumate L96 V, EGG Berthold). The positive control consisted of adding 1 μM TBT and the negative control consisted of the vehicle solvent (isopropanol). The data were expressed as relative light units (RLU) per dry clam tissue extract weight.

2.3. Immunocompetence evaluation

As per the selected method, hemolymphs was collected from the posterior adductor muscle sinus using a 5.0-mL syringe with a 23G needle (Brousseau et al., 2000). The cells were maintained in the hemolymph and stored on ice during the manipulations. An aliquot of 20 μL of each cell suspension was mixed with 180 μL of a Viacount solution (Guava Technologies, Hayward, CA, USA). After ten minutes of incubation at room temperature in the dark, the number of viable and dead cells was assessed by cytometry (PCA Guava Cytometer) (Frouin et al., 2007). Phagocytosis was monitored using a micro plate method modified for flow cytometry (Fournier et al., 2002). For each organism, a volume of 200 μL of hemolymph, was placed in duplicate in a 96-well micro plate and mixed with red latex FluoSpheres (Molecular Probes Inc, Eugene, OR, USA), with a diameter of 2 μm, at a ratio of 1:30 (hemocyte to beads). Samples were incubated at room temperature in the dark. After 18 h, the micro plates were emptied by inversion. Cells with phagocyted beads were resuspended in 200 μL of the fixation solution (50 mL of artificial sea water mixed with 100 mg of sodium azide and 0.250 mL of formaldehyde). A Guava cytometer (Guava Technologies, Hayward, CA, USA) was used to measure active hemocytes for phagocytosis. A total of 2000 events were acquired for each sample and stored in the list mode data format. The data were then analyzed using the red fluorescence (Fl) versus cell size (FSC) dot blot distribution of hemocytes. The results were expressed as the percentage (%) of hemocytes having engulfed fluorescent beads (Brousseau et al., 2000).

2.4. Gonadal integrity

The state of gonads was assessed by following the gonado-somatic index (GSI), the maturation index, and the relative levels of vitellogenin-like proteins. For the maturation index, a gonadal smear was examined at 400× enlargement for grading: stage 1 for undeveloped or immature gonad, stage 2 for maturing gonad (where spermatogenesis or vitellogenesis starts), stage 3 for fully developed or ripe gonad, and stage 4 for spawned gonads. The maturation index was determined by the mean stage value with standard error at each site. The presence of vitellogenin (Vtg)-like proteins was determined by the alkali-labile phosphate assay using the acetone fractionation procedure (Gagné et al., 2002). Vtg-like protein levels were expressed as μg of alkali-labile phosphate (ALP)/mg of gonad protein.

2.5. Energy status

Mitochondrial electron transport (MET) activity was determined in the mitochondria-enriched supernatant (3000 ×*g* supernatant) using the *p*-iodonitrotetrazolium dye method (Smolders et al., 2004; King and Packard, 1975). Briefly, 100 μL of the supernatant was mixed with 100 mM Tris–HCl, pH 8.5, containing 100 μM $MgSO_4$, 0.1% Triton X-100 and 5%

Table 1
Population status of *Mya arenaria* clams in the Saguenay Fjord

Sites	Pollution index 1=clean 2=polluted	Suspected impacts of pollution	Clam density (no./m^2) Mean±S.E.	% live [a]	Total clams [b] (no./m^2) Mean±S.E.	Sex ratio [c] Mean±S.E.
BSC	2	High input Whale-watching wharf	35.75±1.5	89	40±2	1.36±0.06
AL	1	Light input No direct source	30±3	85	35±4	1.45±0.06
BAR	2	Moderate input upstream of ferry	19.5±3.8	93	21+3	1.50±0.07
TAD	2	High input Dock for commercial or pleasure boats	37.5±7	62	61±10	1.60±0.06
BAU	1	Light input No direct source	13.5±2.9	67	20±2	1.46±0.06
ASE	1	Light input No direct source of pollution	18.75±6	51	49±6	1.39±0.06
ASJ	2	High input Municipal effluent	15.5±2	94	16±2	1.39±0.06
PS	1	Light input Small marina	6±1.3	54	11±3	1.48±0.06
BE	2	High input Tourist wharf	11.25±2	60	18.7±4	1.52±0.06
AE	2	High input Close to industries and towns	<10	NA [d]	NA	1.40±0.11

[a] The percentage of living clams was defined by the number of live clams/total number of individuals.
[b] The total number of individuals was estimated by summing the number of (paired) empty shells with the number of live individuals.
[c] Sex ratio: male=1 and female=2.
[d] NA: not analyzed because of the low number of clams per unit area (<5 clams/m^2).

polyvinylpyrrolidone for 1 min on ice. The reaction mixture was mixed with 1 mM NADH and 0.2 mM NADPH on ice and then divided into two portions, one being equilibrated at 4 °C, the other, at 20 °C. The reaction was initiated with the addition of 50 µL of *p*-iodonitrotetrazolium at 5 mM for 30 min. Absorbance readings were measured at 15-min intervals at 520 nm. The data were expressed as the loss of absorbance at 4 °C and 20 °C/30 min/mg total proteins in the corresponding supernatant. Temperature-dependent MET (MET_T) was determined and the rate difference in MET at 20 °C and 4 °C was divided by the temperature change (20−4 °C=16 °C). The data were expressed as the rate change (normalized against total proteins)/unit temperature in °C. Levels of total lipids were also determined in the gonad homogenate according to the phosphovanillin method (Frings et al., 1972). The detergent Triton X-100 was used for calibration. The data were expressed as µg lipid equivalents/mg protein.

2.6. Biomarkers of toxic effects

Pollution-related effects were determined by tracking changes in GST activity, LPO and DNA strand breaks. Glutathione *S*-transferase (GST) activity was determined by the method of Boryslawskyj et al. (1988) using 2, 4-dichloronitrobenzene as the substrate in a microplate format. The assay was performed on the 15,000 ×*g* supernatant using 0.1 mM of the substrate and absorbance readings were made at 340 nm. Data were expressed as the decrease in absorbance/min/mg proteins. LPO was determined in gonad homogenates using the thiobarbituric acid method (Wills, 1987). Standard solutions of tetramethoxypropane were used for calibration and fluorescence was measured at 520 nm excitation and 600 nm emission (Chameleon-II, Multireader, Bioscan, USA). Given that the reagent could react with other aldehydes, results were expressed as µg of thiobarbituric acid reactants (TBARS)/mg of homogenate protein. DNA damage was determined by the alkaline precipitation assay developed by Olive (1988), with fluorescence quantization of DNA strand breaks in the presence of detergents and an alkaline pH (Bester et al., 1994). The assay principle is based on the potassium-detergent (SDS)-assisted precipitation of protein-linked genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. The number of DNA strand breaks results from the DNA repair of DNA adducts and alkali-labile sites. The results were expressed as µg of DNA/mg proteins. Calibration was achieved with salmon sperm DNA (Sigma Chemical Company, USA).

2.7. Data analysis

At each site, 12 clams were collected for the biomarker analyses. The normality of the data distribution was verified using Levene's test. The data were log-transformed when significant deviation from normality occurred. The data were analyzed by factorial two-factor analysis of variance with site and

Table 2
Relative TBT and DBT levels in clam soft tissues

Site	Induction factor [a]	Mann–Whitney *U* test	Comments
AE	2.6±0.25	$p=0.04$	Significant compared to ASE
BE	1.9±0.27	$p=0.19$	No difference
ASJ	1.6±0.14	$p=0.38$	No difference
PS	2.3±0.37	$p=0.08$	Marginal compared to ASE
BAR	3.2±0.2	$p=0.05$	Significant compared to BAU
TAD	3.2±0.1	$p=0.08$	Marginal compared to BAU
		$p<0.001$	Significant compared to ASE
BSC	3.0±0.09	$p=0.09$	Marginal compared to BAU
		$p=0.02$	Significant compared to ASE
BAU	2.7±0.025	–	Estuary reference site
ASE	1.47±0.09	–	Fjord reference site
Sea water	1±0.05	–	Control group; all sites were significant at $p<0.05$ level.

[a] The induction response ratio was calculated as follows: relative light units of sample/ relative light units of control group (sea water).

gender as the major factors. For inter-site trend analysis between population metrics, individual morphology and biomarker responses, a ranked-correlation test was used (Spearman-rank correlation tests). A canonical analysis was also performed to determine the relatedness of groups of measurements: population metrics, individual morphology, immunocompetence, reproduction (gamete activity), toxic stress, and energy status (including temperature-dependent effects). Interrelationships between population characteristics and biomarker data were also studied by non-linear modeling using artificial neural networks. First, the biomarker data were screened against the population metric group (clam density, number of empty shells, age, and growth index) using a genetic selection algorithm to identify the most useful subset of biomarker data. The selected subset of biomarker data was then modeled using the back propagation learning paradigm (Statistica Neural networks module, version 3). Training was performed for nine randomly selected sites for 1000 epochs, where the model was verified with one randomly selected site. The learning paradigm was further trained by repeating the above four more times while randomly changing the verification site for each training time.

3. Results

3.1. Site quality and organotin levels

Mya arenaria clams were collected at various sites along the St. Lawrence Estuary near the mouth of the Saguenay Fjord and upstream of the Saguenay River (Fig. 1). Of the Estuary sites, BSC and TAD came into direct contact with pollution, while BAU and AL were subject to no direct pollution (Table 1). This was confirmed by the relative levels of organotin (TBT+DBT) contamination in soft tissues in the present study (Table 2). All tissue-extract sites contained significantly more organotin compounds than did the control sea water. The relative levels of TBT/DBT were significantly higher at site AE (Mann–Whitney U test: $p=0.04$), site BAR (Mann–Whitney U test: $p=0.05$), and marginally so at site PS (Mann–Whitney U test: $p=0.08$) when compared to the Saguenay Fjord reference site (ASE). The estuarine sites were significantly more contaminated by organotins than were those in the Saguenay Fjord. Indeed, organotin values were significantly higher at the estuary reference site BAU compared to site ASE, the Saguenay Fjord reference site (Mann–Whitney U test: $p=0.049$). The relative levels of organotins in clams from estuary sites BSC and TAD were only marginally higher (Mann–Whitney U test: $p=0.09$ and $p=0.08$, respectively) relative to BAU, but organotin levels at site BSC were significantly increased (Mann–Whitney U test: $p=0.02$) when compared to the fjord reference site ASE. There was a marginally significant correlation between organotin levels in tissues and relative distance from the estuary site BAU ($r=-0.62$; $p=0.08$), suggesting a high TBT contamination trend at sites closer to the estuary.

3.2. Clam population characteristics

Clam density estimates varied among sites, ranging from <10 clams/m^2 at the farthest site upstream of the Saguenay Fjord to 37 clams/m^2 at the TAD site. Clam density significantly separated into three groups (ANOVA $p<0.001$), in decreasing order: (BSC, AL, TAD)>(BAR, ASE, ASJ)>(BAU, PS, BE, AE). There was a significant correlation between distance from site BSC to the farthest site (AE) and clam density ($r=-0.81$; $p=0.005$), where density gradually decreased from the estuary to upstream sites in the fjord. It is noteworthy that the estuarine reference site was part of the groups with low clam densities. The effects of pollution on clam density were significant (Mann–Whitney U test: $p<0.05$) when clam densities were corrected against the distance effect (i.e. residual clam density value not linked to distance, as estimated by the linear regression model). The rank sum of residual clam density at the polluted sites was lower than at the clean sites, indicating that pollution was associated with

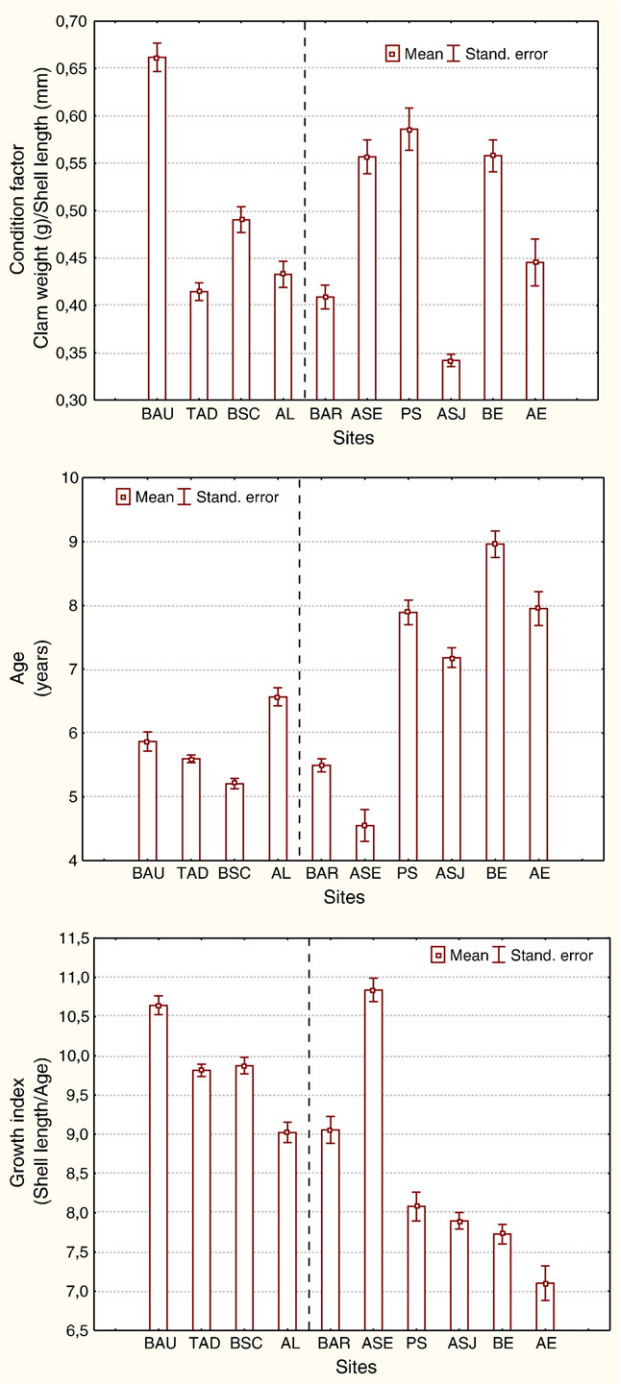

Fig. 2. Morphological characteristic of clam populations. Clams were collected intertidally and analyzed for condition index (total weight/shell length), age and relative growth (shell length/age). The dotted line separates the clam populations of the estuary (left side) from those of the fjord (right side).

low clam recruitment. The proportion of live clams also varied between sites and was marginally lower (Mann–Whitney U test: $p=0.1$) at the polluted sites. As with clam density, the proportion of live clams (relative to the number of empty shells) was significantly separated into three groups, in descending order (ANOVA $p<0.001$): (BAR, BSC, ASJ)>(AL, TAD, BAU)>(ASE, BE, PS). The reference site ASE in the Saguenay Fjord was associated with the low-viability group because of its high number of empty shells, which could indicate predation by aquatic animals and birds and clam consumption at the site because of the strong

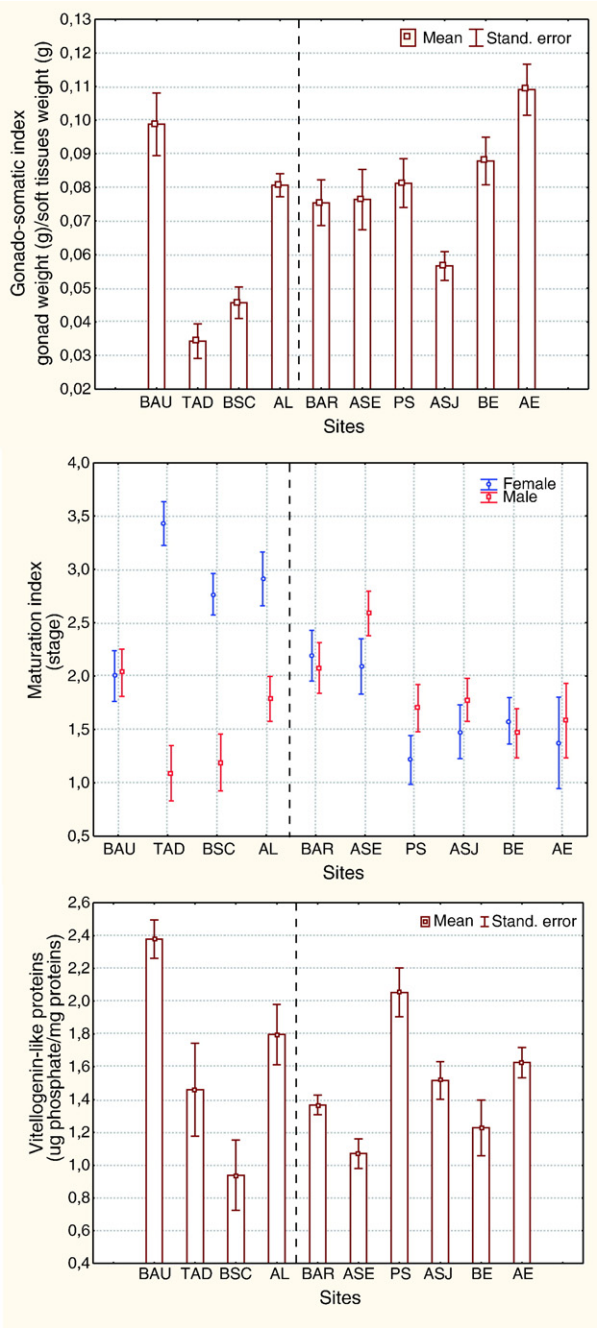

Fig. 3. Gamete activity in *Mya arenaria* clam populations. Clams were collected during low tide and analyzed for gonado-somatic index, maturation index and vitellogenin-like proteins. The dotted line separates the clam populations of the estuary (left side) from those of the fjord (right side).

correlation between the number of live clams and total clam numbers (live and empty shells). The sum of live clams and paired empty shells (i.e. total clam number) differed between sites and three groups were significantly distinguished (ANOVA $p<0.001$), in decreasing order: (ASE, TAD, BSC)>(AL, BAR, BAU)>(ASJ, PS, BE). The total clam number was significantly correlated with clam density ($r=-0.85$; $p=0.01$) and marginally with relative distance ($r=-0.62$; $p=0.07$), as described above. However, the grouping of total clam number was related to relative distance ($r=-0.68$; $p=0.04$), indicating a gradient effect of distance (salinity) on clam density. The variation in sex ratios was more consistent across sites, but three groups differed significantly, in descending order: (BAR, BAU, TAD)>(PS, BE, AL)>(BSC, ASE, ASJ). Sex ratio was not correlated with any of the population parameters described above.

3.3. Morphological characteristics

Individual and morphological characteristics of local clam populations were examined by monitoring condition factor, age and growth index (Fig. 2). The condition factor (weight-to-length ratio) was readily reduced at polluted sites in both the estuary and Saguenay Fjord areas,

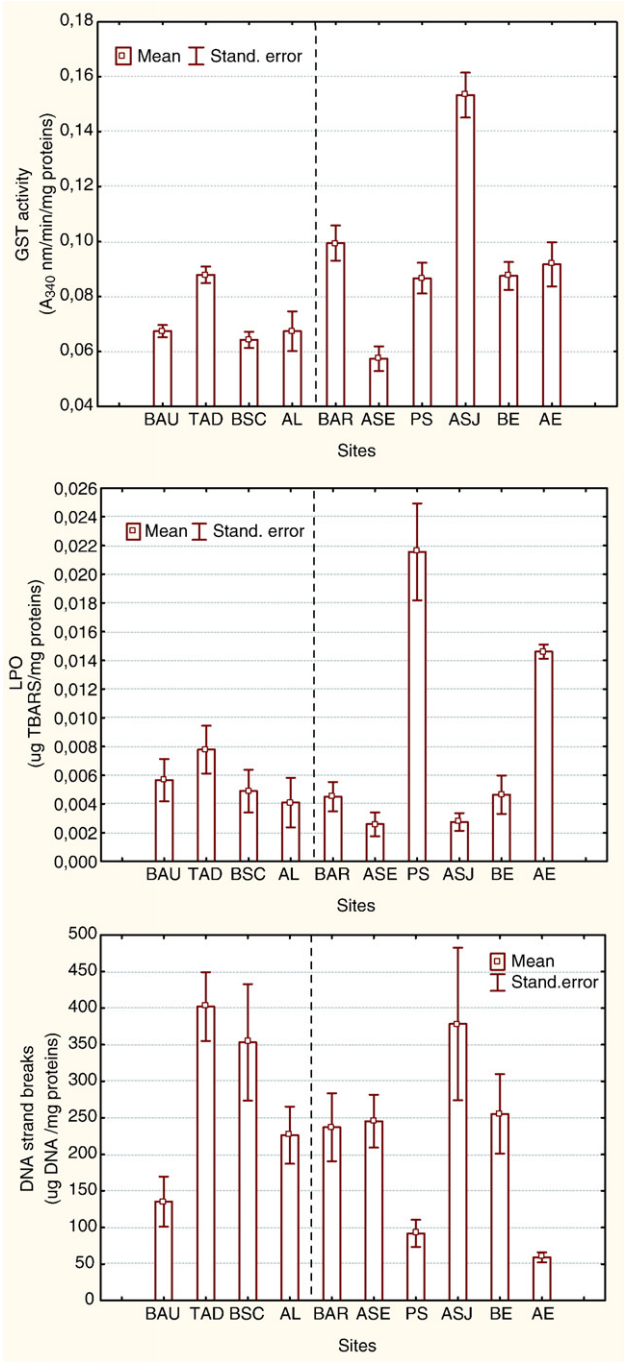

Fig. 4. Stress and tissue damage biomarkers. Clams were collected during low tide and analyzed for glutathione *S*-transferase (GST) actitvity, lipid peroxidation (LPO) an DNA strand breaks. The dotted line separates the clam populations of the estuary (left side) from those of the fjord (right side).

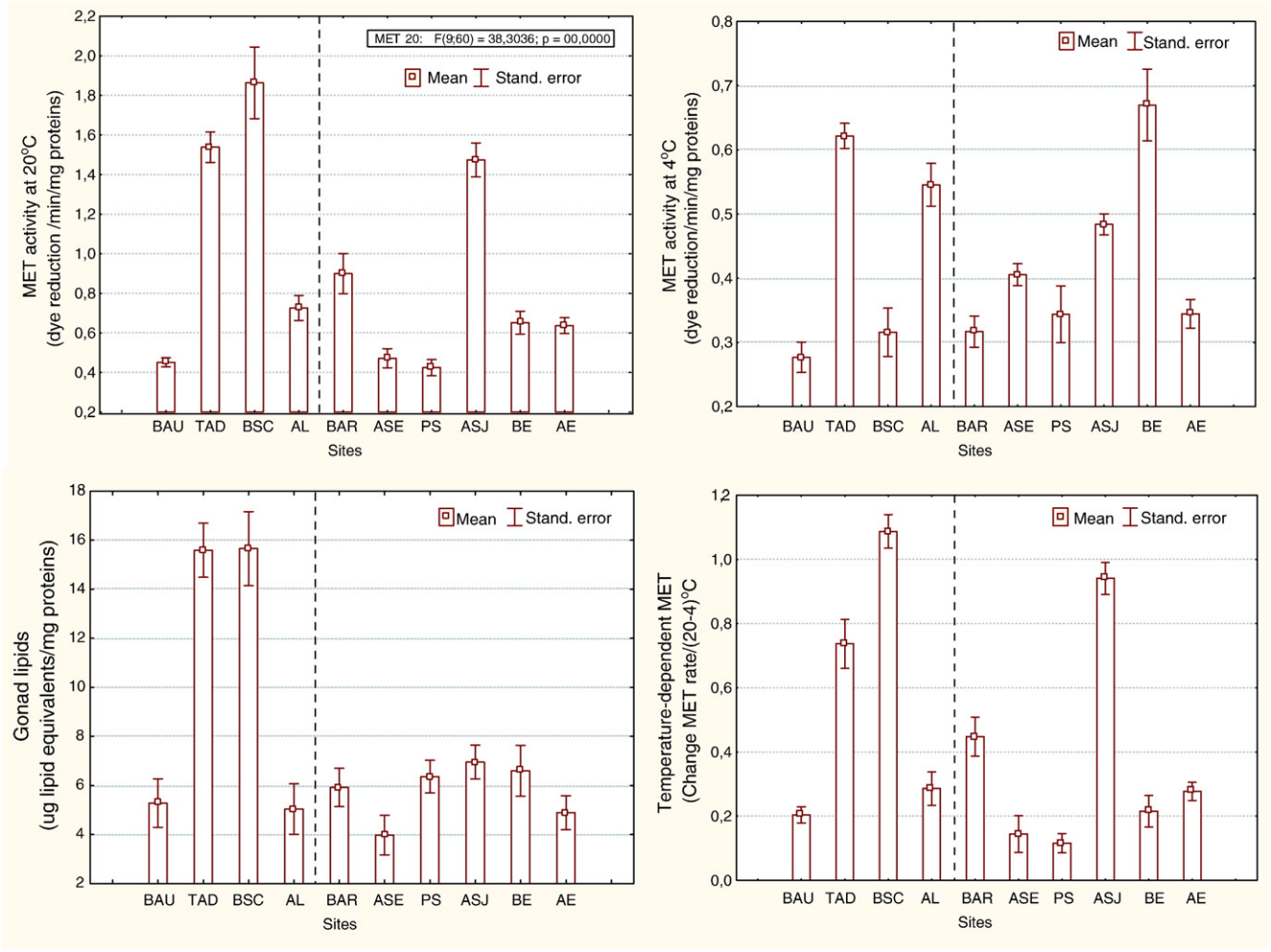

Fig. 5. Spatial variation in energy status and its temperature dependence in *Mya arenaria* clams. Clams were collected during low tide and analyzed for mitochondrial electron transport (MET) activity at 20 °C and 4 °C, temperature-dependent MET and gonad lipids. The dotted line separates the clam populations of the estuary (left side) from those of the fjord (right side).

with no significant sex-based effects (two-factor ANOVA $p<0.001$ for site and $p>0.1$ for gender). In the estuary, the condition factor decreased 1.6-fold at the TAD and AL sites and 1.4-fold at BSC. In the fjord, the condition factor decreased 1.6-fold at ASJ, with moderate drops (1.2–1.3-fold) at sites BAR and AE. No significant changes were observed in clams collected at the PS and BE sites. Although the mean age values were fairly constant, given the biased selection of clams during sampling (clams > 4 years were sampled), significant differences were nevertheless observed spatially (two-factor ANOVA: $p<0.001$ for sites and $p>0.1$ for gender). In the estuarine area, clams from site AL were somewhat significantly older (1.12-fold relative to BAU). In the Saguenay Fjord, the mean age value at the reference site ASE was 4.6 years and clams were generally older at the polluted sites relative to site ASE, ranging from a 1.2- to a 2-fold increase in mean age values. The growth index varied significantly spatially, with a significant interaction between site and gender (two-way ANOVA: $p<0.001$ for sites, $p>0.1$ gender, $p<0.01$ gender-site interaction). The growth index tended to fall at both the estuary and fjord areas, where females were occasionally more affected than males at some sites (TAD and AL). Growth was more strongly correlated with age ($r=-0.7$; $p<0.001$), corroborating the obvious: that growth was more important in younger individuals. Condition factor was also marginally correlated with residual clam density ($r=0.67$; $p=0.07$) and total residual clam estimates ($r=0.64$; $p=0.08$).

3.4. Gametogenesis activity

Gamete activity in clams was studied by tracking GSI, maturation index and Vtg-like proteins (Fig. 3). GSI varied only by site, with no influence for gender (two-way ANOVA; $p<0.001$, gender $p>0.1$). In the estuary, the GSI dropped 2.9-fold at TAD, 2.2-fold at BSC and 1.2-fold at AL. In the Saguenay Fjord, the GSI dropped significantly 1.3-fold at ASJ and increased 1.4-fold at the AE site. The gonad maturation index varied significantly by site, gender and site-gender interaction (two-way ANOVA: $p<0.001$ with all factors). The maturation index in females was significantly higher than in males. In the estuary, the maturation index was significantly affected at TAD, but only where maturation increased in females with a concomitant decrease in maturation in males relative to the BAU site. In the Saguenay Fjord, however, the maturation index was significantly lower for both sexes at sites PS, ASJ, BE and AE, where female gonads were more mature than males'. The levels of Vtg-like proteins, the egg-yolk precursor, changed significantly by site and marginally so by gender (two-way ANOVA: $p<0.001$ for site and $p=0.1$ for gender). At sites with little or no

Table 3
Trend analysis between population metrics and biomarkers

Population metric	Biomarker	Rank correlation (R; p)
Live clam density	Total clam number	0.95; 0.002
	Number empty shells	0.61; 0.06
	Age	0.73; 0.02
–	MET 20 °C	0.60; 0.08
Residual clam density		0.86; 0.01
	LPO gonad	0.60; 0.08
–	Hemocyte viability	−0.67; 0.03
Residual clam density		−0.95; 0.001
–	MET_T	0.55; 0.09
Residual clam density		0.74; 0.03
–	DNA damage gonad	0.64; 0.08
Residual clam density		0.86; 0.006
Residual clam density	Condition factor	0.78; 0.02
	Phagocytic activity	−0.87; 0.005
Total clam number	Number of empty shells	0.86; <0.01
	GST	0.62; 0.1
	MET_T	0.76; 0.03
	Age	−0.83; 0.01
	Gonad maturation	0.68; 0.06
	MET 20 °C	0.62; 0.1
	LPO gonad	0.62; 0.1
	Condition factor	0.7; 0.05
Residual total clam number	Phagocytic activity	0.74; 0.03
	MET 4 °C	0.90; 0.002
	Condition factor	0.64; 0.08
	GSI	0.69; 0.06
Age	Growth index	−0.84; 0.002
	Gonad maturation	−0.27; $p<0.001$
	Vtg-like proteins	0.52; $p=0.1$
Growth index	Gonad maturation	0.31; $p<0.001$
	Phagocytosis index	0.55; 0.09
	GST	−0.45; <0.001
	Condition factor	0.19; $p=0.001$
	Phagocytic capacity	0.2; 0.05
	DNA gonad	0.27; 0.04

pollution exposure, females contained more alkali-labile phosphates than males, but this was not the case at the polluted sites. In the estuary, levels of Vtg-like proteins were significantly decreased 1.9-fold at the TAD and BSC sites and 1.4-fold at site AL sites. In the Saguenay Fjord, Vtg-like proteins were increased 1.3-, 1.2-, 1.7- and 1.3-fold at sites BAR, ASJ, BE and AE, respectively. A correlation analysis revealed that the GSI was significantly related with the proportion of live clams ($r=0.74$; $p=0.04$) and with condition factor ($r=0.6$; $p=0.04$). Gonad maturation was significantly correlated with organotin levels ($r=0.79$; $p=0.01$).

3.5. Bioenergetics characterization in feral clam location

Energy status was examined in *Mya arenaria* clams by measuring mitochondrial electron transport (MET) at cold (4 °C) and warm (20 °C) temperatures and total lipid levels in gonad (Fig. 4). MET at warm temperatures varied significantly by site, with no gender-based influence (two-way ANOVA: $p<0.001$ for sites and $p>0.1$ for gender). In the estuary, MET was readily increased at polluted sites, with 4.3-, 3.5- and 1.9-fold increases at the BSC, TAD and AL sites. In the Saguenay Fjord, MET activity at warm temperatures was increased 3- and 2-fold at sites ASJ and BAR, respectively. MET activity at cold temperatures also varied significantly by site, with no significant effect for gender (two-way ANOVA: $p<0.001$ for sites and $p>0.1$ for sex). In the estuary, MET activity at cold temperatures was readily increased 2.2- and 2-fold for the TAD and AL sites, respectively. In the fjord, MET activity was significantly induced 1.7-fold at ASJ and BE sites. There were spatial changes in total gonadal lipids with no gender-based effects (two-way ANOVA: $p<0.001$ for sites and $p>0.1$ for gender). In the estuary, gonadal lipid levels were significantly increased 3-fold at the TAD and BSC sites. In the fjord, gonadal lipid levels were up 1.8-, 1.7- and 1.6-fold at the ASJ, BE and PS sites, respectively. Temperature-dependent MET (MET_T) was also examined in an attempt to understand the interaction of pollution with temperature (climate change). MET_T changed significantly by site only (two-way ANOVA: $p<0.001$ for site and $p>0.1$ for gender). In the estuary, MET_T was readily increased 5.2- and 3-fold at the BSC and TAD sites, respectively. In the fjord, MET_T activity was increased 7-, 3.5- and 2.5-fold at the ASJ, BAR and AE sites, suggesting increased susceptibility of energy expenditure with changes in temperature at polluted sites. Correlation analyses revealed that MET was correlated with residual clam density ($r=0.86$; $p<0.01$), gonad maturation ($r=0.73$; $p=0.015$), lipids ($r=0.67$; $p=0.03$), MET_T ($r=0.96$; $p<0.001$), and gonadal lipids ($r=0.67$; $p=0.03$) at warm temperatures. MET at cold temperatures was significantly correlated with total residual clam density ($r=0.90$; $p<0.01$), condition factor ($r=0.66$; $p=0.04$), and GSI ($r=0.68$; $p=0.03$). MET_T was significantly correlated with residual clam density ($r=0.74$; $p=0.04$), gonad maturation ($r=0.72$; $p=0.02$), and total lipids ($r=0.64$; $p=0.05$). Total gonadal lipid was significantly correlated with residual clam density ($r=0.81$; $p=0.015$).

Table 4
Canonical analysis of biomarker data and population characteristics

	Population	Morphology	Reproduction	Immunocompetence	Energy status	Toxic stress	Temperature dependent energy
Population	1	$r=0.927$; $p=0.23$	$r=0.945$; $p=0.19$	**$r=0.997$; $p=0.007$**	**$r=0.999$; $p<0.001$**	$r=0.958$; $p=0.354$	**$r=0.90$; $p=0.05$**
Morphology		1	$r=0.933$; $p=0.48$	$r=0.961$; $p=0.28$	$r=0.982$; $p=0.14$	$r=0.912$; $p=0.29$	$r=0.56$; $p=0.64$
Reproduction			1	**$r=0.971$; $p=0.07$**	$r=0.837$; $p=0.54$	**$r=0.972$; $p=0.15$**	$r=0.70$; $p=0.43$
Immunocompetence				1	**$r=0.992$; $p=0.02$**	$r=0.915$; $p=0.34$	$r=0.81$; $p=0.18$
Energy status					1	**$r=0.986$ $p=0.07$**	**$r=0.997$; $p<0.001$**
Toxic stress						1	$r=0.836$; $p=0.14$
Temperature-dependent energy							1

Population: clam density, live clams, age; morphology: CF, growth, sex ratio; reproduction: Vtg, GSI, gonad maturation; immunocompetence: phagocytosis, hemocyte number, viability; energy status: MET 20 °C, MET 4 °C, lipid; Toxic stress: LPO, DNA strand breaks and GST. Groups of biomarkers with $p<0.1$ are shown in **boldface**.

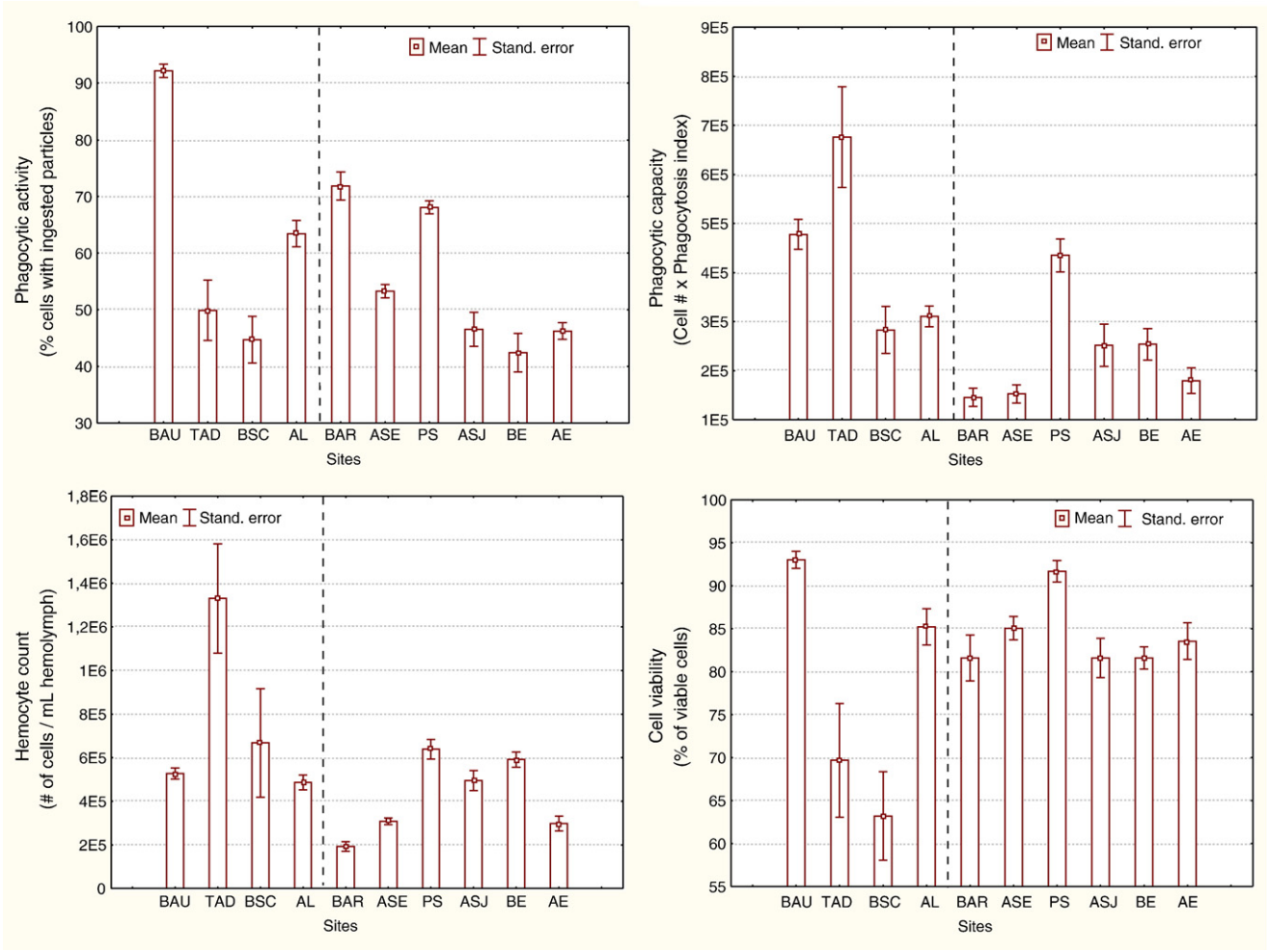

Fig. 6. Alteration of immunocompentence in peripheral clam hemocytes. Clams were collected during low tide and the immune function determined in the hemolymph on the day of sampling using a portable flow cytometer. Hemolymph was analyzed for phagocytosis of latex beads, cell number and cell viability. The dotted line separates the clam populations of the estuary (left side) from those of the fjord (right side).

3.6. Toxic stress biomarker responses

Biomarkers of stress were examined in *Mya arenaria* clams by determining GST activity in the digestive gland, LPO and DNA damage in the gonad (Fig. 6). GST activity varied significantly by site, but not with gender (two-way ANOVA: $p<0.001$ for site and $p>0.1$ for gender). In the estuary, GST was significantly induced 1.3-fold at the TAD site only. In the Saguenay Fjord, GST activity was significantly induced 1.7-, 1.5-, 2.7-, 1.5-, and 1.6-fold at the BAR, PS, ASJ, BE and AE sites, respectively. LPO in the gonad was significantly correlated with total clam density ($r=0.62$; $p=0.05$), organotin levels in tissues ($r=0.72$; $p=0.03$) and phagocytic capacity ($r=0.60$; $p=0.05$). DNA strand breaks in the gonad were significantly correlated with distance-corrected clam density ($r=0.86$; $p<0.01$), condition factor ($r=-0.76$; $p=0.01$), MET at 20 °C ($r=0.77$; $p<0.01$), residual MET at 4 °C ($r=0.65$; $p=0.04$), MET_T ($r=0.65$; $p<0.01$), lipids ($r=0.7$; $p=0.02$) and hemocyte viability ($r=-0.81$; $p<0.01$).

3.7. Immunocompetence of feral clams

The impact of pollution on the immune system of bivalves was also examined (Fig. 5). The percentage of hemocytes that had ingested fluorescent particles (phagocytic activity) changed significantly across the sites when compared to the respective control sites, with no influence for gender (two-way ANOVA: $p<0.001$ for site and $p>0.1$ for gender). In the estuary, phagocytic activity decreased significantly by 1.8-, 2- and 1.4-fold at sites TAD, BSC and AL, respectively relative to the reference BAU site. In the Saguenay Fjord, phagocytic activity declined significantly at site BE, while it increased 1.4- and 1.3-fold at the BAR and PS sites, respectively relative to the reference site ASE. The number of hemocytes was also determined in the hemolymph and varied significantly by site, with a marginal difference for gender, females having somewhat less hemocytes than males (two-way ANOVA: $p<0.001$ for site and $p=0.08$ for gender). In the estuary, the number of hemocytes was significantly increased 2.6-fold at the TAD site. In the estuary, hemocyte numbers rose significantly 2-, 1.4- and 1.3-fold at the PS, BE and ASJ sites, respectively. Cell viability also varied significantly across the sites with no effects for gender (two-way ANOVA: $p<0.001$ for site and $p>0.1$ for gender). In the estuary, cell viability decreased significantly 1.3- and 1.5-fold at the TAD and BSC sites, respectively. In the Saguenay Fjord, no significant changes were observed relative to the reference site ASE. Phagocytic capacity (i.e. number of hemocytes with phagocytic activity) was also examined and it was found that sites were the major factor (two-way ANOVA: $p<0.001$ for sites and $p>0.1$ for gender). In the estuary, the capacity was increased 1.4-fold at TAD while the capacity decreased 1.6- and

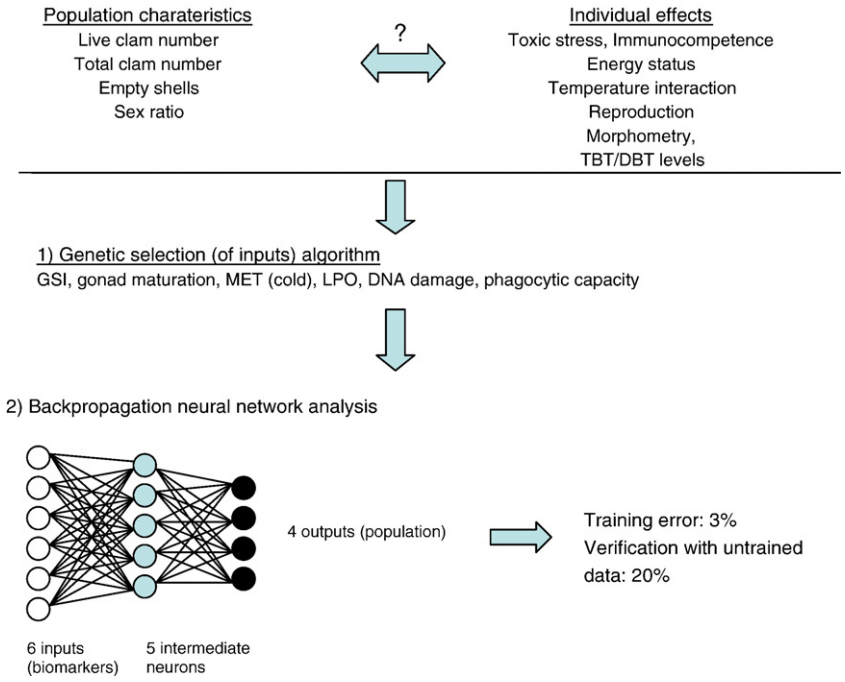

Fig. 7. Ecosystemic effect analysis in *Mya arenaria* clams with artificial neural network analysis. The population parameters (clam number, live and dead clam number, sex ratio) was studied in relation to biomarkers of effects at the individual (condition factor, age, growth), immunocompetence, energy status, toxic stress, reproduction and TBT/DBT contamination levels. After selecting for biomarkers using a genetic selection algorithm, a neural network composed of the six inputs (selected biomarkers) and four outputs (population parameters) was trained using the backpropagation learning paradigm.

1.5-fold at sites BSC and AL, respectively. In the Saguenay Fjord area, only PS displayed a significant increase in phagocytic capacity. Phagocytic activity was significantly correlated with residual clam density ($r=-0.8$; $p<0.01$), total clam density ($r=-0.74$; $p=0.04$), condition factor ($r=-0.64$; $p=0.05$) and hemocyte viability ($r=0.71$; $p=0.02$). Hemocyte counts were significantly correlated with gonadal lipids ($r=0.78$; $p<0.01$) and phagocytic capacity ($r=0.67$; $p=0.03$). Hemocyte viability was significantly correlated with residual clam density ($r=-0.95$; $p<0.001$), condition factor ($r=-0.66$; $p=0.04$), MET at 20 °C ($r=-0.89$; $p<0.01$), MET_T ($r=-0.82$; $p<0.01$), gonadal lipids ($r=-0.73$; $p=0.015$), DNA strand breaks ($r=-0.81$; $p<0.01$) and phagocytic activity ($r=0.71$; $p=0.03$).

3.8. Relationships between population characteristics and biomarker responses

In an attempt to examine the relationships between clam population metrics, both rank and canonical trend analyses were undertaken (Table 3). Residuals were calculated for parameters that followed a significant trend with distance (i.e. live and total clam density estimates). Clam density was significantly correlated with total clam number ($r=0.95$; $p<0.01$), age ($r=-0.73$; $p<0.05$), MET at 20 °C ($r=0.6$; $p=0.08$ marginal), LPO in the gonad ($r=0.6$; $p=0.08$ marginal), hemocyte viability ($r=0.67$; $p=0.03$), MET_T ($r=0.55$; $p=0.09$), and DNA strand breaks ($r=0.64$; $p=0.008$) (Table 3). Residual clam density (clam density not related to distance) was correlated with MET at 20 °C ($r=0.86$; $p=0.01$), hemocyte viability ($r=-0.95$; $p=0.001$), MET_T ($r=0.74$; $p=0.03$), DNA strand breaks ($r=0.86$; $p<0.01$), condition factor ($r=0.78$; $p=0.02$), and phagocytic activity ($r=-0.87$; $p<0.01$). Groups of increasing physiological complexity were sorted based on the various biomarkers (Table 4). Groups representing toxic stress (GST, LPO and DNA damage in the gonad), immunocompetence (phagocytic activity/capacity, hemocyte concentration and viability), energy status (MET at 20 °C, 4 °C and lipid reserves), temperature-dependent MET, reproduction (GSI, gonadal maturation index and Vtg-like proteins), morphological characteristics (condition factor, growth index and sex ratio) and population characteristics (live clam density, number of live and dead clams and age) were examined by canonical analysis. The groups of biomarkers more closely related to population changes were immunocompetence, followed by energy status and temperature-dependent MET changes. Morphological characteristics were more strongly associated with energy status, while reproduction was significantly associated with immunocompetence and toxic stress. An artificial neural network analysis revealed similar observations at the ten study sites (Fig. 6). Genetic selection of biomarkers revealed that the following biomarkers were optimal for population characterization: GSI, gonad maturation, MET (at 4 °C), gonadal LPO, DNA damage and phagocytic capacity. A back propagation analysis revealed that the trained model predicted the population parameters with an error of 3%. Testing this algorithm with randomly selected sites revealed a prediction error between 20 to 30%, suggesting that population metrics were fairly well predicted with biomarker data on immune systems, energy status, toxic stress and reproduction using a non-linear modeling approach. Examination of the input neural activations revealed that phagocytic capacity, DNA strand breaks and gonad maturation had the strongest activation potential across all sites (i.e. these inputs were the major drivers in the transformation of inputs into output signals)(see Fig. 7).

4. Discussion

The integrity of clam populations in ecosystems requires a multifactorial (and multidisciplinary) approach based on the

weight of evidence comprising physical, water/sediment quality, and biological characteristics of habitats. Population condition also requires information on individual health status based on survival (mortality) and reproduction. Field ecotoxicity studies based on a biomarker approach permit measurement of the impact of environmental stressors (i.e. anthropogenic activity) and follow the evolution of the ecosystem toward degradation or rehabilitation (Vasseur and Cossu-Leguile, 2003). Ecosystem health can be examined at the clam population level by the complementary measurement of population metrics such as clam density, the number of dead shells, age, and sex ratio. In the present study, clam density followed an upstream to downstream (salinity) gradient characteristic to estuarine areas, where clam populations close to the St. Lawrence Estuary, at the mouth of the Saguenay Fjord, displayed higher density values than those farther upstream at lower salinity. The impacts of anthropogenic activity (pollution) were apparent when clam density values were corrected against distance effects (i.e. residuals). Indeed, the residual clam density value was significantly lower at the polluted sites BSC, BAR, TAD, ASJ, BE and AE compared to lesser-impacted sites such as ASE, BAU, AL and PS. However, linking early biochemical toxic interactions to toxicity leading to long-term population changes is a challenge. Indeed, many confounding factors in "natural" environments complicate our efforts to circumscribe the impacts of pollution to population changes (Vasseur and Cossu-Leguile, 2006). Population dynamics are influenced not only by pollution-mediated (anthropogenic) effects, but by other factors such as physical habitat and chemical characteristics, prey-predation pressures, and climate change.

Predation represents a major factor that could influence intertidal clam bed density in addition to salinity gradient in the estuary distance as inferred with the effects mediated by distance (Smee and Weissburg, 2006). Perceptive, clams are able to reduce their feeding behavior (pumping) in the presence of predators. It was shown that clam survival was higher in areas containing a caged predator, suggesting that predator-induced alterations in feeding behavior reduce clam mortality in the field. Because the dietary intake of toxicants represents a major exposure pathway, sites under prey-predation pressures could have different sensitivities to environmental contaminants. In the present study, the number of empty shells was positively correlated with GSI and total clam number and marginally so with the number of living clams. This suggests that predation is associated with sites having higher clam densities (higher survival), with energy-rich gonad in areas with low anthropogenic disturbances. Condition factor was marginally related to the number of empty shells, suggesting that a predation-induced decrease in feeding was not a major factor in these ecosystems. Flow was also shown to influence the chemoperceptive ability of consumer and prey aptitudes to detect predators. However, flows do not change much throughout the area, these being intertidal clam populations. Parasitism was also shown to influence the burrowing behavior of clams (Edelaar et al., 2003). The bivalve *Macona balthica* burrowed less when infected by the trematode *Parvatrema affinis*. Less burrowing has been attributed to energy competition between host and parasite, increasing the likelihood of predation by birds, the parasite's final host. Parasitized individuals (trematodes at least) were seldom identified in gonad smears (<2% incidence when observed at some sites). The highest number of empty shells was observed at sites that were relatively less impacted by pollution (ASE, BAU, PS) and also at one polluted site (TAD). The number of dead animals seems to be less related to parasitism than to site attractiveness due to higher condition factors, GSI (more nutritive value) and more accessibility (less anthropogenic disturbances). Future work should characterize infection rates and histopathology changes related to infectious diseases in these clam populations. Another study showed that the loss of lectin-like molecules from the serum of systematically infected animals by *Haplosporidium nelsoni* and the increase in lectin receptors in hemocytes was related to changes in hemocyte composition and loss of serum glycoproteins in diseased animals (Kanaley and Ford, 1990). In the estuary, decreased phagocytic activity was observed at impacted sites, while both decreased and increased phagocytosis was observed at the Saguenay Fjord sites. Moreover, these responses were independent of hemocyte counts, indicating that the increase in hemocyte numbers in diseased animals (i.e. loss of lectins for the agglutination of hemocytes) did not seem to be related to the immune response.

Bivalve population changes should also depend on large-scale processes such as global warming. Inconsistent changes in biomass and bed thickness in local mussel populations have suggested that declines are associated with such global processes (Smith et al., 2006). This finding was supported in the present study by the observed changes in climate characteristics along the California coastline. In the present study, MET_T activity was significantly correlated with residual clam density, total clam number indicating that increased temperature dependence in MET was associated with increased clam densities. MET_T was also negatively correlated with hemocyte viability and positively correlated with DNA damage in the gonad, gonadal lipids and gonad maturation index. The latter raises the issue of genetic alterations in the population over multiple generations. The negative effects of MET_T on hemocyte viability were corroborated elsewhere (Monari et al., 2007). Indeed, it has been found that clams burrow less when maintained at 30 °C compared to 20 or 25 °C. The high temperature significantly increased total hemocyte counts whereas it reduced phagocytic activity. In the Saguenay Fjord, the incoming warmer influent from the rivers stays at the surface where intertidal clams are located. The increase in water temperature with pollution seems less likely since the observed decrease in phagocytosis and increase in hemocyte count were somewhat similar in the Saguenay fjord and the estuarine sites. Resistance to air exposure time was also reduced from 6 days to 4 days in clams kept at 30 °C compared to those held at 20 or 25 °C. The positive relationship of MET_T with lipid energy stores ($r=0.64$; $p=0.05$) indicates an interaction between temperature adaptation and energy reserves and expenditures. Lipid production is physiologically assisted at the molecular level by the so-called lipogenic enzymes (malate dehydrogenase, malate enzyme, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase), which produce reduced NADH and NADPH to support lipid and steroid synthesis.

Malate dehydrogenase (MDH) was shown to be temperature-sensitive where this property could confer success for an invasive species (Fields et al., 2006). MDH from the cold-adapted *Mytilus trossolus* differed from the heat-tolerant MDH of *Mytilus galloprovincialis* by its temperature-sensitive and lower enzymatic affinity for the cofactor NADH. Hence, adaptation to cold temperatures would result in NADH production to support lipid production. This study represents the first evidence that some local *Mya arenaria* clam populations are possibly adapting to warm temperatures (the ASJ and BAR sites) while others (sites BSC and TAD) remained better adapted to cold temperatures: the cold adapted clams would have more lipid reserves relative to MET activity. The most temperature-sensitive sites being the polluted sites (in decreasing order of MET_T): BSC > ASJ > TAD > BAR.

The degradation of water quality stemming from anthropogenic activity also represents a contributing stressor to loss of population density and age structure. Hemocyte viability and phagocytic activity were the physiological markers most strongly associated with clam density. Moreover, phagocytic activity was negatively correlated with condition factor and directly related to Vtg-like proteins. Increased phagocytic activity and decreased hemocyte membrane permeability (i.e. high viability index) were significantly associated with lower clam density (before and after correction for distance), indicating that sustained immunological responses and maintenance of hemocyte integrity were associated with lower clam densities. This suggests that long-term clam survival is lower in situations of sustained immunoactivity and hemocyte cell membrane integrity. Besides its physiological role as a source of energy, Vtg was recently shown to have a role in defense mechanisms against disease, at least in fish (Shi et al., 2006). Vtg purified from the rosy barb possessed both antibacterial and hemagglutinating activities *in vitro* where male fish challenged with *E. coli* synthesized Vtg. However, whether this immunological role for Vtg holds true in bivalves needs further examination in invertebrates, although both hemagglutinating and antibacterial activities were identified in bivalve hemolymph (Olafsen, 1995) and perhaps Vtg contributes to these humoral characteristics. Estradiol-17β, which is implicated in vitellogenesis, was shown to decrease phagocytic activity in *Mya arenaria* injected intramuscularly with 10, 20 and 50 nmol of estradiol-β (Gauthier-Clerc et al., 2006). In the present study, while phagocytic activity was significantly correlated with Vtg-like proteins in gonad ($r=0.62$; $p=0.05$), analysis of covariance of phagocytosis with Vtg-like proteins as the covariate revealed that site-specific effects were more significant than egg-yolk protein levels ($p=0.15$ for Vtg and $p<0.001$ for site-specific effects), suggesting that the effects of estradiol-17β were not a major factor in *Mya arenaria* during late gametogenesis. However, phagocytic activity was inhibited 75% of the time at polluted sites, with phagocytic activity being significantly induced at the PS and BAR sites. Both mercury and organotin compounds were shown to have immunotoxic effects in bivalves (Fournier et al., 2001). Phagocytic activity of hemocytes declined in clams exposed to 1 μM of $HgCl_2$ for 28 days. In another study on clams exposed to contaminants for 12 weeks, suppression of phagocytic activity was observed and this response was significantly correlated with the body burden of polychlorinated biphenyls (Fournier et al., 2002). These studies suggest that contaminant alone seems not to always explain the observed effects in the field. Although temperature-related effects seem a major contributor as well, salinity was shown to reduce cell viability in the present study and this was also found by others (Gagnaire et al., 2006). Salinity at the lower and upper range (i.e., 15 and 50 ppt) increased phagocytosis activity while lowering hemocyte viability.

The strong and negative relationships between clam density with either hemocyte viability or phagocytosis, and energy metabolisms (MET, MET_T and total lipids) indicate that sustained immunostimulation depletes energy stores while reducing temperature-dependent energy expenditures. Energy budgets derived from MET and cellular energy reserves (glycogen and lipids) were the most promising endpoints to extrapolate cellular effects to higher levels of biological organization in zebra mussels exposed to a pollution gradient (Smolders et al., 2004). In daphnia reared in the presence of cadmium for two weeks, reduced body size and scope for growth were observed, while an increase in mass-specific scope for growth in the food ration group suggests that cadmium exposure leads not only to reduced energy assimilation, but to energy expenditure as well (Baillieul et al., 2005). In a transplanted *Pyganodon grandis* mussel population in lakes contaminated with cadmium from mining activity, reduced survival was associated with less cadmium sequestration in the gill cytosol by metallothionein-like proteins (Perceval et al., 2006). Lower survival was consistent with the absence of indigenous mussel populations at these sites, indicating that excessive accumulation of cadmium in the high-molecular-weight pool of the gill cytosol of individuals was related to the impairment of population health status. In the present study, clams were contaminated by organotin compounds, which were correlated with oxidative stress and gonad maturation. Maintaining energy expenditure with those of lipid reserves could reveal an underlying (perhaps genetic) acclimation mechanism in local populations. For example, while clams at the BSC and TAD sites had increased MET and lipids in the gonad, those from the BAR and ASJ sites had less lipid reserves and high, albeit with less intensity, MET activity. An *in situ* experiment on *Daphnia magna* exposed to a metallurgical effluent revealed that organisms exposed downstream of the effluent source had lower energy metabolisms than those at the upstream site (De Coen et al., 2006). These changes in biochemical processes suggest a selective advantage to coping with additional environmental stressors. Indeed, reduced energy metabolism could reduce the production of reaction oxygen species (leading to genotoxicity) by contaminants in mitochondria and prevent tissue damage but at the cost of reduced lipid reserves. These effects of the industrial discharge were perhaps associated with a reduction in long-term survival. However, DNA damage in the gonad was not associated with lower clam numbers in the field suggesting that DNA strand breaks was not a major contributor to intertidal bed clam density.

The multi-biomarker approach in this study permitted the examination of relationships between population characteristics and biomarker data. Canonical and artificial neural network analyses revealed some degree of hierarchy with the biomarker

data relative to population changes. Indeed, population metrics were more strongly associated, in decreasing order, with immunocompetence, energy status and temperature-dependent MET. Toxic stress was, in turn, more strongly correlated with reproduction and energy status. Energy status, as defined by cellular energy expenditure (MET) and energy reserves (gonadal lipids), was the physiological endpoint that predicted most changes at the organism such as survival (Smolders et al., 2004) and population levels (De Coen and Janssen, 2003). Furthermore, non-linear modeling of the biomarker data to predict changes at the population level revealed that the following biomarkers were the most predictive: phagocytic capacity (immunocompetence), MET at 4 °C (energy status), GSI and gonad maturation for reproduction and gonadal LPO and DNA damage for toxic stress. This is the first report that sustained immunoactivity, in addition to cellular energetics, could be a predictor of altered effects in individuals and populations of intertidal *Mya arenaria* clam populations. Toxic stress appears to affect energy status before acting at the population level in the Saguenay Fjord.

Acknowledgments

The authors are grateful for the technical assistance of Sophie Trépanier, Brian Walker, Sylvain Trottier and Chad Boyko from the Aquatic Ecosystem Protection Research Division, Environment Canada. This project was funded by Environment Canada under the St. Lawrence Action Plan.

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