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Energy and Antioxidant Responses of Pacific Oyster Exposed to Trace Levels of Pesticides

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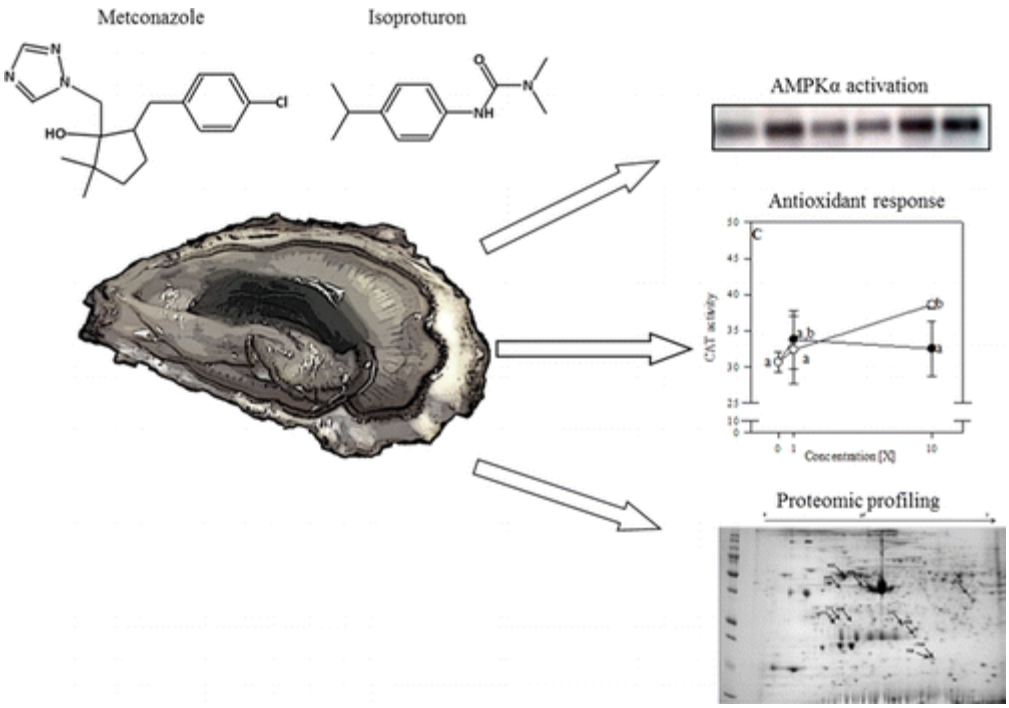
Abstract :

Here, we assess the physiological effects induced by environmental concentrations of pesticides in Pacific oyster Crassostrea gigas. Oysters were exposed for 14 d to trace levels of metconazole (0.2 and 2 mu g/L), isoproturon (0.1 and 1 mu g/L), or both in a mixture (0.2 and 0.1 mu g/L, respectively). Exposure to trace levels of pesticides had no effect on the filtration rate, growth, and energy reserves of oysters. However, oysters exposed to metconazole and isoproturon showed an overactivation of the sensing-kinase AMP-activated protein kinase alpha (AMPK alpha), a key enzyme involved in energy metabolism and more particularly glycolysis. In the meantime, these exposed oysters showed a decrease in hexokinase and pyruvate kinase activities, whereas 2-DE proteomic revealed that fructose-1,6-bisphosphatase (F-1,6-BP), a key enzyme of gluconeogenesis, was upregulated. Activities of antioxidant enzymes were higher in oysters exposed to the highest pesticide concentrations. Both pesticides enhanced the superoxide dismutase activity of oysters. Isoproturon enhanced catalase activity, and metconazole enhanced peroxiredoxin activity. Overall, our results show that environmental concentrations of metconazole or isoproturon induced subtle changes in the energy and antioxidant metabolisms of oysters.

Graphical abstract



INTRODUCTION

Coasts and estuaries are often anthropic environments[1] where pesticides can reach non-target aquatic organisms, such as marine bivalves.[2] The Pacific oyster *Crassostrea gigas* is a benthic suspension feeder widely distributed all over the world. Oysters, like other bivalves, bioaccumulate contaminants in their soft tissues, so that they are often used as sentinel animals in the marine environment.

Only few studies have investigated the effect of environmental concentration of pesticides on the physiological responses of oysters. For instance, oysters exposed to trace levels of pesticides, alone or mixed with other contaminants, could exhibit growth reduction,[3] partial spawning[4] and developmental anomaly.[5,6] At the cell level, hemocytes of oysters exposed to pesticides show a reduced phagocytosis which may impair their immune response[7,8]. At the molecule level, oysters exposed to low concentrations of pesticides present differences in gene expression related to energy production, immune system and xenobiotics detoxification.[9,10,11]

The aim of this study is to determine the metabolic response of oysters exposed to trace levels of commonly used pesticides. We analyzed the whole organism (filtration rate, growth and energetic reserves) and molecular responses (targeted and global proteomic) of oysters. This study focused on metconazole and isoproturon, two pesticides that are detected in surface water along the French coasts.

Metconazole is a fungicide that inhibits the biosynthesis of ergosterol, which is an essential component of cell membranes in fungi. This fungicide has been used for nearly 20 years as a growth regulator and protects cereals against foliar diseases.[12] Isoproturon is an herbicide belonging to the family of substituted urea, which acts as an inhibitor of photosynthesis. It is one of the most extensively used agricultural pesticides in Europe.[13]

The novelty of our work is that we particularly investigate the effects of environmentally relevant concentrations of pesticides on 5' AMP-activated protein kinase (AMPK) and its related glycolytic enzymes, hexokinase and pyruvate kinase. This protein kinase has a catalytic activity that is regulated through phosphorylation at Thr 172 and is involved in energy-sensing of the cell. It is very well conserved across the eukaryotic kingdom.[14] In all species, AMPKα is a Ser/Thr protein kinase that plays a central role in energy homeostasis[15] by activating ATP-producing catabolic pathways such as lipolysis, glycolysis and glucose uptake and deactivates ATP-consuming anabolic pathways such as glycogen synthesis and lipogenesis by modulation of downstream targets.[16]

Also, we particularly looked at antioxidant defense systems and fatty acid composition as potential indicators of oxidative stress and lipid peroxidation.[17]

EXPERIMENTAL PROCEDURES

Animals

Experimental oysters were produced as previously described.[18] Briefly, forty adults from wild oyster population were transferred to a grow-out farm and then moved on January 8th 2013 to the Ifremer facilities in Argenton (Brittany, France) for conditioning. These animals were held in 500-L flow-through tanks with seawater at 16 °C enriched with a phytoplankton mixture. Once the oysters were reproductively mature, gametes from 40 individuals (1/3 males, 2/3 females), obtained by stripping, were mixed. Fertilization and larval rearing were conducted as previously described.[18] When oysters were > 2 mm shell length on April 11th 2013, they were transferred to the Ifremer nursery in Bouin (Vendée, France) and then transferred back to Argenton on mid-June 2013. At the beginning of the experiment, around 7000 four months-old oysters (weight: 0.72±0.02 g) were distributed in 18 tanks (287g of oysters per tank). Here we intentionally used young oysters to limit the likely confounding effect of reproductive stages and other life history traits such as rearing conditions. The tanks used for the

experiment were 800 mL Artemio® Breeding sets (JBL) with an open flow-through seawater system. No mortality was recorded in our experiment.

Experimental design

Pesticides used in this experiment were purchased from Pestanal® (Sigma-Aldrich). Exposure was done during 14 days under experimental concentrations of metconazole (M) and isoproturon (I). Metconazole was used at 1X (0.2 µg/L, 625 µM) or 10X (2 µg/L, 6.25 mM). Isoproturon was used at 1X (0.1 µg/L, 485 µM) or 10X (1 µg/L, 4.85 mM). In the mixture, metconazole was 1X and isoproturon 1X. All oysters were fed *ad libitum* with a daily mixed diet that consisted of *Isochrysis affinis galbana* (T-ISO) and *Chaetoceros gracilis* (1:1 in dry weight). Seawater was treated with UV radiation, filtered at 1 µm, maintained at a temperature of 20±1°C and oxygen saturation of 75±10% (5.54 mg/L of seawater). The flow rate of 20 L/h took into account the expected concentration of each pesticide in tanks. Three tanks were used for each condition: control oysters in seawater (C), metconazole 1X, metconazole 10X, isoproturon 1X, isoproturon 10X, and the mixture (M+I at 1X). Two stock solutions of active substance were prepared at room temperature in ultrapure water: metconazole 62.5 M and isoproturon 48.5 M. Experimental concentrations were obtained by dilution of stock solutions using the sea water flow rate and renewed every 48 hours to ensure a continuous supply of pesticides all along the experiment by a peristaltic pump. Water containing pesticides at the outlet was treated by a 1µm bag filter followed by an active carbon filter. At the end of the experiment, one pool of 16 oysters per tank was sampled in the 3 tanks per condition (3 pools per condition). The shell was quickly removed, and flesh was immediately frozen into liquid nitrogen. Pools of flesh were crushed into a fine powder at -196°C with a Dangoumeau mill under liquid nitrogen and kept under liquid nitrogen for further analysis.

Detection of pesticides in sea-water

A 1 L-sample of sea water was collected at the inlet of every 18 tanks at day 1, 7 and 14, then frozen at -20°C until analysis. Detection of metconazole and isoproturon was performed by the Labocéa laboratory (Plouzané, France) as previously described for other pesticides.[3] The method used for metconazole and isoproturon detection was a solid / liquid extraction (SPE: solid phase extraction) automated and coupled to liquid chromatography with detection by tandem mass spectrometry (MS-MS). The detection limit for metconazole and isoproturon was 0.02 µg/L. Assessing the effective concentrations of pesticides in sea water surrounding animals was an important step necessary to validate that exposure to pesticides was done at trace levels concentrations all along the experiment.

Filtration rate and growth

Feeding supply was expressed in phytoplankton cell biovolume ($\mu m^3/\mu L$), by means of an electronic particles counter (Multisizer 3, Beckman Coulter), and a phytoplankton level of 1500 $\mu m^3/\mu L$ was continuously maintained at the outlet of each tank by modulating the seawater flow. Filtration rate (FR) was determined twice a day and expressed in cell volume per oyster per day (μm^3/oyster/day) as $FR=[(Ci-Co)\times D]/n$, where Ci is the cell volume at the inlet, Co is the cell volume at the outlet, D is water flow (L/h) and n is the number of oysters per tank.

Growth was estimated by the ratio weight of each tank/number of oysters at day 1, 5, 9 and 14 of the experiment.

Carbohydrate content

Analysis of carbohydrates was performed as previously described,[19] using 300 mg of flesh powder that was homogenized with a Polytron® PT 2500 E (Kinemetica) at 4°C in 3 mL milliQ water. Samples were then diluted and mixed with a phenol solution (5% m/v) and 2.5 mL of H_2SO_4 and then incubated for 20 min. Carbohydrates were quantified by a colorimetric

assay on an Uvikon 941 (Kontron instruments) at 490 and 600 nm. A standard calibration curve was used to calculate total carbohydrates concentration.

Lipid analysis

Lipids were extracted as previously described,[20] using 300 mg of flesh powder incubated in 6 mL dichloromethane/methanol (2:1, v/v). Neutral lipid classes (triacylglycerol and sterols) were analyzed by high performance thin layer chromatography (HPTLC) with a CAMAG system, consisting of a sampler (TLC Sampler 4) and a reader (TLC Scanner 3). The lipids were placed at the top of a silica gel microcolumn (30×5 mm internal diameter; Kieselgel; 70–230 mesh [Merck, Lyon, France]; previously heated to 450°C and deactivated with 5% water). Neutral lipids were eluted with 10 mL of chloroform/methanol (98:2, v/v) and polar lipids were eluted with 15 mL of methanol and lipid fractions were stored at -20°C under nitrogen before further analyses. A known amount of tricosanoic acid was added as internal standard. Lipids were transesterified at 100 °C for 10 min with 1 mL of boron trifluoride (12% Me-OH) and analyzed as previously described.[21] After cooling and adding 1 mL of hexane and 1 mL of milliQ water, the organic phase containing fatty acid (FA) methyl esters was washed with 1 mL of water. FA methyl ester were analyzed in a HP6890 GC system (Hewlett-Packard). FA methyl esters identification was performed using a DB-Wax capillary column (30 m×0.25 mm; 0.25 μm film thickness; Agilent technologies), by comparison of their retention time with those of a standard 37 component FAME mix and other standards mix from marine bivalves.[22] FA contents were expressed as the mass percentage of total FA content.

Total protein extraction

Total protein extraction was performed using 500 mg of flesh powder that was homogenized with a Polytron® PT 2500 E (Kinemetica) at 4°C in 5 mL of lysis buffer[23] (150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Igepal; pH 8.8 at

4°C) containing phosphatase and protease inhibitors (1% of Phosphatase inhibitor cocktail II [Sigma-Aldrich], 2% of NaPPi 250 mM, and 1 tablet of complete EDTA free protease inhibitor cocktail [Roche] in 25 mL of lysis buffer). Solubilized proteins were extracted by centrifugations at 4,000 g for 1 h at 4°C for elimination of lipids and cellular debris. The phase containing proteins was then collected and centrifuged at 10,000 g for 45 min at 4°C. The resulting lysates were aliquoted and stored at -80°C for further analysis. Total protein content in each lysate was analyzed using the DC protein assay (Bio-Rad), in 96-well microplates (Nunc) using a microplate reader Synergy HT (Bio-Tek). Concentration was obtained using Gen5 version 2.03 software (Bio-Tek).

Hexokinase, pyruvate kinase and citrate synthase activities

Enzymatic activities of hexokinase (HK; EC 2.7.1.1), pyruvate kinase (PK; EC 2.7.1.40)[24] and citrate synthase (CS; EC 2.3.3.1)[25] were measured using 20 µL of total protein lysates. HK assay buffer contains Tris 100 mM pH 8, EDTA 1 mM, $MgCl_2$ 2 mM, glucose 50 mM, ATP 10 mM, NADP 2 mM, glucose-6-phosphate dehydrogenase 10 UI/mL. PK assay buffer contains Imidazole-HCl 200 mM pH 7.2, $MgCl_2$ 5 mM, KCl 50 mM, phosphoenolpyruvate 100 mM, ADP 20 mM, lactate dehydrogenase 1.2 UI/mL. For HK and PK, the increase in NADPH or decrease in NADH, respectively, was monitored by measuring absorbance at 340 nm for 10 min at 25°C using a Synergy® HT microplate reader (BioTek). CS assay buffer contains Tris/HCl 100 mM pH 8, 5,5′-dithio-bis-[2-nitrobenzoic] acid (DNTB) 0.1 mM, acetyl-Coenzyme A 0.2 mM, oxaloacetate 0.5 mM. CS activity is measured by following the increase in TNB absorbance for 10 min at 412 nm using a Synergy® HT microplate reader (BioTek). All enzymatic activities were measured and related to the total protein concentration of each sample.

Superoxide dismutase, catalase, glutathione S-transferase activities

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined using a SOD assay kit (Sigma Aldrich), following manufacturer instruction. SOD activity was measured by adding 200 µL of Water Soluble Tetrazolium salt (WST-1) to 20 µL of total protein lysates 20 times diluted and reaction was initiated by adding 20 µL of xanthine oxidase (XO) and xanthine mix. After incubation at 25 °C for 20 min, absorbance of samples was read at 450 nm using a Synergy® HT microplate reader (BioTek). A standard inhibition curve was performed using SOD from bovine erythrocytes. SOD activity was expressed in units per mg of protein (U/mg), 1 U of SOD being defined as the amount of enzyme inhibiting by 50% the xanthine/XO complex formation. Catalase (CAT; EC 1.11.16)[26] activity was measured using 8 µL of total protein lysates diluted into 792 µL of H_2O_2 solution. Activity was measured for 1 min at 240 nm using a spectrophotometer Uvikon923 (Kontron instruments) by reading the consumption of hydrogen peroxide 10 mM (H_2O_2). Results were expressed in units per mg of protein (U/mg) corresponding to the consumption of 1 µmol of H_2O_2 per min per mg of protein.

Glutathione S-transferase activity (GST; EC 2.5.1.18)[27] was measured using 15 µL of total protein lysates 10 times diluted. GST buffer assay contains 1-chloro-2,4 dinitrobenzene 1 mM (CDNB) and reduced glutathione 1 mM (GSH) as substrates. After a short incubation of 5 min at 25°C, absorbance was read for 3 min at 340 nm at 25°C using a Synergy® HT microplate reader (BioTek). Results were expressed in units per mg protein (U/mg) corresponding to the production of 1 µmol of product per minute.

SDS-PAGE and Western Blot

Total protein lysates were adjusted to 3 mg/mL, mixed with 4X loading buffer containing 5% β-mercaptoethanol and heated for 5 minutes at 95°C. For mono-dimensional electrophoresis, 40 µg of total protein lysate was loaded on 10% Mini-PROTEAN® TGX™ precast

polyacrylamide gel (Biorad). The quantification of AMPKα Thr^{172} phosphorylation was done by immunodetection in Western-blot, using methods already validated in our model.[28] After electrophoresis, proteins were transferred onto a polyvinylidenedifluoride membrane with a Trans-Blot® Turbo™ Transfer Starter System (Biorad). Membranes were washed in PBS-Tween 1‰ (PBS-T), blocked in 5% w/v BSA in PBS-T for 1 h at 37°C, then incubated overnight at 4°C with the rabbit polyclonal antibody AMPKα Thr^{172} phosphorylation (#2535, Cell Signaling Technology; dilution 1:1000).[28] After washing with PBS-T, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (#7074, Cell signaling technology; dilution 1:2500). The immune-reactivity was revealed by using an ECL kit (RPN 2232, GE Healthcare). Bands detected in blots were visualized with enhanced chemi-luminescence detection system G:box (Syngene) and quantification was done using Genetools software v. 4.03 (Syngene). The obtained value is expressed in OD/mm^2, and represents band intensity. To control for identical amounts of total protein loaded onto gels, membranes were dehybridized for 1 h at room temperature in dehybridizing buffer (glycine 100 mM, NaCl 100 mM, pH 3.2) and rehybridized with the rabbit polyclonal antibody histone H3 (#9715, Cell Signaling Technology; dilution 1:5000).

Two-dimensional electrophoresis (2-DE)

Total proteins were extracted using 50 mg of flesh powder homogenized with a pestle in a urea/thiourea buffer[29] containing urea 7 M, thiourea 2 M, DTT 0.1 M, 4% CHAPS, 0.5% IPG buffer (pH 4–7, GE Healthcare), 1% of Phosphatase inhibitor cocktail II [Sigma-Aldrich] and 2% of NaPPi. Samples were sonicated for 15 s and centrifuged at 15,000 g at 4 °C for 30 min. Protein concentrations were determined using a modified Bradford assay[30] and adjusted to 400 µg of proteins in 250 µl of rehydration solution (urea 7 M, DTT 0.1 M, 4% CHAPS, and 0.2% IPG buffer). Samples were loaded onto an immobilized pH gradient (IPG) strip (ImmobilineDryStrip pH 4–7NL, 11 cm, GE healthcare). Three technical replicates were

carried out to ensure 2-DE reproducibility. IPG strips were passively rehydrated overnight with 250 µl of protein sample. Isoelectric focusing was conducted in a Protean IEF electrophoresis system (Biorad) with the following protocol: 100 V for 2 h 30 min, 300 V for 1 h 30 min, gradient voltage increase to 1000 V for 1 h, gradient voltage increase to 8000 V for 6 h, 8000 V for 10 h, and reduced to 500 V. Focused strips were incubated in an equilibration buffer (Tris–HCl 50 mM pH 8.8, urea 6 M, 30% glycerol, 2% SDS and 0.002% Bromophenol Blue) for two 15 min-periods, with 1 g/L dithiothreitol replaced with 48.2 g/L iodoacetamide. For second dimension electrophoresis, each strip was applied to 8-16% Tris-HCl Criterion® precast gel (Biorad) and separated at 100 V for 35 min and 180 V for 65 min. Gels were stained with Coomassie Blue (PhastGel, GE Healthcare), unspecific coloration was destained with an aqueous solution containing 30% methanol and 7% acetic acid. Colored 2-DE gels were scanned with an imaging system G:box (Syngene) for spot detection and relative quantification. Spots intensities were quantified based on normalized spot volumes, using built-in algorithms included in the Progenesis SameSpots software (version 3.3, Nonlinear Dynamics). Automatic detection was performed by the software, but artifact spots were manually checked and removed. Protein spots with statistically significant (P<0.05) fold induction greater than 1.5 were selected for subsequent identification by mass spectrometry.

Mass spectrometry

These spots were washed with milliQ water, destained in NH_4HCO_3 100 mM/acetonitrile (ACN) (1:1), and dehydrated in 100% ACN. After rehydration in NH_4HCO_3 100 mM and dehydration in 100% ACN, excised spots were air-dried and incubated overnight at 37 °C in a solution of 12.5 ng/µL modified trypsin (Promega) in NH_4HCO_3 50 mM. The resulting tryptic peptides were extracted from the gel spots by several washes in formic acid/ACN/water as previously described.[31] The tryptic digests were then concentrated by vacuum centrifugation to reach a final volume of 30 µL. Mobile A (H_2O/formic acid, 100:0.1) and B (ACN/formic

acid, 100:0.1) phases for HPLC were delivered by the Ultimate 3000 nanoflow LC system (Dionex, LC Packings). 10 µL of peptide mixture was loaded on a trapping precolumn (5 mm × 300 µm i.d., 300 Å pore size, Pepmap C18, 5 µm) for 3 min in 2% buffer B at a flow rate of 25 µL/min. This step was followed by reverse-phase separations at a flow rate of 0.250 µL/min using an analytical column (15 cm × 300 µm i.d., 300 Å pore size, Pepmap C18, 5 µm, Dionex, LC Packings). We ran a gradient ranging from 2% to 35% of B for the first 60 min, 35% to 60% of B from min 60-85, and 60% to 90% of B from min 85-105. Finally, the column was washed with 90% of B for 16 min, and with 2% of B for 19 min prior to loading of the next sample. The peptides were detected by directly eluting them from the HPLC column into the nanoelectrospray ion source of the LTQ-Orbitrap XL (ThermoScientific) mass spectrometer. An ESI voltage of 1.4 kV was applied to the HPLC buffer using the liquid junction provided by the nanoelectrospray ion source and the ion transfer tube temperature was set to 200°C. The LTQ-Orbitrap XL instrument was operated in its data-dependent mode by automatically switching between full survey scan MS and consecutive MS/MS acquisition. Survey full scan MS spectra (m/z 400–2000) were acquired in the OrbiTrap with a resolution of r = 60000 at m/z 400; ion injection times are calculated for each spectrum to allow for accumulation of 106 ions in the OrbiTrap. The 10 most intense peptide ions in each survey scan with intensity above 2000 counts and a charge state≥2 were sequentially isolated and fragmented in the ion trap by collision induced dissociation. Normalized collision energy was set to 35% with an activation time of 30 ms. Peaks selected for fragmentation were automatically put on a dynamic exclusion list for 60 s with a mass tolerance of ±10 ppm.

Data processing protocol

MS data were saved in RAW file format (ThermoScientific) using XCalibur 2.0.7 with tune 2.4. The data analysis was performed with the Proteome Discoverer 1.2 software supported by Mascot (Mascot server v2.2.07; http://www.matrixscience.com) database search engines for

peptide and protein identification. MS/MS spectra were compared to the *Crassostrea gigas* Uniprot Reference Proteome database (UP000005408, July 2014, 25978 sequences, 11649621 residues).[32] Mass tolerance for MS and MS/MS was set at 10 ppm and 0.5 Da, respectively. Trypsine was selected as enzyme with one miscleavage allowed. Protein modifications were fixed carbamidomethylation of Cys and variable oxidation of Met. Identified peptides were filtered based on the Mascot score to obtain a false discovery rate of 1%. Only rank 1 peptides were considered. In the case of peptides shared by different proteins, proteins were automatically grouped. The proteins within a group were ranked according to their score and those reported in the protein table correspond to the top score protein.

Statistical analysis

Two-way ANOVA were performed on all biochemical parameters to investigate the potential effects of a 14 day exposure of metconazole or isoproturon at two concentrations (1X, 10X), and the effect of the mixture at 1X concentration compared with control oysters. Where overall differences were detected, Least squares mean (LSMean) multiple comparison test was used to determine which means were significantly different. All these analyses were performed by using SAS software 9.1.3 (SAS institute). Homogeneity of variances was checked and all values with $P<0.05$ were considered statistically significant.

RESULTS

Detection of pesticides

Metconazole and isoproturon were not detected in control tank (Table 1). There was a deviation between intended and achieved concentrations of both pesticides (Table 1). For example, at day 14, concentrations of metconazole and isoproturon 1X were 0.098 µg/L and 0.138 µg/L while we intended 0.200 µg/L and 0.100 µg/L respectively (Table 1).

Filtration rate, growth and energy reserves

Growth and filtration rate of oysters exposed to pesticides were similar to that of control animals (P>0.05). On average, filtration rate was 3.35±0.04×10^{10} μm^3 of cell per animal per day and body mass of oyster increased of 0.82±0.05 g during the 14-day experiment. Energy reserves of oysters as measured as carbohydrates, proteins and the ratio triacylglycerol/sterol were similar between oysters exposed to pesticides and control (Table 2).

Enzyme activities

HK activity was ca. 25% lower in oysters exposed to pesticides at 1X for 14 days compared to that of control animals. However, HK activity in oysters exposed to pesticides at 10X or exposed to the mixture was not significantly different to that of control (Figure 1A, 1B).

PK activity was 34% to 46% lower in oysters exposed to pesticides compared to that of control (Figure 1C, 1D).

Mean CS activity was 27.4±2.7 mU/mg and was similar between control and exposed oysters, regardless of concentration (data not shown).

SOD activity was similar in oysters exposed to pesticides at 1X compared to that of control, but was ca. 16% higher in oysters exposed to pesticides at 10X (Figure 2A). When metconazole and isoproturon 1X were mixed, SOD activity was similar to that of control oysters (Figure 2B).

CAT activity was 26% higher in oysters exposed to isoproturon 10X than in control (Figure 2C, 2D).

GST activity remained similar between control and exposed oysters, regardless of concentration (Figure 2E, 2F).

AMPKα Thr^{172} phosphorylation

Western-blot analysis of AMPK phosphorylation is presented in Figure 3A. Overall, phosphorylation of AMPKα Thr^{172} was 71% to 132% higher in oysters exposed to pesticides

than in control animals (Figure 3B, 3C). There was no significant difference among exposed oysters, regardless of the type and the number of pesticides and their concentrations.

Global proteomic analyses

2-DE proteomic was performed to identify changes in the proteome of oysters exposed for 14 days to metconazole 10X or isoproturon 10X. Analysis by Progenesis Samespots software detected 405 spots among the different replicates of Coomassie stained gels.

Oysters exposed to metconazole and isoproturon 10X exhibited changes in the expression of 9 and 3 spots compared to control oysters corresponding to 8 and 2 different proteins respectively (Figure 4). The protein contained in each spot was identified using tandem mass spectrometry (Table 3). Oysters exposed to metconazole 10X showed up-regulation of 2 proteins related to energy metabolism (ATP synthase subunit β and fructose-1,6-bisphosphatase 1), one protein involved in antioxidant response (peroxiredoxin-5) and one transcription factor (Transcription factor BTF3-like). Oysters exposed to metconazole 10X also showed up-regulation of proteins related to cytoskeleton: severin, actin 2 and thymosin, and down-regulation of Rho GDP-dissociation inhibitor 1. Oysters exposed to isoproturon 10X showed up-regulation of one protein involved in the antioxidant response (14-3-3 protein ζ) and one protein involved in amino-acid biosynthesis (phosphoserine phosphatase).

Fatty acids analysis

All fatty acids (FA) of polar lipids analyzed are presented in table S1. Overall, unsaturation index of FA remained unchanged irrespective of treatments (Figure 5A, 5B). Total dimethylacetals (DMA) of oysters exposed to isoproturon 1X and isoproturon 10X increased by 9 and 8% respectively compared to that of control animals. Total DMA of oysters exposed to metconazole (1X and 10X) and to the mixture of pesticides were similar to that of control animals (Figure 5C, 5D).

DISCUSSION

Energy metabolism

In our study, AMPKα Thr^{172} phosphorylation increased by between 70 and 130% in oyster exposed to metconazole or isoproturon. Therefore, it is likely that the over-activation of AMPK is a generic response to pesticides. This however needs further testing with other families of pesticides.

Over-activation of AMPK in response to pesticides also suggests that the energy metabolism of oysters is disturbed. Accordingly, energy metabolism of mice was disturbed during exposure to low concentrations of pesticides as reflected by disturbance of the protein kinase B (PKB/Akt).[33] Also, in crayfish *Cherax quadricarinatus* exposed to glyphosate, glucose metabolism was disturbed with a decrease of PK activity.[34]

Concomitantly with over-activation of AMPK, HK and PK activities were lower in exposed oysters compared to that of control. This result appears somewhat paradoxical. Indeed, AMPKα generally stimulates glucose uptake and glycolysis by up-regulating HK and PK activities. In our study, it is likely that glucose signaling pathway was deregulated due to a constitutive activation of AMPKα after the exposure to trace levels of pesticides. In fact, in many species, short-term AMPKα activation of several minutes stimulates downstream targets such as HK and PK activities, but a longer term activation of several days can modify gene expression.[35] We can thus hypothesize that over-activation of AMPKα might down-regulates mRNA expression of HK and PK on the long-term. Alternatively, pesticides may have directly inhibited HK and PK independently from AMPK as reported in human cells.[36]

Regarding oysters exposed to the mixture of pesticides, AMPKα was also over-activated. Although HK activity was lower in oysters exposed to metconazole 1X or isoproturon 1X than in control, no changes were observed in oysters exposed to the mixture of both

pesticides. This could highlight an antagonistic interaction between metconazole and isoproturon, however, this was not observed for PK activity, which is similar when metconazole and isoproturon were applied alone or in mixture.

2-DE proteomics revealed that the fructose-1,6-bisphosphatase (F-1,6-BP) was up-regulated in oysters exposed to metconazole 10X. These results are consistent with the facts that in these animals HK and PK activities were lowered, reflecting a decrease in glycolysis and an enhancement of gluconeogenesis. For instance, F-1,6-BP converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis. This might reflect that gluconeogenesis was enhanced to compensate for the inhibition of glycolytic enzymes induced by AMPKα over-activation, as previously reported in mice.[33]

Oysters exposed to metconazole 10X showed up-regulation of the ATP synthase subunit β. This enzyme catalyzes ATP synthesis from ADP by an electrochemical gradient of protons in the inner mitochondrial membrane during oxidative phosphorylation.[37] Up-regulation of ATP synthase subunit β is consistent with an increase in gluconeogenesis.

Although pesticides clearly influenced the energy metabolism of oysters, they have no effect on filtration rate, growth, and energy reserves. In contrast, growth of oysters is inhibited after a 7 days exposure to diuron at 0.4 µg/L.[38] Discrepancies between studies may reflect differences in experimental conditions. Indeed, in their experiment, the authors used a closed system with intermittent food supply whereas in our experiment, oysters were kept in a flow-through system with a continuous supply of *ad libitum* fresh phytoplankton. Although energy reserves of oysters were not impacted by low dose of pesticides, a long term exposure to high concentrations of pesticides can induce depletion in glycogen, protein and lipid contents in several aquatic animals.[34,39,40]

Antioxidant response

Oysters exposed to a 10X concentration of pesticides showed higher SOD activity than control. This enzyme is involved in management of reactive oxygen species (ROS) and catalyzes the dismutation of the superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Level of ROS generally increases in animals exposed to pollutants such as pesticides.[41,42] A 24 h exposure to trace levels of diuron decreased SOD activity in oyster *C. gigas*.[7] Despite this apparently paradoxical result, our results are consistent with those observed in several bivalve species exposed to contaminants.[43,44]

Oysters exposed to isoproturon 10X showed higher CAT activity than controls. This result agrees well with the fact that the increase in SOD activity in these animals should enhance H_2O_2. Catalase is the main enzyme involved in H_2O_2 detoxification and its activity generally increases with substrate concentration. Similarly, mussels exposed to pesticides or other organic contaminants had higher CAT activity than controls.[45,46,47]

Interestingly, oysters exposed to metconazole 10X used a different antioxidant pathway from those exposed to isoproturon. Indeed, CAT activity of oysters exposed to metconazole 10X was not enhanced but expression of peroxiredoxin (Prx), another enzyme involved in H_2O_2 detoxification, was up-regulated. In support to this result, transcription of Prx gene in shrimp increase when CAT expression was unchanged and reciprocally.[48]

In contrast to increase in SOD, CAT and Prx, activity of glutathione S-transferase (GST), an enzyme involved in cell detoxification system in oysters exposed to pesticides was similar to that of control as observed in oysters exposed to a low-dose pesticide mixture.[49] However a tissue-specific response was observed in oysters, with an increase in GST activity in digestive gland but not in gills. Similarly, tissue specific response of antioxidant system (SOD, CAT, GST) was observed in bivalve exposed to pesticides.[44,49] Moreover, although some synergistic effects were observed in *C. gigas* exposed to a mixture of a herbicide and a fungicide,[3] the

mixture of metconazole and isoproturon used in our study did not lead to increased antioxidant enzymes activity.

Oysters exposed to pesticides showed similar unsaturation index of polar lipids compared to that of control, suggesting that lipid peroxidation was limited.[17] This may reflect that ROS were efficiently managed by the antioxidant system and the lipid bilayer was not altered by trace levels of pesticides. Oysters exposed to the mixture of pesticides showed a lower unsaturated index than that of controls and pesticides alone, which could suggest synergetic effects of the two tested pesticides on this variable.

Total dimethylacetals (DMA) of oysters exposed to isoproturon were higher than that control animals. DMA is a glycerophospholipid class which contains a vinyl ether linkage at the sn-1 position typical of plasmalogens. Cell membrane of bivalves contain high levels of plasmalogens.[50] In the bivalve *Arctica islandica,*[51] plasmalogens were associated with protection against lipid peroxidation. Therefore, the observed increased of plasmalogens in oysters exposed to isoproturon 10X may be correlated with the activation of antioxidant pathway (SOD, CAT), reflecting an increase of ROS and a potential role to avoid lipid peroxidation.

Modification of some cytoskeletal components

Three cytoskeleton proteins were up-regulated in oysters exposed to metconazole 10X (severin, actin 2, thymosin), which confirmed the well-known generic disturbance of cytoskeleton components by marine pollutants, such as heavy metals in *Mytilus galloprovincialis*[52,53] or glyphosate in *Unio pictorum.*[54] Severin is a protein that blocks F-actin and causes the fragmentation and depolymerization of actin filaments in a Ca^{2+} dependent way leading to a disturbance in cytoskeletal remodeling. Thymosin belongs to a group of small proteins that also play a role of actin regulators[55] and can activate the antioxidant enzyme SOD in some species.[56]

In conclusion, environmental concentrations of metconazole and isoproturon induced a metabolic response in oysters. Over-activation of the sensing-kinase AMPKα was associated with a decrease in HK and PK activities that might reflect a decreased glycolysis. A compensatory response could be an increase in gluconeogenesis, as revealed by 2-DE, which might explain the maintenance of energy reserves in oyster during the 14-day exposure. In addition, activation of antioxidant response was observed for both pesticides but not for the lowest concentrations.

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SUPPORTING INFORMATION

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[57] via the PRIDE partner repository with the dataset identifier PXD002116, with reviewer access (Username: reviewer68486@ebi.ac.uk, Password: 9ixKJswa, to access the data please visit: http://www.ebi.ac.uk/pride).

Polar lipid fatty acids composition in Pacific oyster *Crassostrea gigas* exposed to metconazole, or isoproturon, or both, is provided in table S1.

ABBREVIATIONS LIST

AMPK: AMP-activated protein kinase, OsHV-1: Ostreid Herpesvirus 1, PK: Pyruvate kinase, HK: Hexokinase, CS: Citrate synthase, SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, GSH: Glutathione, F-1,6-BP: Fructose-1,6-bisphosphatase, XO: Xanthine oxidase, DTNB: 5,5′-dithio-bis-[2-nitrobenzoic], CDNB: 1-chloro-2,4 dinitrobenzene, ROS: Reactive oxygen species, FA: fatty acids, SFA: Saturated fatty acids, MUFA: Mono-unsaturated fatty acids, PUFA: Poly-unsaturated fatty acids, NMI: Non-methylene-interrupted fatty acids, DMA: Dimethylacetals fatty acids, WST-1: Water Soluble Tetrazolium salt, PKB: Protein kinase B

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TABLES

Table 1. Experimental concentrations of metconazole and isoproturon in µg/L measured in sea water after 1, 7 and 14 d of exposure.

	Control	Metconazole 1X	Isoproturon 1X	Metconazole 10X	Isoproturon 10X
Day 1	<LD	0.113	0.121	1.786	1.208
Day 7	<LD	0.048	0.110	ND	1.355
Day 14	<LD	0.098	0.138	ND	1.405

ND, no data
LD, limit of detection

Table 2. Energy reserves of oysters exposed to metconazole, isoproturon, or both. Values are expressed as µg/mg of flesh ± SD for carbohydrate and protein contents. The ratio between triacylglycerol and sterol contents is presented. Values are means±SD (n=3 tanks). Each sample consists in a pool of 16 oysters collected at day 14.

	Control	Metconazole 1X	Isoproturon 1X	Metconazole 10X	Isoproturon 10X	Metconazole 1X Isoproturon 1X
Carbohydrate (µg/mg flesh)	160.82 ± 30.58	157.56 ± 40.00	141.68 ± 16.35	151.70 ± 33.26	166.89 ± 16.17	149.87 ± 21.42
Triacylglycerol / Sterol	9.00 ± 0.29	9.51 ± 0.21	9.12 ± 0.23	9.21 ± 0.38	9.53 ± 0.20	8.72 ± 0.93
Protein (µg/mg flesh)	194.27 ± 44.54	188.49 ± 16.41	176.02 ± 7.71	162.04 ± 7.44	166.78 ± 13.07	182.83 ± 23.28

Table 3. Identification of proteins by mass spectrometry.

Spot	Uniprot accession	NCBI accession	Protein name	pI/Mr (kDa) theoretical	pI/Mr (kDa) experimental	Anova (p)	Average fold change	Unique peptide matched	Mascot Score
Up-regulated by metconazole 10X									
432	K1RWW5	CGI_10013347	ATP synthase subunit β	44.9 / 5.1	48 / 5.1	6.960e-004	1.5	27	13274.88
449	K1PE57	CGI_10002689	Severin	37.2 / 4.88	40 / 5	0.006	2.0	5	182.64
484	Q8TA69	CGI_10018876	Actin 2	41.7 / 5.48	38 / 5.4	0.004	2.2	13	958.83
490	Q8TA69	CGI_10018876	Actin 2	41.7 / 5.48	38 / 5.3	0.009	1.7	27	11920.34
510	K1QSB0	CGI_10006343	Fructose-1.6-bisphosphatase 1	36.8 / 6.70	35 / 6.6	0.050	1.7	9	400.54
668	K1QM61	CGI_10009700	Thymosin	40.4 / 5.78	22 / 5.9	0.007	3.2	1	36.96
708	K1R3T3	CGI_10019268	Transcription factor BTF3-like protein 4	18.4 / 6.58	16 / 6.1	0.030	1.5	5	56.11
709	K1R7F8	CGI_10027917	Peroxiredoxin-5	16.6 / 5.99	16 / 6.1	0.010	1.6	7	525.46
Down-regulated by metconazole 10X									
636	K1QCM0	CGI_10019175	Rho GDP-dissociation inhibitor 1	23.6 / 5.26	25 / 5.1	0.044	2.2	17	682.45
Up-regulated by isoproturon 10X									
543	K1P9N7	CGI_10014966	14-3-3 protein ζ	35.1 / 4.91	26 / 4.9	0.010	1.6	2	20.60
559	K1P9N7	CGI_10014966	14-3-3 protein ζ	35.1 / 4.91	25 / 4.9	0.053	1.5	2	23.11
547	K1Q596	CGI_10006178	Phosphoserine phosphatase	26 / 5.74	25 / 5.8	0.047	1.5	1	29.03

FIGURE LEGENDS

Figure 1. Activity of HK and PK in Pacific oyster *Crassostrea gigas* expressed as mU/mg of protein. (A,C) Oysters were exposed to metconazole (black dot) or isoproturon (white dot). 0: no pesticides in control; 1: concentration 1X; 10: concentration 10X (B,D) C: control oysters; M+I: mixture of metconazole 1X and isoproturon 1X. Letters indicate significant differences (n.s: no significant). Values are means±SD (n=3 tanks). Each sample consists in a pool of 16 oysters collected at day 14.

Figure 2. Activity of SOD, CAT and GST in Pacific oyster *Crassostrea gigas* expressed as mU/mg of protein. (A,C,E) Oysters were exposed to metconazole (black dot) or isoproturon (white dot). 0: no pesticides in control; 1: concentration 1X; 10: concentration 10X. (B,D,F) C: control oysters; M+I: mixture of metconazole 1X and isoproturon 1X. Letters indicate significant differences (n.s: no significant). Values are means±SD (n=3 tanks). Each sample consists in a pool of 16 oysters collected at day 14.

Figure 3. AMPKα Thr^{172} phosphorylation and Histone H3 content in Pacific oyster *Crassostrea gigas*. (A) C: control oysters; M 1X: metconazole 1X; I 1X: isoproturon 1X; M 10X: metconazole 10 X; I 10X: isoproturon 10X; M+I: mixture of metconazole 1X and isoproturon 1X. Representative image of western blot of AMPKα Thr^{172} phosphorylation (62kDa) and Histone H3 (10kDa). (B,C) Relative quantification of bands intensity corresponding to AMPKα Thr^{172} phosphorylation (expressed as % of the control). (B) Oysters were exposed to metconazole (black dot) or isoproturon (white dot). 0: no pesticides in control; 1: concentration 1X; 10: concentration 10X. (C) C: control oysters; M+I: mixture of metconazole 1X and isoproturon 1X. Letters indicate significant differences. Values are means±SD (n=3 tanks). Each sample consists in a pool of 16 oysters collected at day 14.

Figure 4. Reference image showing a gel of a 2-DE protein profile of oyster *Crassostrea gigas*. Excised spots are marked with corresponding spot number: in bold, under metconazole 10X and in italic, isoproturon 10X. Each sample consists in a pool of 16 oysters collected at day 14. The name of the protein identified is shown in Table 3 and details of the corresponding mass spectrometric data are given in the supplementary data. Mr: molecular marker.

Figure 5. Unsaturated index and total dimethylacetals (DMA, expressed in % of total FA) content in polar lipids. (A,C) Oysters were exposed to metconazole (black dot) or isoproturon (white dot). 0: no pesticides in control; 1: concentration 1X; 10: concentration 10X. (B,D) C: control oysters; M+I: mixture of metconazole 1X and isoproturon 1X. Letters indicate significant differences (n.s: no significant). Values are means±SD (n=3 tanks). Each sample consists in a pool of 16 oysters collected at day 14.

FIGURES

Figure 1

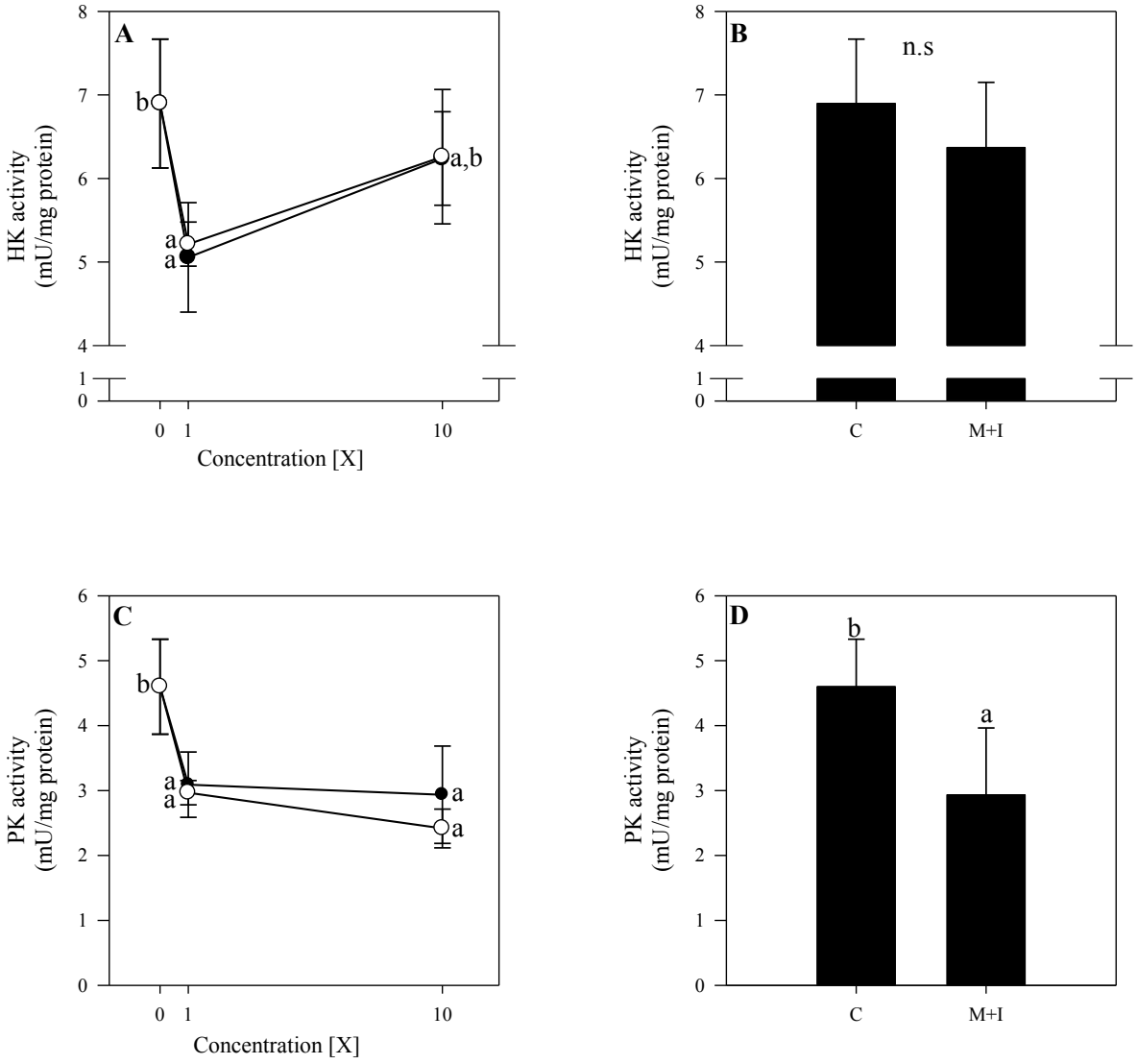


Figure 2

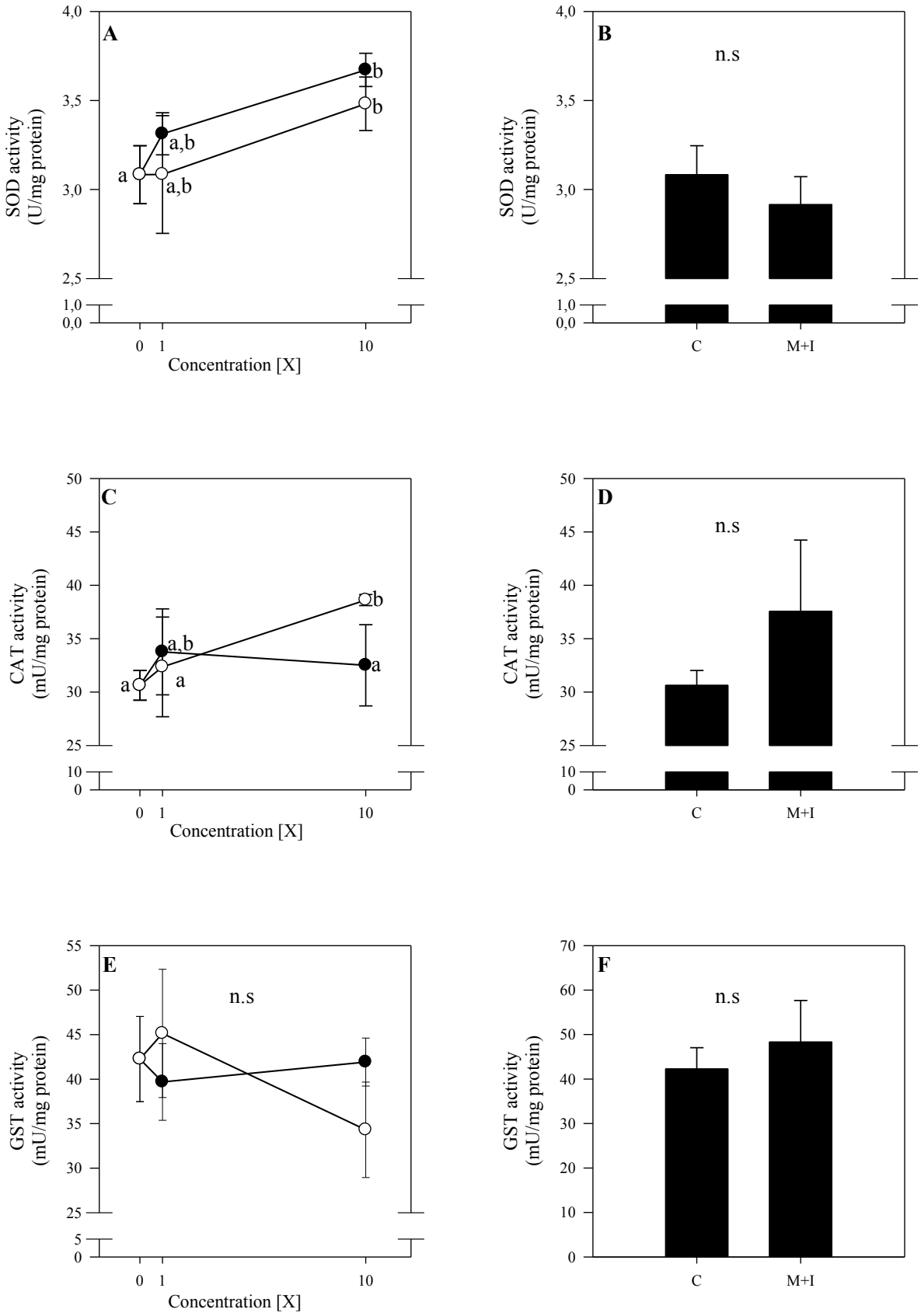

A
SOD activity (U/mg protein)
4,0
3,5
3,0
2,5
1,0
0,0
a
a,b
a,b
b
b
0
1
10
Concentration [X]
B
n.s
C
CAT activity (mU/mg protein)
50
45
40
35
30
25
10
0
a
a,b
a
b
a
D
n.s
E
GST activity (mU/mg protein)
55
50
45
40
35
30
25
5
0
n.s
F
n.s
70
60
50
40
30
20
10
0
C
M+I

Figure 3

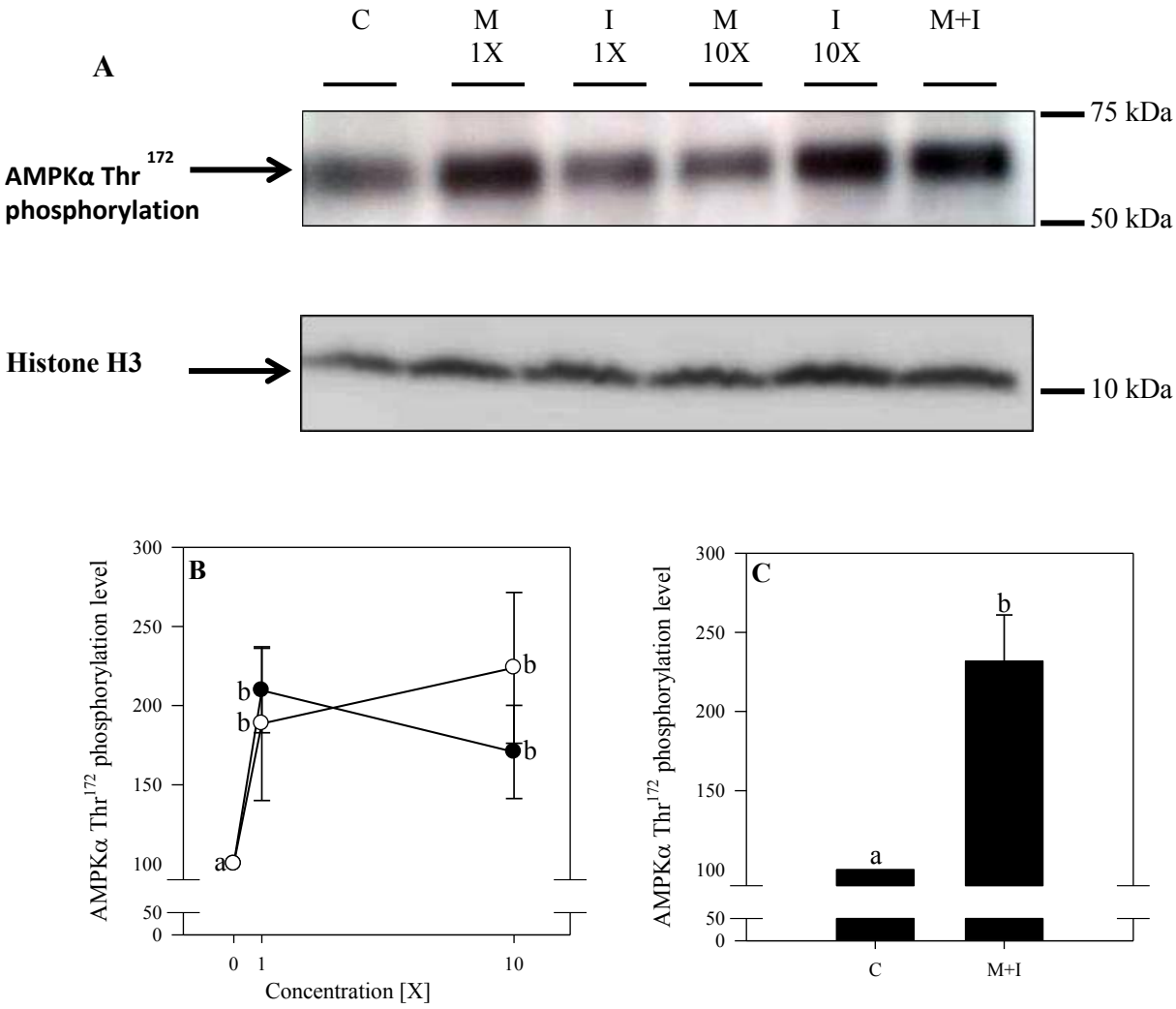

A
C
M 1X
I 1X
M 10X
I 10X
M+I
75 kDa
50 kDa
AMPKα Thr172 phosphorylation
Histone H3
10 kDa
B
AMPKα Thr172 phosphorylation level
Concentration [X]
C
M+I

Figure 4.

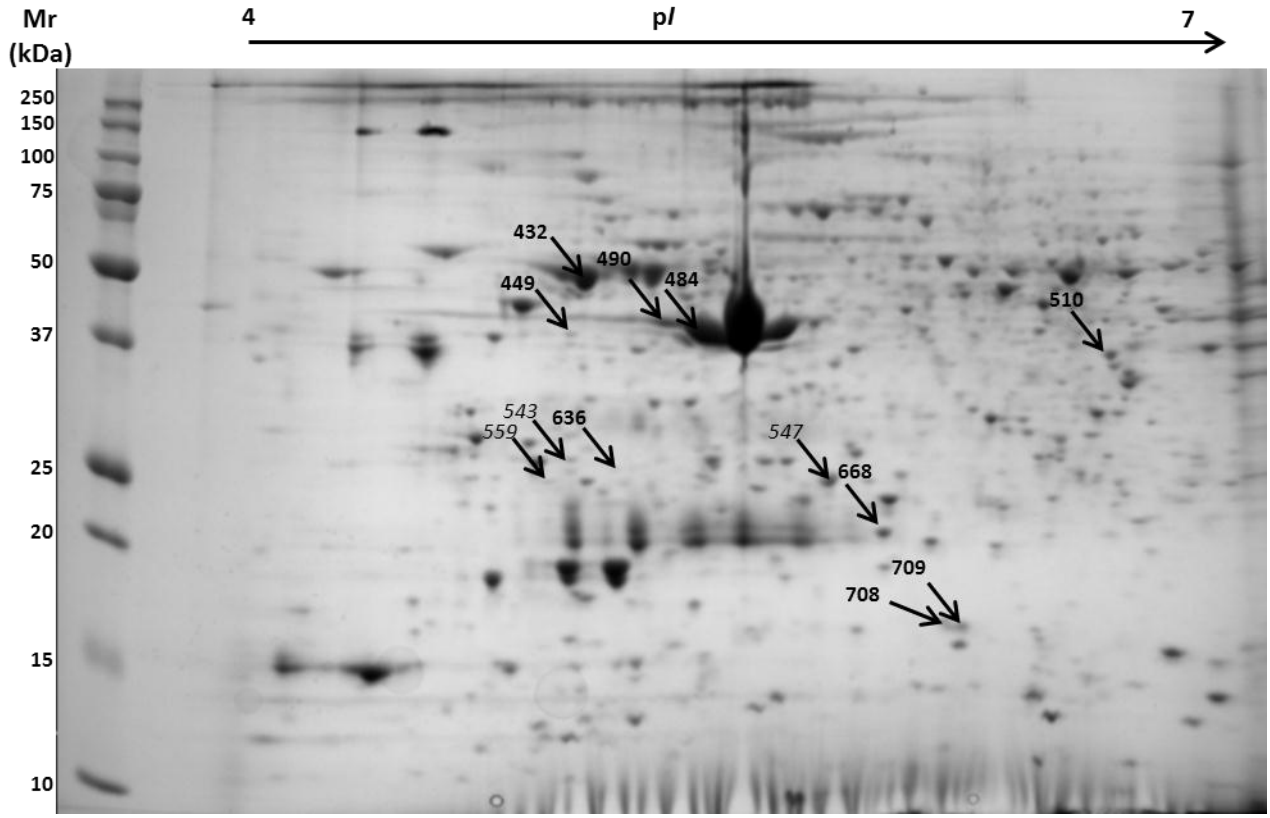
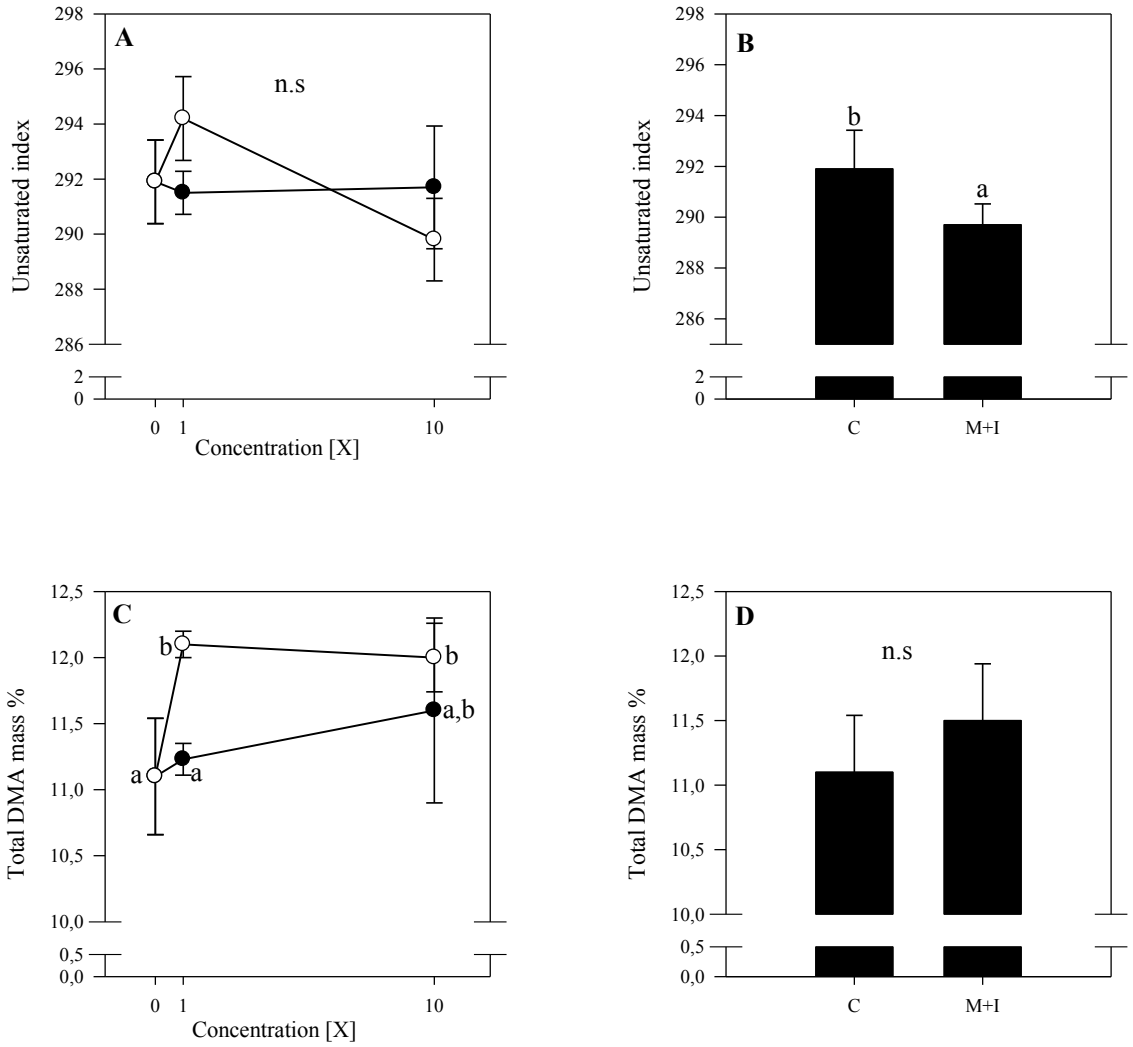

Mr
(kDa)
4
pI
7
250
150
100
75
50
37
25
20
15
10
432
490
449
484
510
543
559
636
547
668
709
708

Figure 5


A
n.s
Unsaturated index
298
296
294
292
290
288
286
2
0
0
1
10
Concentration [X]
B
b
a
Unsaturated index
C
Total DMA mass %
b
b
a,b
a
a
12,5
12,0
11,5
11,0
10,5
10,0
0,5
0,0
Concentration [X]
D
n.s
Total DMA mass %
C
M+I