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Title: Effect of reproduction cycle stage on energy metabolism responses in a sentinel species (*Dreissena polymorpha*) exposed to cadmium: what consequences for biomonitoring?

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Keywords: energy metabolism, ATP, Cd, biomonitoring

Highlights:

- OXPHOS and anaerobic metabolism-related markers varied between seasons in *D. polymorpha*
- Mussel responses to Cd and warming depended on the reproduction stage.
- The reproductive cycle period accounted for 92% of marker responses variance.

Abstract: Metal trace elements such as cadmium (Cd) are commonly present in ecosystems and could lead to impairment of mitochondrial functions and energy imbalance in aquatic organisms including molluscs. Combined exposure to increasing temperatures and Cd could enhance such an impact on animals. Seasonal fluctuations, such as temperature, and the

corresponding reproduction cycle can affect biomarker responses. However, the reproduction cycle stage is rarely taken into account in ecotoxicological studies. Thus, this work aimed at understanding energy metabolism responses in a sentinel species, *Dreissena polymorpha*. Mussels were collected during the rest and the reproduction periods and were exposed to 10 $\mu\text{g.L}^{-1}$ of cadmium (Cd) at two temperatures (*in situ* temperature and *in situ* temperature + 5°C) during 7 days. Energy metabolism was monitored by measuring reserves and energy nucleotides charge and by assessing aerobic and anaerobic metabolism markers, and upstream regulation pathways. Markers related to OXPHOS activity revealed seasonal variations under laboratory conditions. Conversely, adenylate nucleotides, glycogen, lipid and transcript levels of AMP-activated protein kinase, citrate synthase, ATP synthase and cytochrome b encoding genes remained steady after the acclimation period. No evident effect of Cd on energy metabolism markers was noticed for both exposures although the transcript level of succinate dehydrogenase and citrate synthase encoding genes decreased with Cd during the rest period. Cellular stress, revealed by lipid peroxidation and catalase mRNA levels, only occurred in Cd and warming co-exposed mussels during the reproduction period. These results suggest that contaminant impact might differ according to the reproduction cycle stage. The effect of

confounding factors on biomarker variations should be further investigated to have a deeper knowledge of metabolism responses under laboratory conditions.

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1. Introduction

Dreissena polymorpha is a freshwater model species commonly used in biomonitoring (Binelli et al., 2015, 2004; de Lafontaine et al., 2000; Faria et al., 2011, 2009; Kerambrun et al., 2016; Pain and Parant, 2007; Palais et al., 2011). Its reproduction cycle, with gametogenesis occurring in winter, maturation and release of gametes in spring and summer, and post-reproduction and rest periods in autumn, was described to be energy-costly since this species allocates 30 to 45% of its energy to reproduction (Palais et al., 2011; Sprung, 1995). The gonadal development of *D. polymorpha*, similarly to other bivalves, depends on several environmental factors, in particular temperature and food availability (Haag et al., 1993; McMahon, 1996; Ram et al., 1996). Several studies characterised annual fluctuations of diverse physiological responses in *D. polymorpha*. A transcriptomic study revealed seasonal variations with higher transcripts related to gametogenesis and mitochondrial metabolism in the rest period than in the reproduction period (Navarro et al., 2013). Energy reserve storage such as lipids and glycogen were shown to increase and be stored during winter, and used during the reproduction period (Palais et al., 2011). Oxidative phosphorylation markers, such as electron transport system (ETS) activity and ATP synthase mRNA expression level, were higher during summer than during the winter period (Fanslow et al., 2001; Kerambrun et al.,

2016). These seasonal fluctuations are considered as confounding factors and challenge the link between marker responses and the health status of model species (Faria et al., 2014; Minguéz et al., 2009; Pain-Devin et al., 2014; Viarengo et al., 2007).

Temperature and trace metals are common environmental stressors in ectotherm species, which are directly exposed to environmental variations and anthropogenic pollution. High temperatures, such as those resulting from seasonal fluctuations, strongly affect the physiology of ectothermic aquatic bivalves and may decrease their tolerance to toxic substances like metals (Sokolova and Lannig, 2008). Cadmium (Cd) is a widespread toxic trace metal in aquatic environments (Simpson, 1981) and is known to be accumulated in bivalves' soft tissues (Kraak et al., 1992; Marie et al., 2006). Cd concentrations ranging from 1 to $90\mu\text{g}\cdot\text{g}^{-1}$ dry tissues were measured in *Dreissena polymorpha* during biomonitoring surveys (Kraak et al., 1999; Kwan et al., 2003). From $25\mu\text{g}\cdot\text{L}^{-1}$, *in vivo* Cd exposures lead to significant physiological disturbances (apoptosis, respiration rate decline, AMP accumulation) in aquatic organisms including molluscs (Hanana et al., 2019; Simpson, 1981; Sokolova, 2004; Sokolova et al., 2005).

Mitochondria are considered as a major target of temperature and trace metals effects, since mitochondrial metabolism plays an important role in energy balance, stress and

adaptation (Salin et al., 2015; Sokolova and Lannig, 2008). Several studies reported an effect, which is enhanced at high temperatures, of Cd on energy metabolism. The additive effect of Cd and temperature results in an energy deficit in molluscs through a decline of respiration rate (Cherkasov et al., 2010; Ivanina et al., 2012; Lannig et al., 2006; Mubiana and Blust, 2007; Sokolova, 2004). The exposure to Cd coupled to high temperatures also inhibits mitochondrial enzymes such as citrate synthase activity in *Crassostrea virginica* (Cherkasov et al., 2010; Ivanina et al., 2012; Sokolova, 2004). Other metabolism-related markers, such as energy balance and respiratory chain complexes, have already been targeted in metal and thermal stress studies. The adenylate energy charge (AEC) and cytochrome c oxidase (*cox*) mRNA expression decreased after 3 weeks of Cd exposure ($25\mu\text{g.L}^{-1}$) in *Crassostrea gigas* (Sokolova et al., 2005). Besides their combined effect, Cd and high temperature impacts could differ according to the season of the exposure. Cherkasov et al. (2010) exposed *Crassostrea virginica* to a thermal and metal cross-stress at two different seasons (winter and summer) and observed a higher impact of temperature and Cd on energy metabolism during the reproduction period (summer) than during rest (winter).

The influence of confounding factors on energy metabolism is largely described in marine species but is still poorly studied in freshwater species. In the context of freshwater

pollution biomonitoring, the purpose of this work was to study how the reproduction cycle stage influenced the effects of a short-term Cd and warming co-exposure on energy metabolism responses in the sentinel species *Dreissena polymorpha*. Therefore, two Cd exposures at two different temperatures were conducted on mussels harvested during the rest period (November 2018) and during the reproduction period (June 2019). Several markers associated with cellular ATP production and management were measured after 24 hours and 7 days of Cd exposure. Key processes involved in energy metabolism such as energy nucleotides and their regulation (AMP kinase and AMP deaminase), anaerobic (lactate dehydrogenase) and aerobic (citrate synthase, succinate dehydrogenase, cytochrome b, cytochrome c oxidase and ATP synthase) metabolism markers were targeted by analysing mRNA levels and/or enzymatic activities. The potential cellular damage induced by Cd or reactive oxygen species (ROS) produced by the respiratory chain was also assessed through lipid peroxidation and catalase mRNA expression.

2. Material and Methods

2.1 Mussels collection and acclimation

Dreissena polymorpha specimens were hand-collected in the Meuse channel (Commercy, France, E05°35'32'', N48°45'50'') in October 2018 and May 2019. Physicochemical parameters of water at the sampling points are presented in Table S1. Only individuals between 15 and 25mm in length were selected and were quickly transported to the laboratory in aerated field water. Mussels were acclimated for 15 days at the field temperature (11°C in November 2018 and 14°C in June 2019) in two separate 4.5L aquaria. Water was continuously aerated and an artificial photoperiod corresponding to the one at the sampling time (8 a.m. – 6 p.m. in November and 6 a.m. – 10 p.m. in June) was applied. Water from the field was progressively replaced by spring water (Cristaline®, Aurèle spring, 1/3 per day). After 15 days, one water bath was maintained at the field temperature and the temperature of the second was increased by 5°C on three days (2°C per day the two first days and 1°C the third day).

2.2 Exposure design

After acclimation, mussels were exposed for 7 days to $10\mu\text{g.L}^{-1}$ of Cd or kept in spring water (control). Both conditions were repeated at two different temperatures (*in situ* temperature and *in situ* temperature + 5°C), in November 2018 and in June 2019. Cadmium chloride ($\text{CdCl}_2\cdot\text{H}_2\text{O}$; ref 45-23/25-33) was purchased from Sigma-Aldrich. A stock solution of Cd (10mg.L^{-1} , expressed as total ion concentration) was prepared and acidified to 1% with nitric acid to limit metal adsorption. All exposure media (4.5L) were renewed every day with a prediluted Cd solution to ensure a homogenous exposure to Cd. After each renewal, mussels were fed with 15.10^3 cells.mL⁻¹ of fresh algae (*Chlorella vulgaris*). Cadmium concentration was measured twice a day in all tanks. The actual concentrations of Cd in exposure water were $7.51\mu\text{g.L}^{-1} \pm 2.73$ (November 2018) and $7.93\mu\text{g.L}^{-1} \pm 2.25$ (June 2019) during the experiment. Physicochemical parameters (pH, temperature, conductivity and dissolved oxygen) were monitored twice per day in each tank (Table S2). Gonadal development stage was analysed on mussels ($n = 12$) before the start of the exposure and 10 other individuals were frozen for metal tissue concentration measurements. After 24 hours and after 7 days of exposure, mussels were also sampled and dissected to perform all other analyses. For transcript level of the genes and enzymatic activity analyses ($n = 10$ for each measurement),

digestive glands were dissected and shock-frozen. For reserve content measurements ($n = 10$), the whole organisms were dissected and shock-frozen. Mussels were entirely shock-frozen with their shell for energy nucleotide contents analysis ($n = 10$).

2.3 Gonadal development analysis

Dissected mussels (without byssus and shell) were fixed in Bouin's aqueous solution for 24h followed by successive alcohol dehydration baths from 50° to 100°. After dehydration, samples were placed in a butanol solution to permeate all tissues. Samples were then paraffin-embedded, and 7 μ M sections were prepared from the foot to gonads of mussels. Sections were stained with picro-indigo carmine and solid nuclear red and observed with a microscope to sex the gonads and to assign them to a development stage (Gist et al., 1997; Palais et al., 2011). The assignment was based on the size and the number of present oocytes (females) or spermatogenesis steps (males).

2.4 Cd concentration in tissues

Mussel soft tissues were dried at 60°C for 24h and digested in nitric acid 65% for 48h at 80°C. Metal quantifications were performed in the resulting acid solutions (adjusted to 3mL with deionized water) by flameless atomic absorption spectrophotometry (flameless AAS:

Varian SpectrAA 220 Zeeman), as described in Palais et al. (2012). Results are expressed in $\mu\text{g}\cdot\text{g}^{-1}$ of dry weight (DW).

2.5 Enzymatic activities

The digestive glands were weighed and crushed in phosphate buffer containing protease inhibitors (50mM pH 7.6 supplemented with 1mM phenylmethylsulfonyl fluoride and 1mM L-serineborate) at 8:1 (v/w) ratio. Samples were centrifuged (3000g, 10min, 4°C). The supernatant was collected for the enzymatic activities' measurement. Assays were performed with an automated spectrophotometer (Gallery, ThermoScientific). Electron transport system (ETS) and lactate dehydrogenase (LDH) activities, protein content, and lipid peroxidation (LOOH) were measured according to Garaud et al. (2016) and Potet et al. (2016).

For cytochrome c oxidase (COX) activity, cytochrome c ($10\text{ mg}\cdot\text{ml}^{-1}$) was dissolved in potassium phosphate buffer (10 mM, pH 7.0) and reduced by the addition of 6% of ascorbate. The absorbance of cytochrome c was measured at 550nm and 565nm to calculate the ratio A550/A565, which indicates a correct reduction when values are comprised between 10 and 20. No purification step was realised after ascorbate addition. Sample supernatant was diluted to 1:45 in 100mM Tris buffer (pH 8.1). Reduced cytochrome was added to the previous

dilution. Cytochrome c oxidation was then monitored for 3min at 550nm. For citrate synthase (CS) activity, supernatant was diluted to 1:55 in 0.1M Tris buffer (pH 8.1) supplemented with 0.15mM DTNB (5,5'-dithiobis-2-nitrobenzoate; Merck) and 0.2mM acetyl-coenzyme A (Merck). The activity was first monitored for 3min at 420nm to subtract the background due to the buffer absorbance. A second measure was realized for 3min at 420nm after the addition of 4mM oxaloacetate (Merck). Enzymatic activity is expressed in $\text{U}\cdot\text{g}^{-1}$ proteins (molar extinction coefficient (ϵ) reduced cytochrome c = $7040\text{M}^{-1}\cdot\text{cm}^{-1}$, ϵ DTNB = $14140\text{M}^{-1}\cdot\text{cm}^{-1}$).

2.6 Energy reserve contents

Mussel soft tissues were weighed and crushed in 50mM phosphate buffer (pH 8) at 5:1 (v/w) ratio and centrifuged (3000g, 5min, 4°C). Protein content was determined by Bradford's method. Lipid and glycogen contents were measured according to the method of Plaistow et al. (2001).

2.7 Energy nucleotide contents

Nucleotides extraction was realized as described in Sokolova et al. (2005). Briefly, frozen mussels were quickly dissected and lyophilized for 48h. Soft tissues were then crushed in 0.6M perchloric acid (Merck) solution, centrifuged (3000g, 10min, 4°C) and the supernatant was collected. The pH of each extract was adjusted to 5 - 6.5 with 5mM

potassium hydroxide and 1M bicine solution. No EDTA was added to perchloric acid solution leading to a potential underestimation (10-20%) of nucleotide contents. Precipitated potassium perchlorate was removed by a second centrifugation. Extracts were then filtered (0.45µm) and stored at -80°C. Concentrations of ATP, ADP, AMP and IMP were measured with a high-performance liquid chromatography (HPLC) system and Nucleosic C18 column (Nucleosil) according to Sokolova et al. (2000). The separation was performed at a flow rate of 0.8mL.min⁻¹ using a mobile phase of 1M phosphate buffer with 5% methanol (pH 6) for 15min. Nucleotide contents were monitored at 254nm and nucleotide concentrations were calculated using ATP, ADP, AMP, and IMP standards (Sigma) of 20, 10, 2 and 10µM, respectively. Adenylate energy charge (AEC) was calculated using the following formula:

$$AEC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]}$$

2.8 mRNA expression of metabolism-related genes

Total RNA was extracted from digestive glands using TriReagent (Euromedex) following the manufacturer's protocol. Reverse transcription was performed on 400ng of total RNA using Verso cDNA Synthesis Kit (Thermo Scientific) and random hexamer/oligo dT

primers (v/v 3:1) according to the manufacturer's instructions. The reaction was conducted at 42°C for 30min using a PCR Mastercycle (Eppendorf).

Transcript levels of the genes were analysed by real-time quantitative PCR using Absolute Blue qPCR SYBR Green ROX Mix (Thermo Scientific) according to the manufacturer's protocol. Specific primers are given in Table 1. Energy nucleotides regulation was studied through AMP-activated protein kinase (*ampk*) and AMP deaminase (*ampd*) mRNA expressions. Concerning the OXPHOS, genes encoding the beta subunit of the ATP synthase (*atp*), cytochrome b (*cytb*), cytochrome c oxidase (*cox*), but also another unit of the respiratory chain, the alternative oxidase (*aox*) were selected. Citrate synthase (*cs*), lactate dehydrogenase (*ldh*), and catalase (*cat*) transcript levels were also measured. The optimal primer quantity was evaluated across a range of concentrations between 150 and 600nM. The PCR steps consisted in 15min initial denaturation at 95°C followed by 40 cycles of heat denaturation at 95°C for 10s, annealing at 60°C for 60s and were carried out in a CFX Manager (BioRad) on 1/10 diluted complementary DNA (cDNA). The relative transcript expression level was calculated using the Pfaffl method (Pfaffl, 2001) taking into account the PCR efficiency and normalized by the geometric mean of two housekeeping genes (encoding

actin and ribosomal protein S3) according to Navarro et al. (2011). A standard curve using dilutions of cDNA between 1:10 and 1:1000 was performed to calculate the PCR efficiency.

2.9. Statistical analysis

Marker responses were analysed by a two-way ANOVA to assess temperature, exposure duration, and contamination effects. Parametric (Tukey) or non-parametric (Kruskal-Wallis) post-hoc tests were used to determine the significant differences between conditions. Marker responses measured on mussels sampled after the acclimation were compared by a t-test to assess differences between the two reproduction cycle steps. Previously, normality and homoscedasticity of the data were tested with Shapiro and Bartlett tests respectively. A global analysis of marker results was also performed with a Partial Least Square Discriminant Analysis (PLS-DA) to identify the overall capacity of markers to discriminate the different conditions and periods as well as to define the most relevant markers. The relevance of marker responses was based on the individual role of each marker in the overall model, estimated by the VIP (Variable Importance in the Projection). Only markers with a $VIP > 1$ were considered significant in the model. Partial redundancy analysis was also performed on the total dataset to see whether well-characterised variables (months) with strong effects obscure the effects of other explanatory variables. A threshold of $p < 0.05$

was considered significant. Statistical analyses were performed using R software (R Development Core Team, 3.5.0).

3. Results

3.1. Gonadal development

Gonadal development was analysed on mussels before exposure for both seasons (Fig. 1). In November, 6 females and 2 males were identified, and sex could not be determined in 4 individuals due to complete gonad regression. The histological analysis revealed that 82% of mussels were in the resting stage and 18% in developing one. In June, there were 7 females and 5 males which were either in pre-spawn (33.4%) or post-spawn period (66.6%).

3.2. Metal tissues concentration

No Cd accumulation was observed in control mussels after 24h or 7 days. Mussels exposed to Cd accumulated metal in both experimentations compared to control mussels, with a significantly higher accumulation after 7 days of exposure compared to 24h (Fig. 2). Temperature effects on Cd accumulation were observed in Cd-exposed mussels with higher Cd concentration at the elevated temperature after 7 days. While Cd accumulation after 24h

was similar between the two months, mussels' tissues presented a significantly higher Cd concentration in November than in June after 7 days of exposure.

3.3. Cellular energy metabolism

3.3.1. Seasonal variations

Several markers showed differences between seasons after the acclimation period under laboratory conditions (Table 2). All energy nucleotide contents (ATP, ADP, and AMP) remained stable after 15 days of acclimation (T0) in both months. Only IMP content was higher in November than in June (Table 2). Glycogen and lipid contents were similar between November and June, while protein content increased significantly in June (Table 2). The mitochondrial density, which is revealed by *cs* relative mRNA expression and CS activity, did not vary between the two reproduction periods (Table 2). The oxidative phosphorylation was assessed by measuring ETS and COX activities and relative transcript levels of *cytb*, *cox*, *sdh*, *atp* and *aox* genes. Most of these markers did not show differences between seasons except for *aox*, *sdh* and *cox* mRNA expressions and COX and ETS activities (Table 2). COX activity, *sdh* and *aox* transcript levels were higher in November than in June, while the opposite was observed for ETS activity and *cox* mRNA expression. The increase of *ldh* transcript level and LDH activity in June suggested that the potential capacity for anaerobic

metabolism was enhanced in summer period (Table 2). The regulation of energy nucleotides was affected by the sampling period. Transcript level of *ampd* was significantly increased in June compared to November, while no difference was noticed in *ampk* relative mRNA expression (Table 2). No difference between the two seasons was observed in cellular stress markers, as revealed by *cat* transcript level and lipid peroxidation measurement (Table 2).

3.3.2. Exposure effects

The effects of Cd exposure on available energy were assessed by analysing energy nucleotide and reserve contents. In November, levels of adenylates (ATP, ADP and AMP) showed no variation between control and Cd-exposed mussels (Fig. 3A and Table 3). In June, ATP content significantly increased at 14°C in Cd-exposed mussels for 24h while no variation was observed in AMP and ADP contents (Fig. 3B, Table 3). AEC values remained unchanged in all conditions in November (Fig. 3C), while a significant decrease in AEC values was observed in control mussels in June after 7 days at the highest temperature (Fig. 3D). Although no effect of Cd on AEC values was observed, inter-individual variability increased in mussels co-exposed to Cd and elevated temperature for both sampling times (Fig. 3C and D). In November, energy reserves did not show any significant variation during exposure (Fig. 3E and Table 3). During the reproduction period, 24h of Cd exposure led to a

significant decrease in glycogen at both temperatures (Fig. 3F), whereas no effect was observed after 7 days of exposure. Protein and lipid contents remained unchanged in all conditions (Table 3).

Mitochondrial responses were evaluated by the activity and mRNA levels of OXPHOS-related enzymes and the citrate synthase (Fig. 4 and Table 3). In November, only *sdh* relative mRNA expression significantly decreased after 7 days of exposure at the highest temperature (Fig. 4A). The other OXPHOS related genes did not display any variation in their transcript level under Cd exposure (Fig. 4C and Table 3). In June, Cd did not affect the relative mRNA expression of the genes involved in OXPHOS (Fig.4B-D and Table 3). A Cd effect was observed on *cs* relative mRNA expression in both exposures. In November, after 24 hours at 11°C (Fig. 4E), *cs* relative transcript level significantly increased in Cd-exposed mussels. In June, it decreased in Cd and warming co-exposed mussels after 7 days compared to control (Fig. 4F). ETS, COX and CS activities remained stable in all conditions (Table 3).

3.4. Stress markers

In November, no difference was observed between the different experimental groups neither on *cat* relative mRNA expression nor on lipid peroxidation measurement (Fig. 5 A-C). In June, no Cd effect was observed after 24h of exposure. After 7 days, *cat* transcript level

and lipid peroxidation significantly increased in Cd and warming co-exposed mussels compared to the corresponding control (Fig. 5 B-D).

3.5. Global analysis

The PLS-DA model explained 74% of the variance of the entire data set with the two first axes (Fig. 6). The identified structure was tested against a null model which revealed its significance ($p = 0.001$, 999 permutations). According to the results, energy nucleotides and reserves (left side) as well as energy nucleotides regulation and OXPHOS markers (right side) mainly defined the primary axis. The secondary axis was mainly characterised by the mRNA expression of genes involved in mitochondrial energy metabolism (*cs*, *sdh* and *atp*). The transcript levels of these three genes were VIP markers and discriminated the overall data set. The two reproduction periods were separated along the primary axis. In November, responses between controls and Cd-exposed mussels were distinguished along the secondary axis while in June the same axis separated the experimental groups according to the exposure duration. According to the redundancy analysis results, 92% of the variance was due to the exposure period. Conditions (control and Cd) represented only 0.1% of the observed variance while cumulated temperature and exposure duration variance was 1.1%. Only 7.1% of the total variance was unexplained.

4. Discussion

4.1 Seasonal variations

Biomarker development encounters some locks. Confounding factors such as reproduction cycle could bias marker responses interpretation (Amiard-Triquet et al., 2012). This study provided evidence for the potent effect of seasonality on metabolism-related marker responses in biomonitoring. Contrasted marker behaviours emerged after acclimation period to laboratory conditions (15 days), with markers presenting similar or different levels between November 2018 and June 2019. In the present study, all adenylate nucleotide contents and AEC values were similar between November and June after 15 days of acclimation. However, we previously showed that adenylate energy charge values significantly decreased *in situ* between November 2018 (mean AEC = 0.8) and June 2019 (mean AEC = 0.2) in the same population of mussels (Louis et al., 2020). Although sampling, transport and handling were stress factors, mussels were able to stabilise their energy balance during the acclimation period under laboratory conditions.

Lipid and glycogen levels did not vary between November and June after the acclimation period in the present study although mussels spawned their gametes during acclimation in June. Glycogen and lipids are the main energy storage in organisms, and are

largely known to be used for gametes and gonadal tissues formation, leading thus to strong seasonal variations (Anacleto et al., 2013; Andrade et al., 2017; Cordeiro et al., 2017, 2016; Louis et al., 2020 ; Palais et al., 2011; Tourari et al., 1988). By contrast, a seasonal variation was observed in protein contents with an increase in June after the acclimation period. In reproduction period, the synthesis of specific proteins of interest could be enhanced to ensure gametes development (Li et al., 2011).

Aerobic metabolism markers such as *cs*, *cytb* and *atp* mRNA transcript levels and CS activity did not differ between November and June in our study. However, several authors showed a global aerobic metabolism activity enhancement in summer compared to winter season (Louis et al., 2020; Majed et al., 2002; Soren et al., 2018). Of note, ETS activity was reported to show seasonal variations in the natural environment with an increase in summer months (Fanslow et al., 2001; Louis et al., 2020), which is consistent with the increased ETS activity that we observed in June compared to November. Cytochrome c oxidase relative mRNA expression increased in June, while its activity decreased. The discrepancy between these two levels of response might result from diverse regulations steps at the transcriptional but also at the post-translational level (Gedeon and Bokes, 2012).

The regulation of energy nucleotides was partially affected by the sampling period. While no seasonal variation was noticed in *ampk* transcript level, *ampd* mRNA expression significantly increased in June compared to November. Interestingly, *ampd* transcript level increased while IMP content decreased in June. AMP deaminase converts AMP into IMP to avoid AMP accumulation in cells (Składanowski et al., 2005). However, only *ampd* mRNA expression was analysed in this work restricting thus an extrapolation to the enzymatic level.

Lactate dehydrogenase activity and transcript level showed a higher level in June than in November after the laboratory acclimation period. Seasonal variations in LDH activity or *ldh* mRNA expression have been observed in different fish species and in *D. polymorpha* with an increase during summer months (Koenig and Solé, 2014; Louis et al., 2020; Vornanen, 1994). This enhancement could result from a higher energy demand during reproduction period (Sokolova et al., 2012).

This study brought out few markers, which presented stable levels after the acclimation period under laboratory conditions at both exposure periods. These markers include *ampk*, *cs*, *cytb*, *atp* mRNA expression, glycogen, lipid, adenylate nucleotide contents and citrate synthase activity. Markers with stable level present an interest for risk assessment

since they could be used whatever the period of the reproduction cycle, limiting thus bias in response interpretation.

4.2 Exposure effects

The results of the present study highlighted an increased Cd bioaccumulation in mussels exposed to Cd at the highest temperature. Mussels accumulated twice more Cd in November than in June. Nardi et al. (2017, 2018) showed the same results in *Mytilus galloprovincialis* exposed to Cd during winter and summer months. In November, the increase of Cd bioaccumulation under warming-stress could result from a temperature-dependent enhancement of metabolic rate in mussels whose metabolic activity is reduced during winter months (Louis et al., 2020; Nardi et al., 2017, 2018).

No clear impact of Cd was highlighted on energy-related markers in the present study. Adenylate energy charge values remained unchanged and no mitochondrial activity variation was noticed in both exposures. However, inter-individual AEC variability increased in June in Cd-exposed mussels. Energy nucleotides are tightly regulated and continuously balanced (Storey, 2004). Exposure to pollutants may lead to an increase of AEC variability without a significant decrease of the average value (Le Gal et al., 1997; Marazza et al., 1996).

Variability changes could be as informative as significant difference between means (Devin et al., 2014). This variability increase suggests a disparity of physiological status between mussels that could be due to an energy reallocation to compensatory mechanisms. Regarding mussels' energy reserves which are very labile energy storage, only a decrease in glycogen content was observed in Cd-exposed mussels after 24 hours at both temperatures in June. Glycogen could be used to ensure energy demand (Koukouzika and Dimitriadis, 2008). Glycogen is a very labile energy source and can be degraded very quickly in response to a stress, which is supported by the simultaneous increase of ATP content after 24h of Cd exposure in June.

A slight negative Cd effect was here noticed on transcript level of two OXPHOS complexes (*sdh* and *atp*) and citrate synthase mRNA expression in *D. polymorpha*. During the rest period, the transcript levels of these genes trended to decrease under Cd exposure. To the best of our knowledge, the transcript levels of these genes have never been analysed after warming and Cd multi-stress although their activities were previously investigated. A decrease in OXPHOS activity under Cd exposure has already been shown in a *D. polymorpha* sister species, *Dreissena rostriformis bugensis* (Hanana et al., 2019). In addition, Kurochkin et al. (2011) showed that mitochondrial complex II (succinate dehydrogenase) was the most

sensitive to Cd among all electron transport complexes in *Crassostrea virginica* isolated mitochondria. Temperature increase enhanced its sensitivity since a more pronounced decrease of complex II and citrate synthase activities was observed at 30°C compared to 20°C in Cd-exposed oysters (Ivanina et al., 2008). However, no variations of CS and ETS activities were observed in our study suggesting that Cd did not affect mitochondria activity in *D. polymorpha* whatever the season.

In ecotoxicological investigations on *D. polymorpha*, induction of defence mechanisms and cellular damage are usually observed under metal stress (Garaud et al., 2016; Marie et al., 2006; Potet et al., 2018, 2016; Schäfer et al., 2012). The appearance of cellular damage in June but not in November could suggest that mechanisms involved to overcome a contamination could differ over the reproduction cycle. Nardi et al. (2017, 2018) showed a greater efficiency of antioxidant protection mechanisms in winter than in summer in *Mytilus galloprovincialis* exposed to 20µg.L⁻¹ of Cd. This difference in protection efficiency between seasons could lead to a higher sensitivity of mussels exposed to pollutant during summer months.

Marker variations in response to stress are essential to assess before considering it as a potential biomarker for risk assessment. This study brought out several markers being both

stable whatever the period after acclimation under laboratory conditions and sensitive to contaminant exposure. These markers are ATP synthase and citrate synthase mRNA expression, energy nucleotides and glycogen, which may be considered as promising biomarkers.

4.3 Global response overview

The global analysis performed on control and Cd-exposed mussels' responses allowed the consideration of the whole panel of markers at each period. According to the profile of responses, the two reproduction cycle stages were separated along the first axis while the conditions (control vs Cd-exposed in November and 24h vs 7 days in June) were separated along the second axis. Exposure conditions were discriminated by the transcript levels of the mRNA expression of three genes involved in mitochondrial energy metabolism (*cs*, *sdh* and *atp*). After the acclimation to laboratory conditions, two of these markers (*cs* and *atp*) presented similar mRNA transcript level between November and June, while *sdh* mRNA expression varied between the two experimentation periods. This variation of *sdh* mRNA transcript level could result from a "laboratory effect" and lead to a misinterpretation of marker response. In the present study, the period factor represented more than 92% of the total variability of the dataset while the exposure factor represented only 0.1%. These results

comfort the potent influence of seasonality on biomarker response interpretations in ecotoxicological studies.

4. Conclusion and perspectives

This study brings a deeper understanding of how responses to chemical stress depend on the reproduction cycle in *D. polymorpha*. Strong differences were highlighted in energy metabolism responses under laboratory conditions between seasons. However, several markers (*atp*, *cs* mRNA expression, glycogen and adenylate nucleotide contents) were shown to be season-independent with the same level after laboratory acclimation period, but also sensitive to the contamination. These markers could be potentially used in contaminant effect studies in laboratory. Complementary studies on several *D. polymorpha* populations would be necessary to confirm their potential use in a biomonitoring perspective. Although energy metabolism carries vital functions out, it is also a complex and tightly regulated process. More studies considering intrinsic factor effects on energy metabolism in long-term controlled exposures are required to confirm the potential of energy-related markers as early contamination biomarkers.

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Table 1. Summary of the primers used for mRNA expression measurements. Abbreviations, NCBI accession numbers of the original sequences of the genes targeted in *D. polymorpha*.

Gene	Abbreviation	NCBI	F primers	R primers
Actin	<i>act</i>	AF082863	CCTCACCCCTCAAGTACCCCAT	TTGGCCTTTGGGTTGAGTG
Alternative oxidase	<i>aox</i>	Q9P959	GTGAACCATAACCCTCGCCTC	TGTAGTACTGCGGATGGTC
AMP deaminase	<i>ampd</i>	Q9DBT5	ACCCAGCCAGTGTGTTTCA	TTTGCTCGTGGACTCAACGT
AMP kinase α subunit	<i>ampk</i>	Q8BRK8	TGCTAAATGAAAGAGGTGACGC	CATGTGGTGGCATATACGATGT
ATP synthase	<i>atp</i>	JQ781135	GACTCCACCTCCCCTATCCT	TCCTCTGAAAGCTCGTCCAT
Catalase	<i>cat</i>	EF681763	ACGGCTATGGAAGCCACACG	AGGTCGCGCATCGCATAGTC
Cytochrome c oxidase I	<i>cox</i>	AAL55513	AGGCCCTGCGATAGATTTTT	AAAGGGACCCGGTAAAATTG
Citrate synthase	<i>cs</i>	MN579511	GTGCCCTGTGACACCCTTAC	CTGCCTGCCTTGAGTGTGT
Cytochrome b	<i>cytb</i>	DQ072120.1	CGCCACTTTAAAGCGATTTTT	TGGGTCAGCAAATAGATCTGG
Lactate dehydrogenase	<i>ldh</i>	Q7TNG8	TGCCCAAACACTACATTGCCAAAT	ATTGGGATTGGCAAACGGGA
Ribosomal protein S3	<i>s3</i>	AJ517687	CAGTGTGAGTCCCTGAGATACAAG	AACTTCATGGACTTGGCTCTCTG
Succinate dehydrogenase	<i>sdh</i>	Q9YHT2	TAGACCGTCCAGTTTAGCGC	CCTCTGCCACACATGTACGT

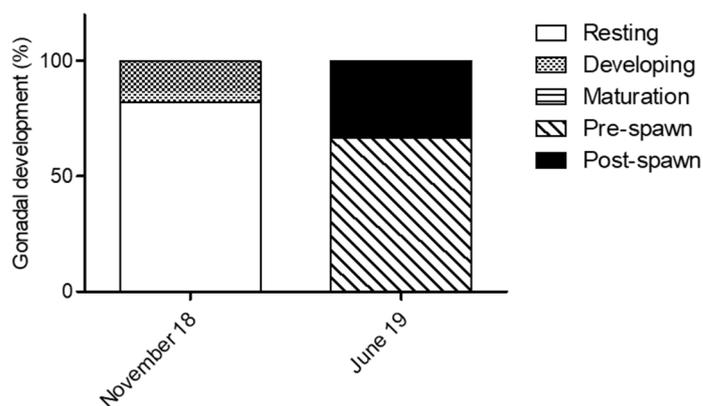


Figure 1. Gonadal development stage in November 2018 and June 2019 observed in overall male and female mussels ($n=12$). Different development stages are represented in percentage of each stage.

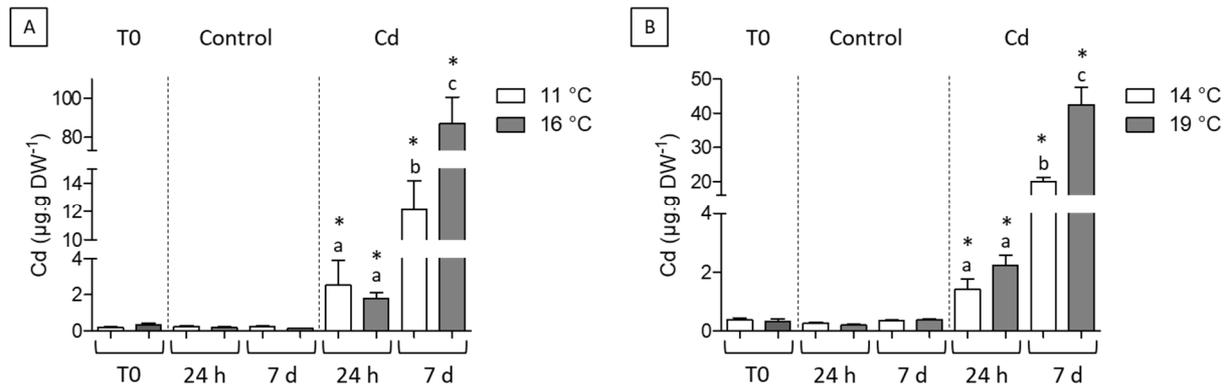


Figure 2. Metal tissues concentrations before (T0) and during exposure (24h and 7d) in control and Cd-exposed mussels in November 2018 (A) and June 2019 (B) at *in situ* temperature (white) and *in situ* temperature + 5°C (grey). Data are expressed as mean ± SD. Asterisk indicates significant difference with controls. Different letters indicate significant difference between Cd-exposed mussels ($p < 0.05$) ($n=10$).

Table 2. Inter-seasonal variations of marker responses measured on mussels sampled after the acclimation period (T0). Results were expression as mean \pm SD ($n = 10$, $p < 0.05$).

	November 2018	June 2019	<i>p</i> value
<i>ldh</i>	0.01 \pm 0.005	0.02 \pm 0.004	0.001
<i>aox</i>	0.6E⁻³ \pm 0.3E⁻³	0.3E-3 \pm 0.3E⁻³	0.048
<i>cox</i>	1.21 \pm 0.36	4.2 \pm 2.5	0.002
<i>ampd</i>	0.0043 \pm 0.02	0.01 \pm 0.006	0.001
<i>ampk</i>	0.01 \pm 0.009	0.02 \pm 0.01	0.05
<i>sdh</i>	5.38E⁻⁵ \pm 1.3E⁻⁵	2.42E⁻⁵ \pm 1.3E⁻⁵	0.048
<i>cytb</i>	0.40 \pm 0.25	0.18 \pm 0.14	0.06
<i>atp</i>	0.15 \pm 0.03	0.016 \pm 0.06	0.64
<i>cs</i>	0.05 \pm 0.01	0.04 \pm 0.01	0.08
<i>cat</i>	0.02 \pm 0.01	0.02 \pm 0.01	0.58
ETS	0.74 \pm 0.11	0.99 \pm 0.09	1.40E⁻⁸
LDH	89.61 \pm 25.03	105.4 \pm 18.3	0.03
COX	5.40 \pm 2.1	1.62 \pm 0.62	2.10E⁻¹⁰
CS	88.04 \pm 24.47	100.93 \pm 35.03	0.09
IMP	0.33 \pm 0.17	0.21 \pm 0.17	0.04
ATP	5.18 \pm 2.28	4.30 \pm 2.10	0.42
ADP	2.82 \pm 0.72	2.01 \pm 0.64	0.05
AMP	1.47 \pm 0.59	0.99 \pm 0.59	0.06
AEC	0.68 \pm 0.12	0.71 \pm 0.06	0.43
Lipids	0.61 \pm 0.12	0.50 \pm 0.19	0.08
Glycogen	1.09 \pm 0.77	1.38 \pm 0.60	0.46
Proteins	35.70 \pm 6.57	48.67 \pm 14.46	0.001
LOOH	22.37 \pm 15.13	21.82 \pm 5.51	0.34

Table 3. *D. polymorpha* marker responses after 24 hours and 7 days of exposure to Cd at *in situ* temperature and *in situ* temperature + 5°C. Data are expressed as mean ± SD (*n* = 10). Abbreviations: Temp. (temperature); ETS (electron transport system); CS (citrate synthase); COX (cytochrome c oxidase); LDH (lactate dehydrogenase); *cytb* (cytochrome b gene); *ampd* (AMP deaminase gene); *ampk* (AMP-activated protein kinase gene) *aox* (alternative oxidase gene). Units: μmol.g DW⁻¹ (ADP, AMP and IMP), mg.g FW (proteins, lipids), mmolO₂.g proteins⁻¹.h⁻¹ (ETS), U.g proteins⁻¹ (CS and COX), μmolNADH.g proteins⁻¹.h⁻¹ and relative mRNA expression (*ampk*, *ampd*, *ldh*, *cytb*, *cox* and *aox*).

Month	Time	Temp.	Condition	ADP	AMP	IMP	Proteins	Lipids	ETS	CS	COX	LDH	<i>ampk</i>	<i>ampd</i>	<i>ldh</i>	<i>cytb</i>	<i>cox</i>	<i>aox</i>
November 2018	24 hours	11 °C	Control	2,68 ± 0,28	1,18 ± 0,55	0,23 ± 0,12	37,56 ± 3,23	0,55 ± 0,09	0,77 ± 0,09	87,09 ± 19,04	4,81 ± 1,54	86,87 ± 27,89	0,013 ± 3,7E-03	0,006 ± 2,8E-03	0,009 ± 4,4E-03	0,45 ± 0,15	1,58 ± 1,29	0,0005 ± 1,8E-04
			Cd	2,56 ± 0,63	1,19 ± 0,34	0,24 ± 0,09	35,31 ± 4,78	0,53 ± 0,11	0,76 ± 0,08	91,98 ± 13,14	5,14 ± 1,65	76,71 ± 16,68	0,014 ± 6,3E-0,3	0,004 ± 1,8E-03	0,009 ± 1,5E-03	0,51 ± 0,29	1,28 ± 0,62	0,0004 ± 1,8E-04
		16 °C	Control	2,67 ± 0,34	0,83 ± 0,29	0,24 ± 0,13	39,83 ± 8,76	0,65 ± 0,17	0,74 ± 0,07	88,54 ± 13,16	5,78 ± 1,65	89,62 ± 20,31	0,012 ± 3,9E-03	0,004 ± 1,6E-03	0,009 ± 2,9E-03	0,37 ± 0,14	0,91 ± 0,41	0,0004 ± 1,8E-04
			Cd	2,82 ± 0,57	1,16 ± 0,5	0,26 ± 0,07	34,92 ± 8,11	0,58 ± 0,16	0,80 ± 0,1	89,17 ± 15,11	3,96 ± 1,56	75,33 ± 17,20	0,018 ± 9,1E-03	0,003 ± 1,1E-03	0,01 ± 2,9E-03	0,40 ± 0,21	1,16 ± 0,65	0,0006 ± 3,4E-04
	7 days	11 °C	Control	2,91 ± 0,87	1,27 ± 0,75	0,19 ± 0,11	42,34 ± 11,3	0,57 ± 0,09	0,70 ± 0,04	78,08 ± 10,85	4,27 ± 1,47	70,84 ± 20,67	0,011 ± 8,2E-03	0,004 ± 2,1E-03	0,006 ± 2,9E-03	0,34 ± 0,14	0,93 ± 0,51	0,0005 ± 2E-04
			Cd	3,10 ± 0,82	1,40 ± 0,46	0,26 ± 0,13	37,48 ± 11,8	0,68 ± 0,15	0,71 ± 0,07	78,76 ± 13,23	5,31 ± 1,43	79,32 ± 13,66	0,010 ± 3,9E-03	0,005 ± 1,5E-03	0,005 ± 1,7E-03	0,27 ± 0,07	1,10 ± 0,52	0,0006 ± 3,2E-04
		16 °C	Control	2,89 ± 0,63	1,40 ± 0,58	0,21 ± 0,11	45,54 ± 9,83	0,63 ± 0,10	0,79 ± 0,09	82,02 ± 17,22	5,73 ± 1,19	78,43 ± 13,43	0,015 ± 8,7E-03	0,004 ± 1,2E-03	0,01 ± 4E-03	0,44 ± 0,20	1,05 ± 0,66	0,0006 ± 2,8E-04
			Cd	2,93 ± 0,54	1,38 ± 0,95	0,27 ± 0,15	43,90 ± 6,88	0,61 ± 0,08	0,75 ± 0,08	77,20 ± 11,12	5,88 ± 2,6	78,73 ± 8,53	0,013 ± 5,3E-03	0,004 ± 1,3E-03	0,007 ± 2,2E-03	0,38 ± 0,13	0,86 ± 0,33	0,0005 ± 2,7E-04
June 2019	24 hours	14 °C	Control	1,11 ± 0,27	0,37 ± 0,13	0,09 ± 0,04	52,94 ± 13,34	0,49 ± 0,1	0,92 ± 0,14	107,63 ± 25,01	1,76 ± 0,82	103,41 ± 20,93	0,022 ± 4,4E-03	0,0137 ± 5,7E-03	0,018 ± 6,9E-03	0,19 ± 0,10	5,97 ± 4,03	0,0003 ± 1,2E-04
			Cd	1,07 ± 0,68	0,30 ± 0,12	0,06 ± 0,02	48,13 ± 10,61	0,59 ± 0,57	0,83 ± 0,24	118,56 ± 73,00	1,34 ± 0,81	90,75 ± 16,90	0,029 ± 2,1E-02	0,011 ± 3,9E-03	0,02 ± 8,3E-03	0,21 ± 0,12	5,24 ± 2,47	0,0003 ± 3,4E-04
		19 °C	Control	1,10 ± 0,41	0,35 ± 0,11	0,1 ± 0,06	47,82 ± 12,27	0,40 ± 0,15	0,83 ± 0,16	89,79 ± 18,75	1,59 ± 1,11	118,21 ± 34,24	0,031 ± 2,3E-02	0,010 ± 3,6E-03	0,016 ± 6,1E-03	0,21 ± 0,14	5,75 ± 3,91	0,0002 ± 1,2E-04
			Cd	1,09 ± 0,16	0,42 ± 0,27	0,07 ± 0,03	56,19 ± 11,60	0,46 ± 0,23	0,84 ± 0,25	99,97 ± 46,70	1,37 ± 0,41	94,42 ± 28,43	0,023 ± 9,3E-03	0,01 ± 4,2E-03	0,017 ± 6,8E-03	0,23 ± 0,12	7,90 ± 3,97	0,0003 ± 2,2E-04
	7 days	14 °C	Control	0,84 ± 0,21	0,33 ± 0,20	0,04 ± 0,02	53,04 ± 17,30	0,47 ± 0,39	0,90 ± 0,20	89,34 ± 28,39	1,69 ± 0,65	103,47 ± 20,80	0,036 ± 2,3E-03	0,016 ± 6,2E-03	0,02 ± 1,2E-02	0,29 ± 0,21	6,54 ± 3,69	0,0004 ± 3,6E-04
			Cd	0,98 ± 0,40	0,50 ± 0,10	0,05 ± 0,02	52,80 ± 11,41	0,54 ± 0,24	0,89 ± 0,32	85,17 ± 19,89	1,13 ± 0,32	112,15 ± 32,55	0,025 ± 1,1E-02	0,013 ± 6,2E-03	0,018 ± 7,5E-03	0,24 ± 0,08	7,42 ± 3,81	0,0002 ± 9,7E-05
		19 °C	Control	1,34 ± 0,36	0,74 ± 0,12	0,08 ± 0,06	52,01 ± 12,71	0,39 ± 0,13	0,80 ± 0,13	83,42 ± 33,49	1,19 ± 0,43	75,90 ± 25,43	0,032 ± 1,2E-02	0,017 ± 1,3E-02	0,03 ± 1,2E-02	0,30 ± 0,13	13,53 ± 7,10	0,0005 ± 4,0E-04
			Cd	0,87 ± 0,47	0,43 ± 0,37	0,04 ± 0,07	56,15 ± 20,08	0,52 ± 0,14	0,91 ± 0,19	101,98 ± 39,44	1,77 ± 0,89	96,40 ± 32,20	0,04 ± 2,3E-02	0,0158 ± 6,0E-03	0,019 ± 9,3E-03	0,22 ± 0,10	6,45 ± 3,08	0,0003 ± 1,6E-04

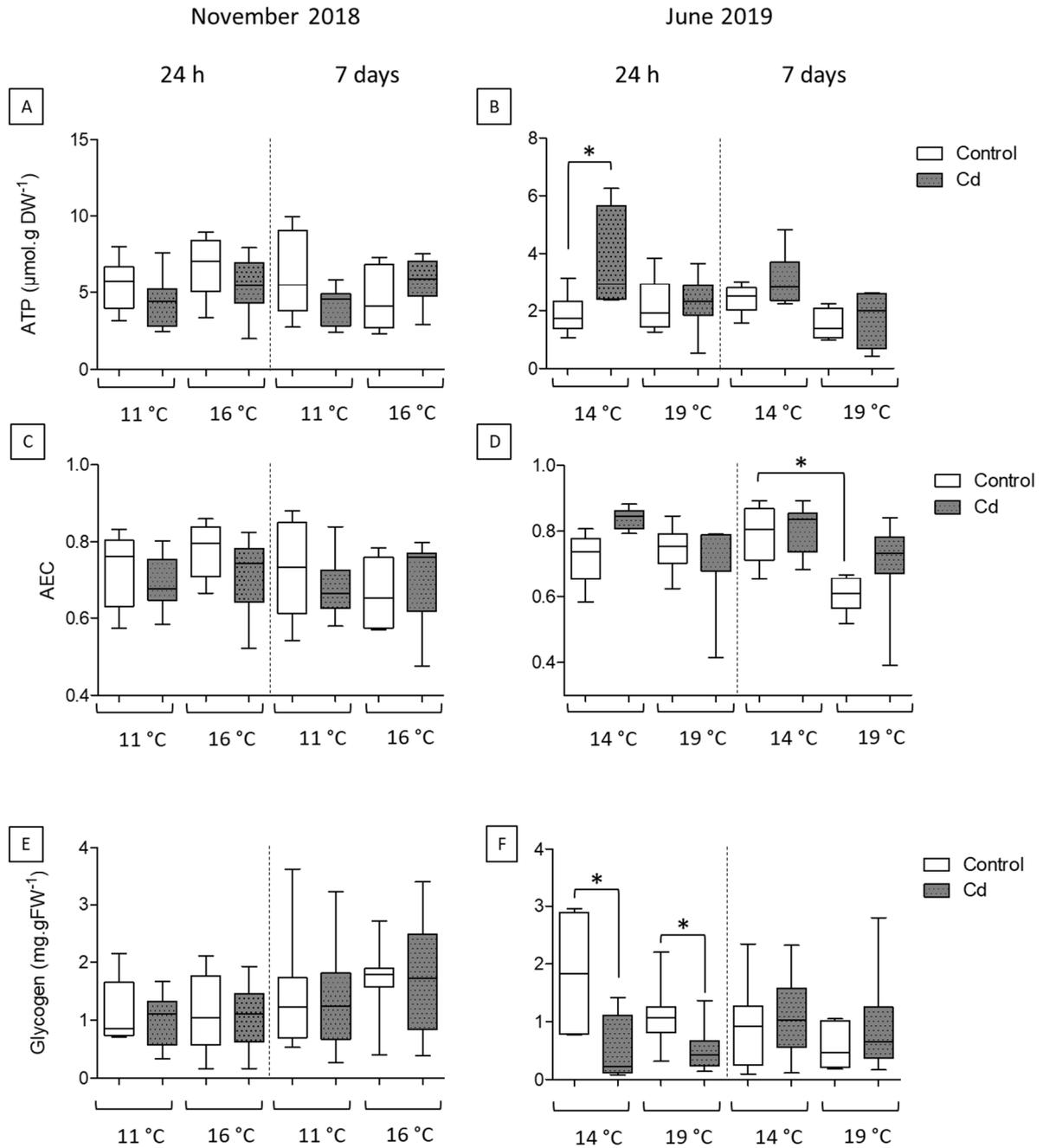


Figure 3. ATP contents, AEC and glycogen contents measured in November 2018 (A, C and E respectively) and June 2019 (B, D and F respectively) in control mussels (white boxes) and Cd-exposed mussels (grey boxes) at *in situ* temperature (11°C and 14°C) and *in situ* temperature + 5°C (16°C and 19°C) ($n=10$). Data are expressed as median \pm SD. Asterisk represent significant difference between two conditions ($p < 0.05$).

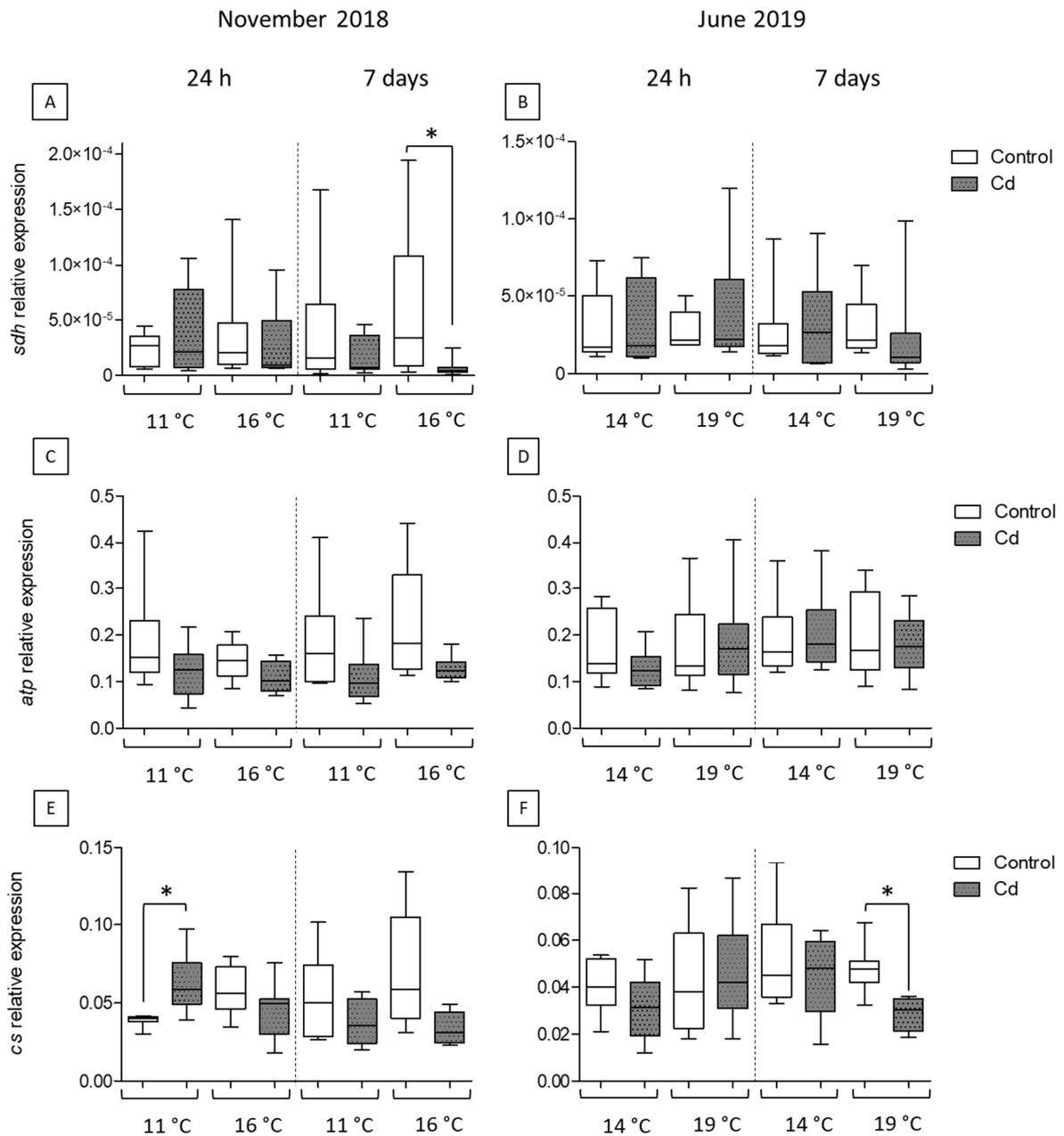


Figure 4. *sdh*, *atp* and *cs* relative transcript expression levels measured in November 2018 (A, C and E respectively) and in June 2019 (B, D and F respectively) in control mussels (white boxes) and Cd-exposed mussels (grey boxes) at *in situ* temperature (11°C and 14°C) and *in situ* temperature + 5°C (16°C and 19°C) ($n=10$). Data are expressed as median \pm SD. Asterisk represent significant difference between two conditions ($p < 0.05$).

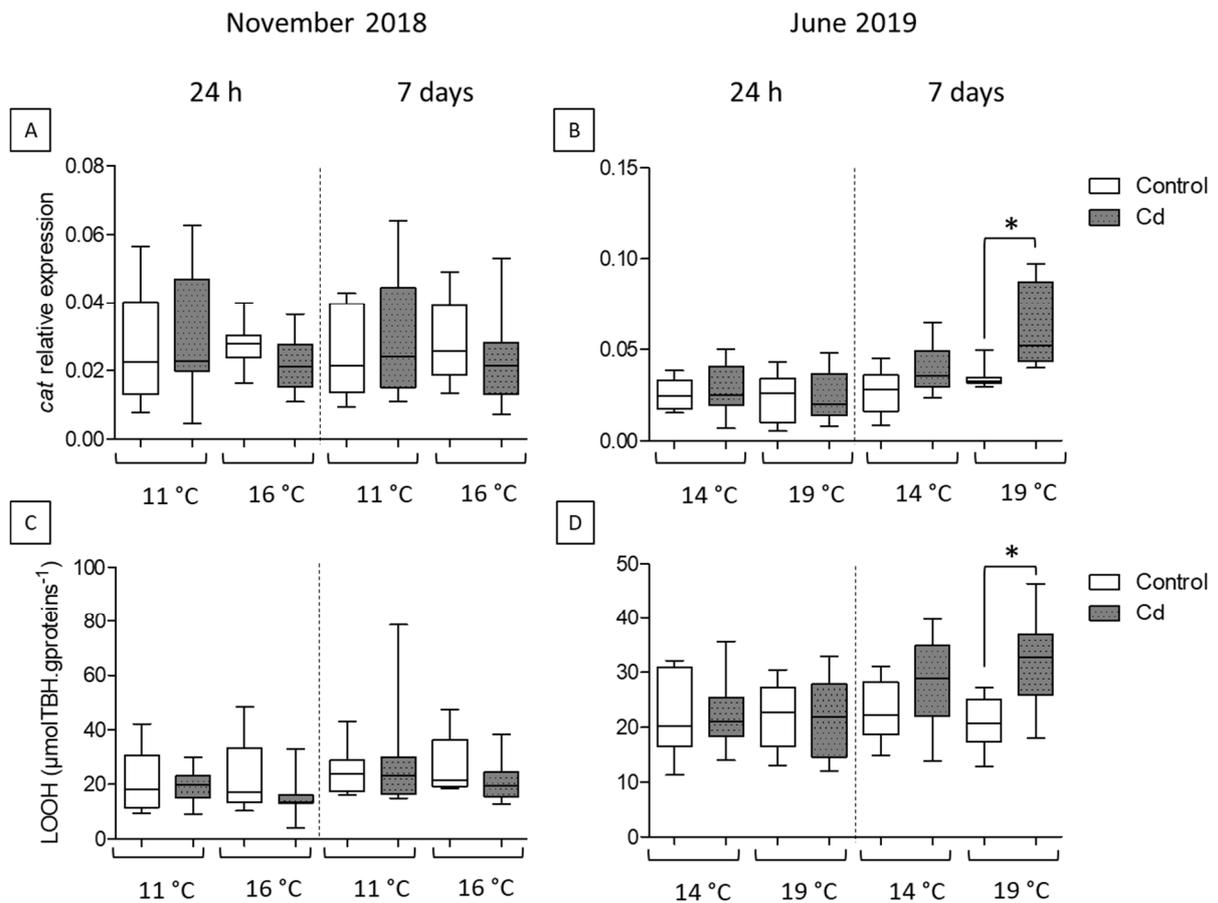


Figure 5. *cat* relative transcript expression level and lipid peroxidation measured in November 2018 (A and C respectively) and June 2019 (B and D respectively) in control mussels (white boxes) and Cd-exposed mussels (grey boxes) at *in situ* temperature (11 °C and 14 °C) and *in situ* temperature + 5 °C (16 °C and 19 °C) ($n=10$). Data are expressed as median \pm SD. Asterisk represents significant difference between two conditions ($p < 0.05$).

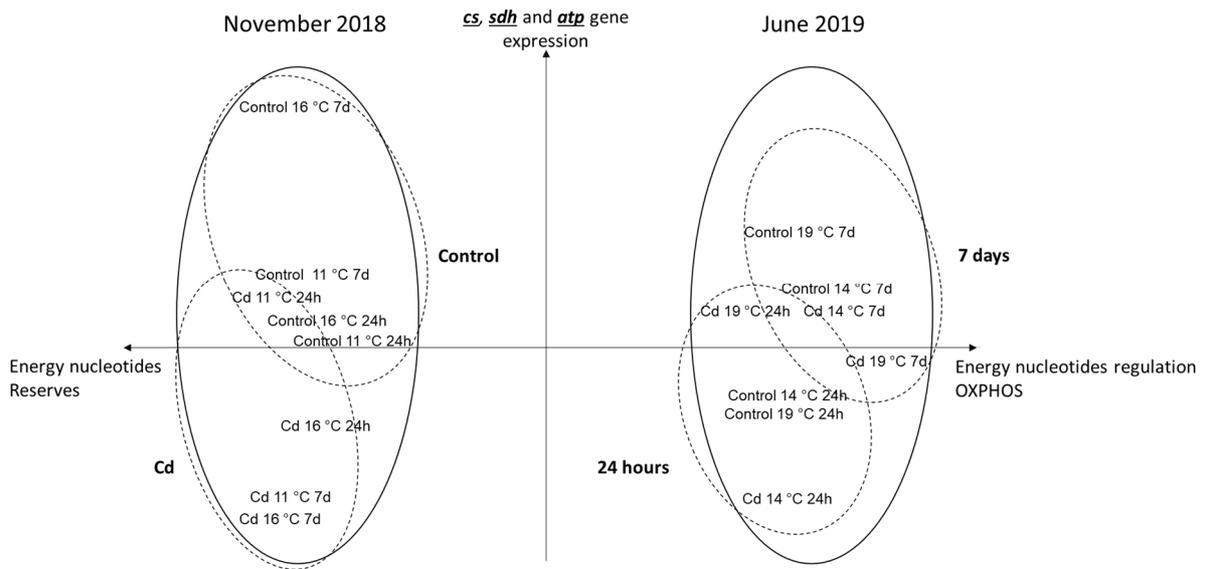


Figure 6. Partial Least Square Discriminant Analysis (PLS-DA) representing biomarkers responses in November 2018 and June 2019 for controls and $10\mu\text{g.L}^{-1}$ Cd-exposed mussels at *in situ* temperature and *in situ* temperature + 5°C . The F1-F2 axes represent 62.4% and 11.5% of the total variance of the data set respectively. Markers in bold and underlined indicate VIP markers.

Supplementary data

Table S1. Physicochemical parameters of water at the two sampling months. N.D: not detected.

Date	Temperature (°C)	Oxygen (%)	pH	Conductivity	Cd ($\mu\text{g.L}^{-1}$)
10/23/2018	11.9	83	7.562	578	N.D
05/15/2019	15.1	101	7.747	562	N.D

Table S2. Physicochemical parameters of exposure media (mean \pm SD).

Month	Condition	Temperature (°C)	pH	Oxygen (%)	Conductivity (μS)
Nov. 18	Control 11 °C	11.2 \pm 0.2	8.2 \pm 0.2	109.1 \pm 6.6	546 \pm 12
	Cd 11 °C	11.2 \pm 0.2	8.1 \pm 0.2	107.1 \pm 6	543 \pm 13
	Control 16 °C	16.4 \pm 0.5	8 \pm 0.2	109.3 \pm 5.2	555 \pm 4
	Cd 16 °C	16.4 \pm 0.5	8 \pm 0.3	108 \pm 7.5	559 \pm 7
June 19	Control 14 °C	13.7 \pm 0.4	7.6 \pm 0.4	95.9 \pm 0.7	522 \pm 6
	Cd 14 °C	13.8 \pm 0.5	7.8 \pm 0.3	92.7 \pm 0.4	534 \pm 5
	Control 19 °C	19 \pm 0.4	7.7 \pm 0.2	90.6 \pm 0.5	535 \pm 5
	Cd 19 °C	18.8 \pm 0.5	7.8 \pm 0.3	92 \pm 0.5	525 \pm 5