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The impacts of cadmium on circadian redox homeostasis in freshwater mussels

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ABSTRACT

The purpose of this study was to examine the effects of cadmium (Cd) on the daily pattern of energy metabolism and the redox status in *Dreissena bugensis*. Mussels were exposed to 50 and 100 µg/L $CdCl_2$ for 7 days at 15 °C under constant aeration. At the end of the exposure period, the mussels were collected at different times of the day, ranging from 09:00 to 24:00 for metallothioneins (MT), lipid peroxidation (LPO), peroxidase, thioredoxin reductase (TrxR), NAD(P)H oxidase and NAD^+ kinase activity assessments. The results revealed that all of the above followed a circadian rhythm in their activity/levels where MT, TrxR and LPO peaks occurred in the morning between 09:00 to 12:00 and the others peaked at 21:00. Exposure to Cd readily increased MT levels in mussels up to 6-fold at 09:00, although the daily variation was quenched. LPO levels remained high at 18:00–21:00 compared with the controls and they lost their cycle. Frequency analysis of the oscillatory behavior of the above revealed the presence of circadian (24-h) frequency, with the exception of NAD^+ kinase, which showed a 12–14 h frequency. The cyclic activity in TrxR, peroxidase, NAD^+ kinase, MT and LPO were altered by Cd exposure while NAD(P)H oxidase and NAD(H)P levels were not affected by Cd. In conclusion, a daily pattern of biomarkers involved in mitochondrial activity and redox status was found in mussels, and exposure to contaminants such as Cd disrupted the oscillatory pattern in redox status during energy metabolism.

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INTRODUCTION

Circadian rhythms exist within a wide range of biological processes and control many aspects of physiology, including fasting/eating, energy metabolism, reproduction, and locomotor activity. The integration of cyclic rhythms in physiological functions has represented an evolutionary advantage for unicellular and multicellular organisms, allowing them to anticipate recurrent cyclical changes in the environment. Moreover, cyclic daily changes are commonly found in most organisms including mollusks [1, 2]. Most canonical clock genes are active in oysters and involve *clock*, *bmal/cycle*, *period*, *timeless* and *vertebrate type cry*, *rev-erb* and *ror* genes which are also found in vertebrates. Redox homeostasis is such an example of integration of cellular energy metabolism and respiration given that mitochondria are the major generator of reactive oxygen species (ROS). Mitochondria are major suppliers of cellular energy through oxidative phosphorylation (aerobic glycolysis) and have to adapt to a transient nutrient supply and energy demands for optimal cell functioning. It has been shown that the inability of mitochondria to adapt to environmental changes is associated with metabolic diseases leading to excessive loss or gain of energy in the form of carbohydrates and lipids respectively [3]. Mitochondria oxidize carbohydrates and lipids to generate ATP during the coordinate process of β-oxidation, the tricarboxylic acid cycle and electron

transport activity. During the tricarboxylic acid cycle, glucose is gradually oxidized to form NADH and CO_2. NADH, in turn, supplies electrons to a series of electron transport proteins leading to the formation of a transmembrane proton gradient needed for the synthesis of ATP. During that process, ROS are formed and require anti-oxidative measures to preserve the redox status and integrity of mitochondria. Recent evidence suggests that circadian clocks regulate daily energy metabolism demands [4, 5]. During the day, changes in food availability and energy demands need to be addressed by the mitochondria, which are the central nexus for energy metabolism. Given that mitochondria are the major source of production in ROS and also exhibit a circadian rhythm [6], the presence of contaminants during these processes could pose a risk to organisms.

It was found that more of 40% of the transcriptome in *Mytilus californianus* mussels exhibits cyclical gene expression [2]. Both tidal and circadian rhythms were observed where circadian rhythm dominated. The production of NADH during the tricarboxylic acid cycle was also shown to oscillate in yeast and rat muscle mitochondria, which are considered major cell oscillators [7]. Mitochondria activity also oscillates during the day in a circadian manner [8]. The majority of cycling mitochondrial proteins peaked in the day and the rate-limiting mitochondrial enzymes that process lipids and carbohydrates accumulate in a circadian manner. They were also associated with clock PERIOD proteins (Per1/2), which are involved in circadian control. The expression of clock-associated genes was also shown to be influenced by seasons [9]. For example, Clock, Cry1 and ROR/HR3 changes occurred seasonally within gender while timeout-like gene expression was invariant. This suggests that circadian gene expression could be modulated by other cues involved in seasonal changes such as temperature, food availability and gametogenesis. Oscillations in mitochondria are also tightly synchronized with the circadian rhythm in redox status [6, 10]. Peroxiredoxins (Prx) are major antioxidant enzymes with peroxidase activity in mitochondria and cytoplasm that follow a diurnal activity pattern. Cysteines in the active center of Prx are oxidized by ROS and their reduction depends on reduced thioredoxin levels which are maintained by NADH-dependent thioredoxin reductase (TrxR). This process is the main pathway for handling ROS production during mitochondria activity (respiration and ATP production). The oxidized state of Prx oscillates during the day and this process is seemingly independent of genetic control [11]. Prx are found in both eukaryotes and prokaryotes and represent perhaps one of the earliest modes of redox control since the Great Oxidation Event that occurred some 2.5 billion years ago [12]. Prx are considered an internal timekeeping mechanism independent of transcription and are intimately associated with the control of ROS production during mitochondrial activity. Hepatic glutathione (GSH), a major peptide in redox homeostasis, also presented a marked circadian pattern along with GSH S-transferase (also involved in phase II biotransformation of xenobiotics) and GSH-peroxidase in mice [10]. Other antioxidant enzymes such as Cu/Zn-superoxide dismutase, catalase, cyclooxygenase-2 and heme oxygenase also displayed a circadian behavior where their activities were highest during the early period of the day. Interestingly, acetaminophen toxicity was more severe when administered in the afternoon when these antioxidant activities were at their lowest. Cadmium (Cd) was shown to produce oxidative stress leading to increased ROS production and lipid peroxidation (LPO) [12, 13]. Cd is a potent inducer of metallothioneins (MT), which are involved in the sequestration of ionic Cd and ROS [14, 15]. MT levels were also shown to have a circadian rhythm [16] which should be in phase with ROS production in cells. In a recent review of the influence of contaminants on circadian rhythms and other oscillators in cells, it was shown that freshwater mussels exhibit a circadian pattern in MT levels and this pattern was altered when exposed to Cd [17]. This raised the issue of the potential effects of environmental contaminants on the circadian control of internal biochemical processes related to oxidative stress and energy metabolism.

The purpose of this study was therefore to examine the effects of Cd on the daily changes of energy metabolism and redox status in quagga mussels (*Dreissena bugensis*). This mussel species is ubiquitous in the Saint-Lawrence River and a good candidate as bioindicator species for water quality monitoring. Energy metabolism was examined following changes in mitochondrial NAD(P)H oxidase activity, endogenous NAD(P)H levels and

cytosolic NAD^+ kinase activity, while redox status was examined by following diurnal changes in thioredoxin reductase, peroxidase and LPO activity in mitochondria. MT levels were also examined given their cyclic behavior and their role in Cd detoxification. The heavy metal Cd was selected as a model compound because of its presence in many contaminated environments which induces oxidative stress and strong detoxication responses such as MT induction.

MATERIALS AND METHODS

Mussel handling and exposure

Adult quagga mussels (*Dreissena bugensis*) were collected in August 2017 at a reference site in the St. Lawrence River near Montréal, Quebec, Canada (45°19′50″N, 73°58′12″W). Mussels (2–3 cm long) were gently cut off from the rocks and quickly transferred to the laboratory in bags filled with river water at 4 °C. Before exposure to Cd, mussels were acclimated for a period of 4 weeks in 50 L aquaria filled with dechlorinated and UV-treated tap water at 15 °C on a 16-h light/8-h dark cycle under constant aeration. The light cycle was operationally set with no assumptions for mussels; it consisted of light between 6:00 to 22:00 followed by an 8-h dark phase between 22:00 and 6:00. The mussels were fed 3 times per week with concentrates of phytoplankton (Phytoplex, Kent Marine, WI) and *Pseudokirchneriella subcapitata* algal preparations (2 L of 100 million cells/mL).

The mussels were exposed to 50 and 100 µg/L Cd^{2+} in 4-L containers lined with polyethylene bags. The Cd concentrations were selected to elicit changes in detoxication mechanisms and oxidative stress in mussels and do not pertain to any particular polluted environment. Each container held N = 28 mussels and 3 containers were used for each treatment (i.e., 3 control tanks and 3 tanks for each Cd concentration). The mussels were exposed to these conditions for 7 days at 15 °C under constant aeration. On Day 3, the mussels were fed once by adding the algae feed as described above. After feeding, the aquarium water was replaced and Cd was added again. The control group consisted of mussels exposed to tap water dechlorinated using carbon filters and UV irradiation. Before the treatment, we assessed the baseline levels (t = 0) of biomarkers on mussels collected from the acclimation aquarium. At the end of the exposure time, the mussels were collected at different times of the day to determine circadian changes in the biomarkers. At each sampling time (09:00, 12:00, 15:00, 18:00, 21:00, 24:00 and 32:00), 4 mussels were removed and immediately stored at -80 °C until analysis to avoid variability due to the dissection time.

Biomarker analyses

Soft tissues were individually dissected, weighted and homogenized at 1:5 (w/v) ratio in ice-cold 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM ethylenediaminetretraacetic acid (EDTA) and 1 µg/mL apoprotinin. Homogenization was performed using a plastic Polytron with one 30-second burst at 5,000 rpm. The homogenates were centrifuged at 2,000 g for 10 min at 2 °C; the resulting supernatant was centrifuged at 10,000 g for 20 min at 2 °C and the supernatant was collected for determination of NAD^+ kinase activity (NADK), peroxidase, TrxR and MT. The pellet was resuspended in 250 mM sucrose, 10 mM tris-HCl, pH 7.5, 0.1 mM $MgCl_2$ and 1 mM KCl and used as a mitochondrial fraction to measure lipid peroxidation (LPO), NAD(P)H levels and NAD(P)H oxidase. The supernatant (S10) and the mitochondrial fraction were stored at -80 °C until analysis. Concentrations of the studied biomarkers were normalized with the total individual protein concentration according to the protein-dye binding methodology [18] using standard solutions of serum bovine albumin for calibration.

Activity of thioredoxin reductase (TrxR) was assayed in 100 mM potassium phosphate, pH 7.0, containing 10 mM EDTA according to Tedesco *et al*. [19]. In the presence of NAD(P)H, the TrxR reduces 5,5'-dithiobisnitrobenzoic acid (DTNB) to produce 2-nitro-5-thiobenzoate. The reaction was therefore initiated by the addition of DTNB and the change in absorbance caused by TNB formation was monitored at 412 nm for 30 min in a plate reader. As DTNB can also react with glutathione reductase and glutathione peroxidase, the assay was performed in the presence and absence of aurothiomalate (ATM), a specific TrxR inhibitor, at 20 µM, thus allowing specific TrxR activity to be determined. Enzymatic activity was expressed as nmoles of TNB formed/min/mg of proteins.

The total MT levels were measured using the silver saturation assay [20] with the modification for non-radioactive silver [21]. The silver concentration in the supernatant fraction was determined using an atomic absorption spectrophotometer equipped with a Zeeman-effect background correction. A ratio of 12 moles of bound Ag to 1 mole of MT was assumed for mussel MT [13]. Results were expressed as nmoles of MT equivalent mg-1 of proteins and normalized at 09:00. Lipid peroxidation (LPO) was evaluated in the mitochondrial fraction using the thiobarbituric acid method [22]. The formation of thiobarbituric acid (TBARS) reactants was measured by fluorescence at 540 and 590 nm for excitation and emission wavelengths, respectively. Standard solutions of tetramethoxypropane were used for instrument calibration. The data were expressed as nmoles of TBARS per mg of mitochondrial proteins and normalized to 09:00. The activity of NAD(P)H oxidase activity was determined in mitochondria. The mitochondria suspension was diluted to 0.1 mg/mL in 250 mM sucrose, 10 mM tris-HCl, pH 7.5, 0.1 mM $MgCl_2$ and 1 mM KCl. After 5 min, 0.1 mM NADH was added to the suspension and fluorescence at 360 nm excitation and 460 nm emission measurements were taken at each 5 min for 30 min at 30 °C. The total volume of the assay was 200 µL and the reaction was performed in a 96-well dark microplate for fluorescent microplate measurements (Synergy 4, USA). The activity was reported as a decrease in fluorescence/min/mg proteins and normalized to the activity at 09:00.

The measurement of the NAD^+ kinase activity was performed in the S10 fraction according to the protocol described by Pollak *et al.* [23] with slight modifications. The assay is based on the production of $NADP^+$ from NAD^+ by the enzyme NAD^+ kinase. $NADP^+$ is then determined by monitoring the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the phenazine methosulphate intermediate [24]. The reduction of MTT was monitored at 600 nm for 30 min. First, a 20 µL sub-sample of the S10 fraction was added to a reaction mixture containing 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM NAD^+ and 10 mM ATP in a final volume of 100 µL followed by incubation for 20 min at 30 °C for the conversion of NAD^+ to $NADP^+$. The amount of NADP produced was then determined using a second reaction to convert $NADP^+$ to NAD(P)H. A 20-µL sample of the mixture was transferred to 2 microplate wells containing 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 unit of NADP-specific yeast glucose-6-phosphate dehydrogenase, 0.5 mM MTT and 1.5 mM phenazine methosulfate (PMS). The amount of NAD(P)H ($NADP^+$) was quantified by determining the change in absorbance/min/mg of proteins and normalized to 09:00 time.

Peroxidase activity was determined using the dichlorofluorescein assay as explained elsewhere [25]. Briefly, in the presence of hydrogen peroxide and peroxidase, the non-fluorescent dichlorodihydrofluorescein (DCFDA) probe is oxidized into a fluorescent dichlorofluorescein intermediate. The reaction mixture containing 50 mM KH_2PO_4, 0.5 mM EDTA, pH 7.0, 1 mM aminotriazole, 10 µM DCFDA and 0.1 mM H_2O_2 was added to 25 µL of S10. The activity was expressed as nmole fluorescein formed using a standard curve of fluorescein and normalized against total protein content in the S10 fraction. The formation of fluorescein was measured by fluorescence at 485 and 520 nm for excitation and emission wavelengths, respectively. The data were normalized against the activity at 09:00. NAD(P)H oxidase activity was determined by adding 0.2 mM NADH to a 0.1 mg/mL mitochondrial suspension in 50 mM Tris-HCl, pH 7.4, in 200 mM sucrose at 30 °C. NADH fluorescence was measured at 360 nm excitation and 450 nm emission using a microplate reader every 5 min for 30 min. The activity was expressed as a decrease in NADH fluorescence/min/mg proteins and normalized to 09:00. For endogenous NAD(P)H levels, fluorescence was made at 360 nm excitation and 450 nm emission in a 0.1 mg/mL mitochondrial suspension. Fluorescence values were normalized to 09:00 values.

Data analysis

The experiment was repeated 3 times with N = 28 mussels in each Cd exposure and the control tanks. The data were subjected to normality and variance homogeneity testing using the Shapiro-Wilks and Brown-Forsyth tests, respectively. The data were then subjected to 2-way factorial analysis of variance (ANOVA) with circadian time (09:00, 12:00, 15:00, 18:00, 21:00, and 24:00) and Cd exposure concentration (0, 50 and 100 µg/L) as the main factors. Critical differences between groups were

confirmed by the Fisher Least Square Difference test. Correlations between biomarkers were determined using the Pearson moment correlation test. Frequency analysis of the biomarker data was determined using Fourier transformation analysis to obtain the periodogram values. Briefly, Fourier transformation consists in finding the sine and cosine functions of the data: $g(w) = a0 + \sum(A\sin(wt) + B\cos(wt))$ where w is the frequency and t is time (discrete Fourier transformation). The coefficients A and B are related to the amplitude of changes and are used to obtain the periodogram value (which is the square sum of A and B and represents the variance of the amplitude at each frequency signal). All tests were performed using Statistica (version 13 TIBCO scientific, USA) and the significance was set at $\alpha = 0.05$ in all cases.

RESULTS

The MT levels were followed at different times of the day in control mussels and in those treated with Cd (Figures 1A and 1B). In the control mussels, the MT levels oscillated during the day (ANOVA $p < 0.001$) with the maximum (acrophase) at 12:00 (day) and minimum (bathyphase) at 24:00. When exposed to Cd, a significant interaction between Cd exposure concentration and circadian time (factorial ANOVA $p = 0.01$) was observed. In the mussels collected at 09:00, the MT levels were readily induced at 3.5- and 4.2-fold relative to control mussels exposed to 50 and 100 µg/L Cd, respectively. The daily rhythm of MT levels in the mussels exposed to Cd was still present but enhanced compared with that of the control mussels. Indeed, the MT levels rose 1.4-fold at the acrophase in the control mussels and the mussels exposed to 50 µg/L while they rose 1.6 fold in the mussels exposed to 100 µg/L Cd. The circadian MT levels in the control mussels were significantly correlated with circadian MT levels in the mussels exposed to 50 µg/L ($r = 0.69$; $p < 0.001$) and 100 µg/L ($r = 0.62$; $p < 0.001$). The difference between the 2 slopes was not significantly different, which suggests that the circadian rhythm was not significantly altered. The LPO levels were also examined in quagga mussels (Figure 1C). The daily changes in LPO were similar for MT with the acrophase at 12:00 (noon) followed by a drop at 21:00 in the control mussels (ANOVA $p < 0.01$) (Figure 1C). In the presence of Cd, the LPO levels were no longer significantly affected by the time of day for the 100 µg/L Cd-exposure group (Figure 1D). LPO levels were higher only during the bathyphase for the 100 µg/L Cd group compared with the control mussels. A significant correlation between circadian LPO levels in the controls and the 50 µg/L Cd group was obtained ($r = 0.43$; $p < 0.05$), which suggests that mussels were still able to maintain the daily pattern at this concentration. However, this was lost at the 100 µg/L Cd concentration. In the controls, LPO levels were significantly correlated with MT levels ($r = 0.42$, $p < 0.05$), but this trend was not seen in the mussels exposed to Cd (Table 1, only 100 µg/L Cd is shown).

TrxR activity peaked between 15:00 and 18:00, while peroxidase activity peaked at 21:00 in the control mussels (Figure 2A). In the mussels exposed to Cd, both Cd concentration and circadian time influenced TrxR activity (2-way ANOVA $p < 0.01$ for Cd concentration–circadian time interaction). The normal cyclic pattern in TrxR activity was lost at 50 µg/L Cd (i.e., no correlation between the controls and the 50 µg/L exposure group) and were significantly higher at 09:00, 21:00 and 24:00 relative to the controls (Figure 2B). The circadian TrxR cycle was also lost at 100 µg/L Cd, although the pattern was seemingly similar. This was corroborated by the lack of significant correlations between the control mussels and the mussels treated with 50 µg/L Cd ($r = 0.26$; $p > 0.1$) and 100 µg/L Cd ($r = 0.31$; $p > 0.05$). A correlation analysis revealed correlations with neither MT nor LPO in the controls and the mussels exposed to Cd. Peroxidase activity was also examined (Figure 2C). A significant interaction between Cd concentration and circadian time was observed (2-way ANOVA $p = 0.05$). The cyclic pattern in peroxidase activity was lost at 100 µg/L and the activity was lower between 18:00 and 21:00 compared with the controls. Although the 50 µg/L Cd group showed a similar pattern for circadian time changes in peroxidase activity, no significant correlations were obtained between the control mussels and the 50 µg/L Cd group ($r = -0.37$; $p > 0.1$) and 100 µg/L Cd group ($r = 0.22$; $p > 0.1$). This suggests that Cd disrupted the circadian rhythms in peroxidase activity.

The NAD(P)H oxidase rate and endogenous NAD(P)H levels were also determined in

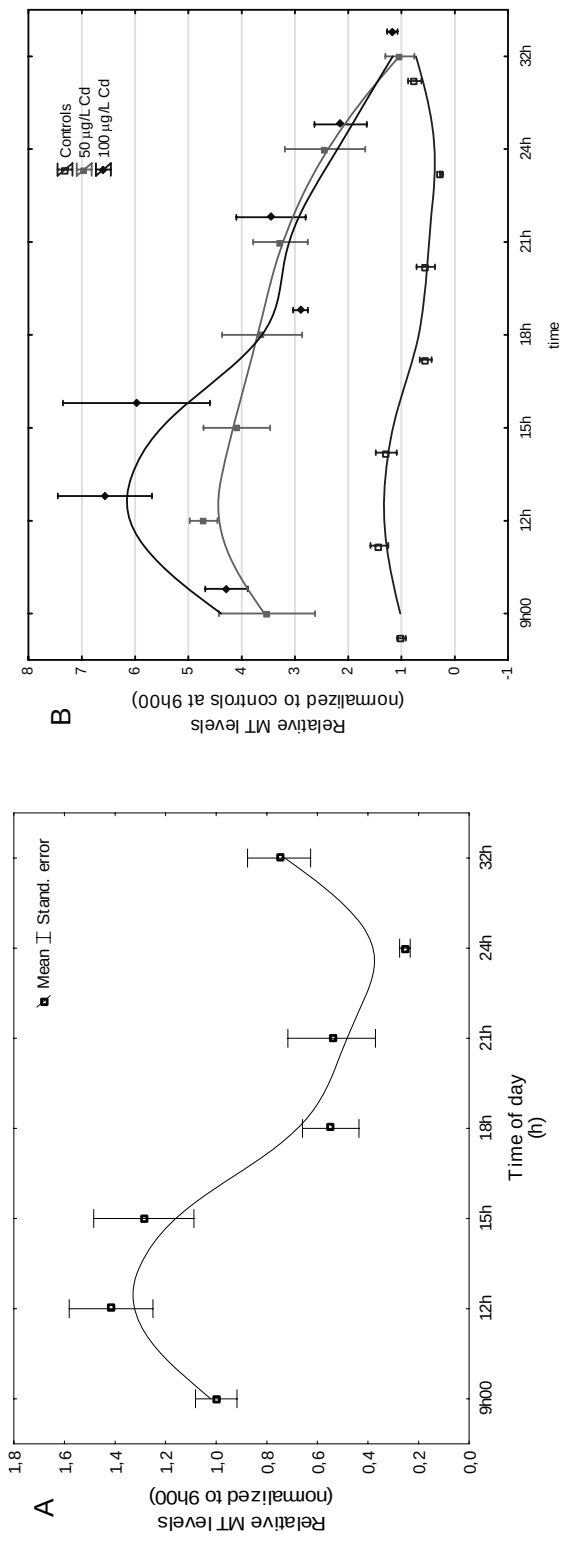

Figure 1. Daily changes in MT and LPO levels and the influence of Cd. MT and LPO levels were determined in the control mussels (A, C) and the mussels exposed to Cd (B and D). The data represent the mean with the standard error (n = 4) and the lines were obtained using the least square difference.

Table 1. Correlation analysis of biomarker data.

Controls	**Perox**	**MT tem**	**Thioredox**	**NAD(P)H oxidase**	**NAD(P)H endo**	**LPO**	**NAD kinase**
Perox	1	0.14 p > 0.1	-0.17 p > 0.1	-0.47 p < 0.05	-0.24 p > 0.1	0.41 p = 0.05	-0.30 p > 0.1
MT		1	0.32 p < 0.1	-0.44 p < 0.05	-0.49 p <0.01	0.42 p <0.05	-0.17 p > 0.1
Thioredox			1	0.15	-0.13	0.04	-0.24
NAD(P)H oxidase				1	0.66 p < 0.001	-0.57 p < 0.01	0.32 p = 0.1
NAD(P)H endog					1	-0.48 p < 0.01	0.49 p < 0.01
LPO						1	-0.44 p < 0.05
Cadmium (100 µg/L)	**Perox**	**MT**	**Thioredox**	**NAD(P)H oxidase**	**NAD(P)H endo**	**LPO**	**NAD kinase**
Perox	1	-0.04 p > 0.1	-0.22 p > 0.1	-0.01 p > 0.1	0.11 p > 0.1	0.36 p < 0.1	**-0.54 p > 0.01**
MT		1	0.22 p > 0.1	**-0.50 p < 0.01**	**-0.52 p < 0.01**	0.18 p > 0.1	0.15 p > 0.1
Thioredox			1	**-0.37 p = 0.05**	**-0.38 p = 0.05**	0.002 p > 0.1	0.31 p = 0.1
NAD(P)H oxidase				1	**0.75 p < 0.001**	-0.04 p > 0.1	-0.09 p > 0.1
NAD(P)H endo					1	0.21 p > 0.1	-0.14 p > 0.1
LPO						1	-0.05 p > 0.1

Significant correlations are highlighted in bold.

mitochondria (Figure 3). In the control mussels, NAD(P)H oxidase and NAD(P)H levels in mitochondria reached a maximum (acrophase) at 21:00 compared with the bathyphase at 09:00, reaching a 1.5-fold increase for NAD(P)H oxidase activity (Figure 3A). Endogenous levels of NAD(P)H were also elevated at 21:00, reaching 3.5-fold of controls at 09:00. NAD(P)H oxidase activity was significantly correlated with endogenous NAD(P)H levels ($r = 0.66$; $p < 0.001$), peroxidase ($r = -0.47$; $p < 0.05$), MT ($r = -0.44$; $p < 0.05$) and LPO ($r = -0.57$; $p < 0.01$) (Table 1). Endogenous NAD(P)H levels were correlated with MT ($r = -0.49$; $p < 0.01$) and LPO ($r = -0.48$; $p < 0.01$). There was no indication that Cd disrupted the circadian rhythm in NAD(P)H oxidase activity as shown by the significant correlations between NAD(P)H oxidase activity in the control mussels and the 50 µg/L Cd-exposure group ($r = 0.81$; $p < 0.001$) and the 100 µg/L Cd-exposure group ($r = 0.83$; $p < 0.001$). However, in the 100 µg/L Cd group, the acrophase was shifted to 18:00 with a lower amplitude of 1.4-fold of controls at 09:00. However, the activity tended to be higher than the controls at 15:00, 18:00 and 32:00, which suggests enhanced oxidation rates in NAD(P)H throughout the day. NAD(P)H oxidase activity in mussels exposed to 100 µg/L Cd was also significantly correlated with MT ($r = -0.5$; $p < 0.01$) and TrxR activity ($r = -0.37$; $p = 0.05$). Endogenous NAD(P)H levels were seemingly not affected by Cd exposure, with the exception of the appearance of a

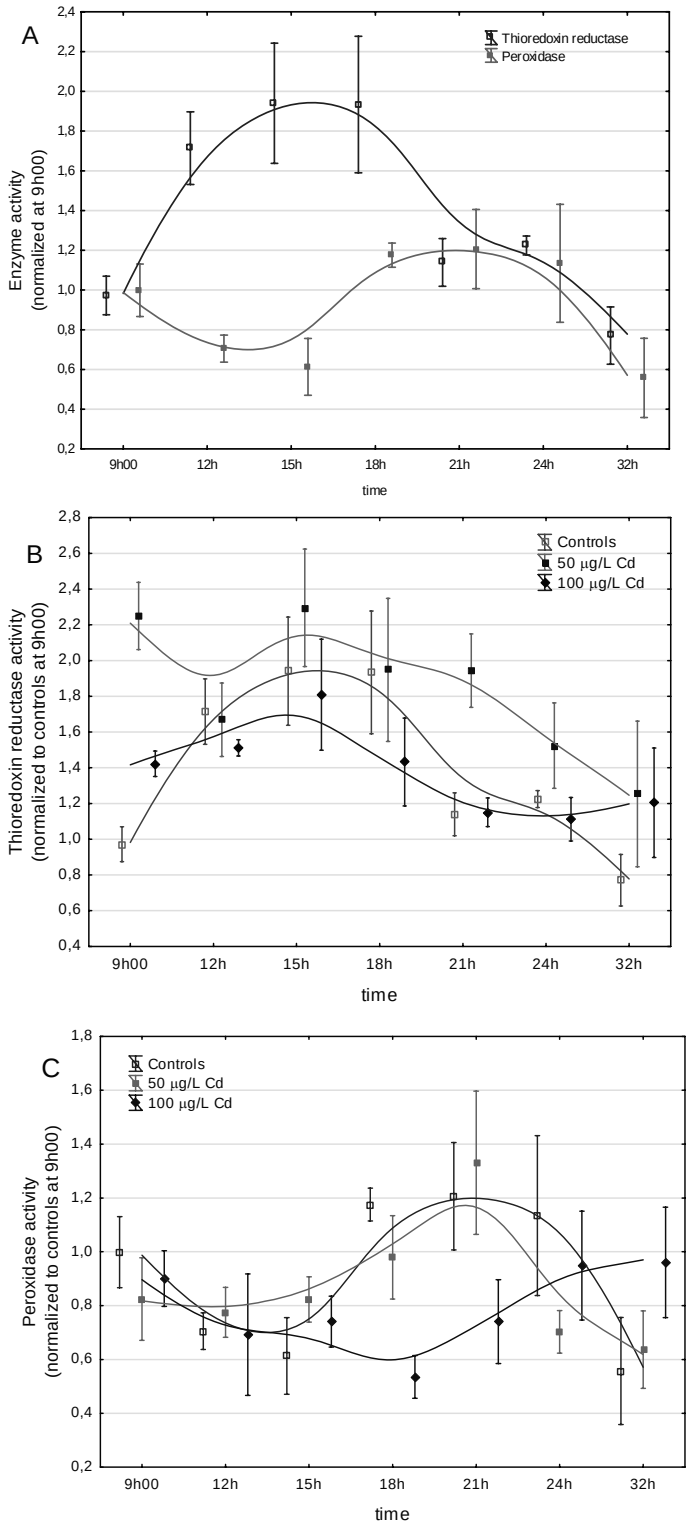

Figure 2. Change in peroxidase and thioredoxin reductase activity during the day and influence of Cd. The levels of thioredoxin reductase and peroxidase activity were determined in the soft tissues of the control mussels (**A**). The activity of thioredoxin reductase (**B**) and peroxidase activity (**C**) were determined in the quagga mussels exposed to Cd. The data represent the mean with the standard error (n = 4) and the line the least square difference fit.

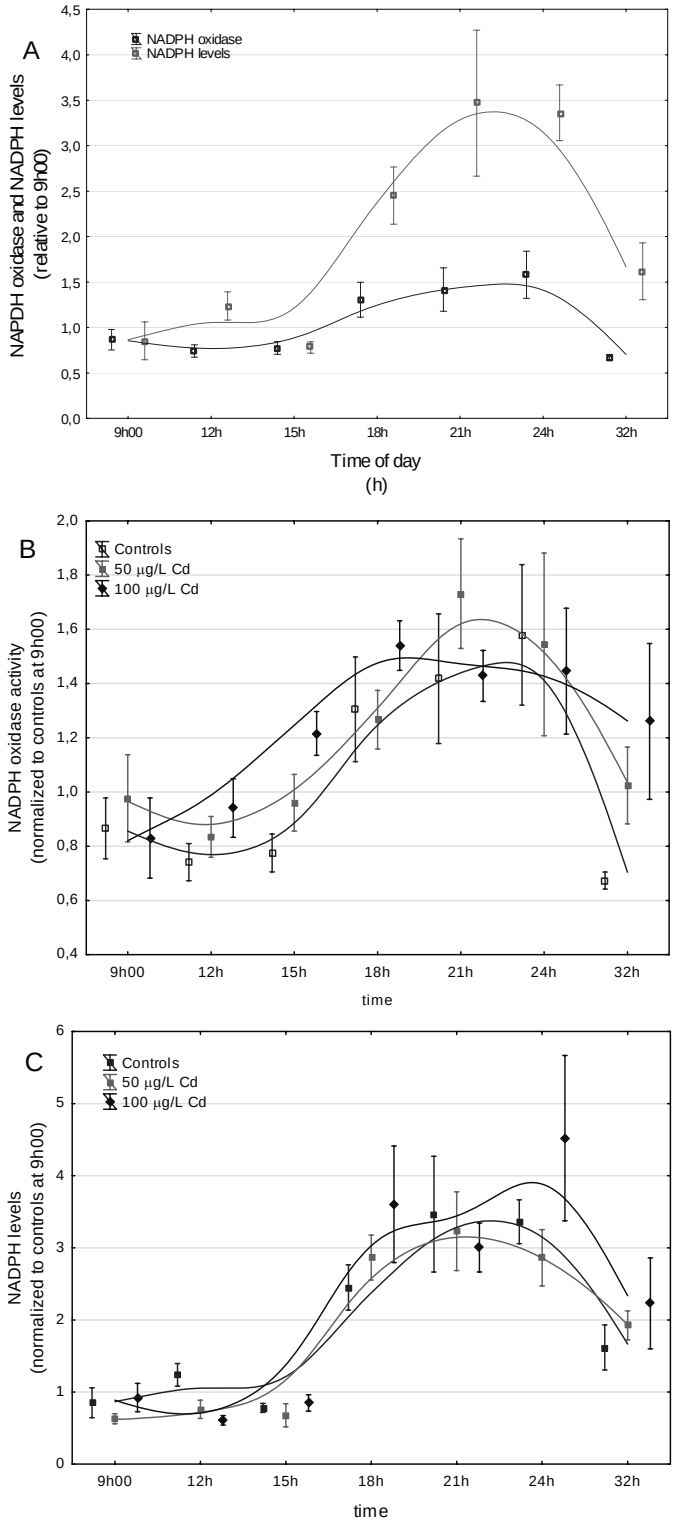

Figure 3. Change in NAD(P)H oxidase and NAD(P)H levels in mussels exposed to Cd. The activity of NAD(P)H oxidase and NAD(P)H levels were determined in the soft tissues of the control mussels (**A**). The activity of NAD(P)H oxidase (**B**) and NAD(P)H levels (**C**) were determined in quagga mussels exposed to Cd. The data represent the mean with the standard error (n = 4) and the line the least square difference fit.

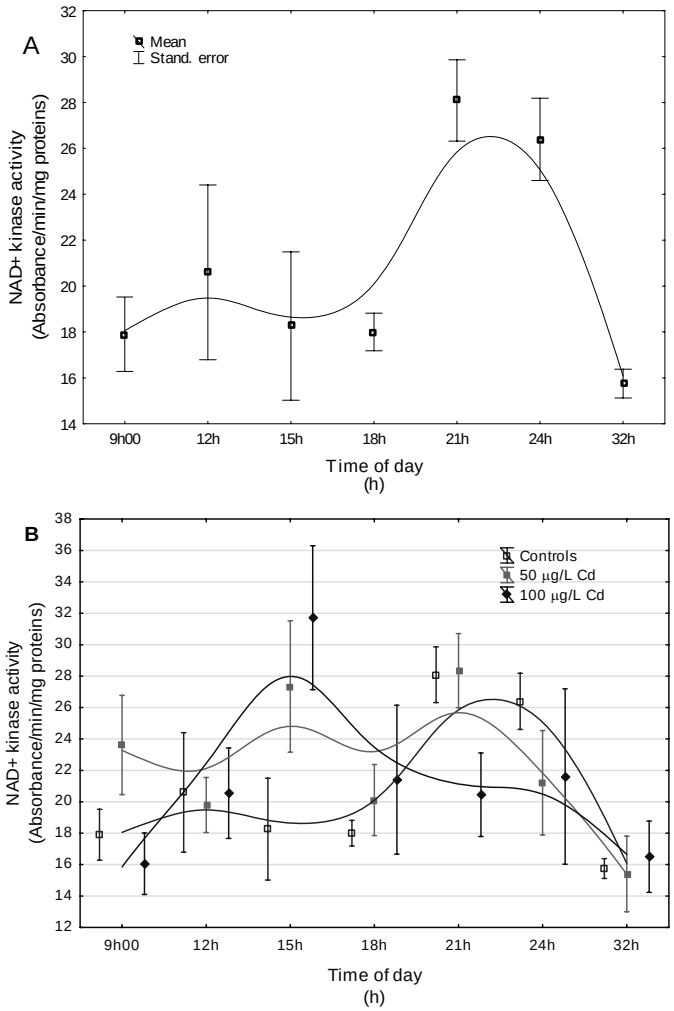

Figure 4. NAD^+ kinase activity in mussels exposed to Cd. The activity of NAD^+ kinase was determined in the soft tissues of the control mussels (**A**). The activity of NAD^+ kinase (**B**) was determined in the quagga mussels exposed to Cd. The data represent the mean with the standard error (n = 4) and the line the least square difference fit.

second peak at 18:00. This was supported by significant correlations of the control mussels' NAD(P)H levels with the 50 µg/L Cd-exposure group ($r = 0.71$; $p < 0.001$) and 100 µg/L Cd-exposure group ($r = 0.54$; $p < 0.01$). Moreover, endogenous NAD(P)H levels were correlated with NAD(P)H oxidase activity ($r = 0.75$; $p < 0.001$), MT ($r = -0.52$; $p < 0.05$) and TrxR ($r = -0.38$; $p = 0.05$). The activity in NAD^+ kinase was also determined in the mussels (Figure 4). In the control mussels, NAD^+ kinase activity also followed a circadian rhythm with the acrophase between 21:00 and 24:00, reaching 1.6 fold of the activity at 09:00 (bathyphase). The activity was also significantly correlated with endogenous NAD(P)H levels ($r = 0.49$; $p < 0.05$) and LPO ($r = -0.44$; $p < 0.05$). In the mussels exposed to Cd, the cyclical nature of the enzyme activity was readily altered. Indeed, we found no correlations between NAD^+ kinase activity in the controls with either the 50 µg/L Cd or 100 µg/L Cd-exposure groups. The acrophase was shifted from 21:00 to 15:00 in the mussels exposed to Cd at the same intensity as the 100 µg/L Cd group relative to the controls. Correlation analysis revealed that the NAD^+ kinase activity was only correlated with peroxidase activity ($r = -0.54$; $p < 0.01$) and marginally so with TrxR activity ($r = 0.31$; $p = 0.1$).

In the attempt to gain an understanding of the effects of Cd on the circadian rhythm of the

examined endpoints, a discrete Fourier transformation analysis of the biomarker data was performed (Figure 5). Fourier transformation essentially transforms the time domain (time of day here) into the frequency domain to highlight at which frequencies changes in biomarker signals are

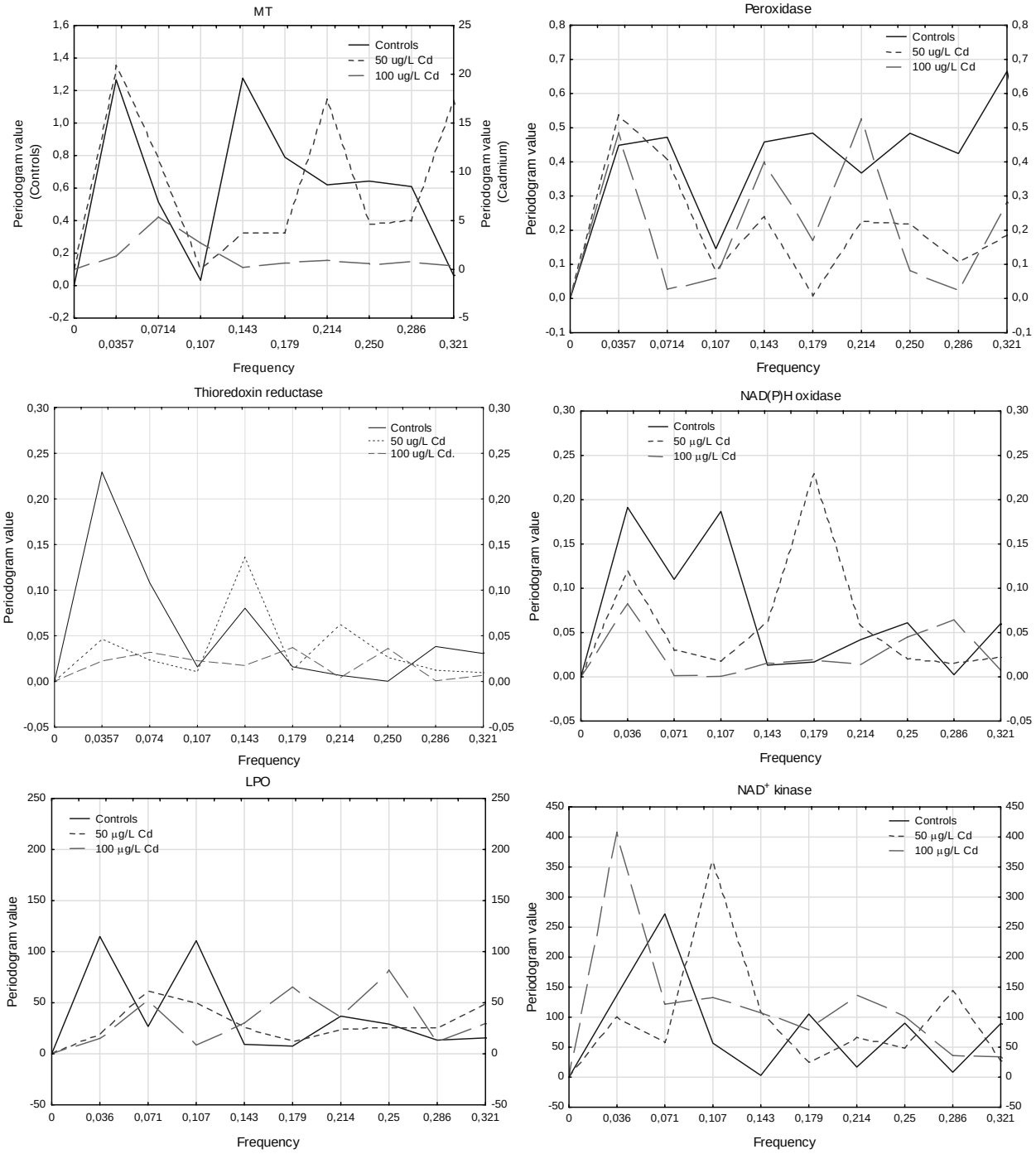

Figure 5. Fourier transformation of biomarkers in the mussels exposed to Cd. The kinetic data in NAD(P)H levels were analyzed by Fourier transformation for the following biomarkers: MT, peroxidase, ThR, NAD(P)H oxidase, LPO and NAD^+ kinase. The x axis represents the frequency domains and the y axis the amplitude changes (periodogram values are described in the Materials and Methods' section).

observed. For the MT levels, the analysis revealed 2 major frequencies at 0.04 and 0.143, which correspond to periods of 24 h and 7 h. The first value consists of the 24-h circadian cycle, while the 7-h period consists of an internal fluctuation in MT levels corresponding to roughly 1/3 of the 24-h cycle. This cycle could represent the influence in NAD(P)H and oxidative stress (LPO) changes, which have an opposite influence on MT levels. Exposure to 50 µg/L displaced the 7-h period to a 5-h period, which suggests the appearance of higher frequencies at the expense of the intensity of the 0.143 frequency. There were no noticeable changes in the circadian rhythm (frequency 0.04) compared with the control mussels. At 100 µg/L Cd, the circadian frequency signal was lost with the appearance of a change at frequency 0.074 (14-h period), which suggests a loss of circadian rhythm in MT levels. The second frequency was also abolished with no apparent displacement in the frequency domain. For peroxidase activity, a Fourier transformation of the data revealed 3 major frequencies in the control mussels: 0.04, 0.179 and 0.31, corresponding to periods of 24 h, 5.6 h and 3 h. This suggests that peroxidase activity changes at many frequencies in mitochondria and other non-circadian regulation in peroxidase activity might be at play. Exposure to 50 and 100 µg/L Cd decreased the signals at 0.179 and 0.31 frequencies while the circadian frequency at 0.04 was not affected. In the 100 µg/L Cd group, the manifestation of a signal at frequency 0.214 (4.7-h period) was observed. Coherency analysis revealed that this frequency was not related with the same frequency as the controls, thus indicating the formation of a new frequency by Cd. For TrxR activity, the control mussels showed 2 major frequencies at 0.04 (24 h) and 0.146 (6.8 h). In the presence of Cd, the periodogram value of circadian frequency (0.04) was reduced in a Cd concentration-dependent manner. The signal at frequency 0.146 was lost at the highest Cd concentration tested only. For NAD(P)H oxidase activity, the control mussels showed 2 major frequencies at 0.04 and 0.107, corresponding to the circadian time of 24:00 and 09:00. In the presence of Cd, a Cd concentration-dependent decrease in the intensity of the 24h signal was observed, suggesting that Cd impairs the circadian amplitude but not its frequency. The second 0.107 frequency was seemingly shifted to a higher frequency at 0.178 (5.6 h) in the presence of 50 µg/L Cd and was lost at 100 µg/L Cd. For LPO levels, the analysis revealed 3 major frequencies at 0.04, 0.107 and 0.25, which correspond to periods of 24 h, 9 h and 4 h. This suggests that 3 underlying control processes are contributing to LPO levels, which include circadian time (24 h). Finally, for NAD^+ kinase activity, which is responsible for the conversion of NAD^+ to $NADP^+$ for lipid metabolism, a major frequency was obtained at 0.071 corresponding to a period of 14 h in the control mussels. In the presence of Cd, the intensity of the major frequency was higher than that in the controls, but occurred at different frequencies. The major peak was displaced to a higher frequency of 0.107 (9 h) in the 50 µg/L Cd-exposure group, while it was displaced at 0.04 with the 100 µg/L Cd-exposure group.

DISCUSSION

The redox status of organisms oscillates in a circadian manner with respect to respiration and energy metabolism. This is best shown by the circadian changes in LPO levels in mitochondria with other periodicities of 9 h and 4 h in quagga mussel mitochondria. The circadian oscillation could be observed over phylogenetically distant organisms ranging from unicellular organisms to complex eukaryotic cell organizations, such as neurons in brain tissues [26]. It is though that redox rhythmicity is a fundamental mechanism to avoid oxidative stress. Indeed, mitochondria LPO and MT levels were in phase with each other (i.e., significantly correlated), which suggests that MT was involved in the sequestration of oxygen radicals, in part at least, for the prevention of LPO exacerbation in mitochondria. TrxR activity is involved in the restoration of peroxidase activity in Prx, which also follow a circadian redox cycle [10]; the enzyme is involved in the reduction of thioredoxin which in turn reduces Prx for optimal activity. This is in keeping with the increased activity of TrxR activity which precedes (i.e., is out of phase) the increase in peroxidase activity in mussel tissues in the present study. TrxR requires NADH to maintain its activity, which also occurs (in phase) with the period of low NAD(P)H oxidase activity. Interestingly, high TrxR activity was also associated with high MT and LPO levels, which suggests that this enzyme could contribute to the

control of ROS production. This raises the hypothesis that low NAD(P)H oxidase activity could favor the production and release of more ROS during ATP synthesis. The production of H_2O_2 in mitochondria is eliminated by peroxidases such as Prx, which abound in this organelle. Prx are kept in a reduced state by thioredoxin (TrxR) and sulfiredoxin, which also follow a circadian rhythm [27]. As with TrxR, the activity of peroxiredoxin is antiphasic with sulfiredoxin reductase activity. It appears that metabolic rates are higher during the later part of the day i.e., during the acrophase of peroxidase, NAD(P)H oxidase activities and NAD^+ kinase activities. This is followed by increased MT levels and TrxR activity, which are involved in the control of ROS production in mitochondria. This was also shown in the suprachiasmatic nucleus of the rat brain [28]. Mitochondrial aggregation (a marker of mitochondria oscillation in membrane potential and cytochrome c oxidase activity) was also observed during the light hours of the day.

The activity in NAD(P)H oxidoreductase activity exhibited a 12-h period during the day in sexually immature rats which followed the levels of cytochrome P450 in microsomes [29]. We found a similar pattern (i.e, 12-14 h period) with NAD^+ kinase activity, which is involved in the conversion of NAD+ to NAPD+ for lipid and xenobiotic metabolism in microsomes. NAD^+ kinase activity showed a period of 12–14 h that was similar to the reported 12-h period. It was shown that NAD^+ cycles could influence oxidative phosphorylation in mice mitochondria [30]. The clock transcription feedback loop produces cycles of NAD^+ biosynthesis with ATP during mitochondrial respiration where ROS are concurrently produced. These cycles synchronize with the daily cycle in fasting and feeding. This is consistent with the presence of changes in peroxidase activity at the same frequency of NAD^+ kinase activity where increased mitochondrial respiration/ROS production is accompanied by peroxidase activity.

Exposure to Cd produced changes in the circadian rhythm in the following biomarkers: MT, peroxidase, TrxR, NAD^+ kinase and LPO. The cyclical levels in NAD(P)H and NAD(P)H oxidase activity were remarkably resilient to Cd exposure. MT levels were shown to oscillate during the day, which could be associated with its role in scavenging ROS and metals during this process [15, 31]. This is evidenced by the significant correlation with LPO ($r = 0.42$; $p < 0.05$) and NAD(P)H levels ($r = -0.49$; $p < 0.05$) in the control mussels. In another study, 1-month exposure to Cd abolished the 24-h rhythmicity in circulating prolactin in rats with increased pituitary LPO and redox enzyme expression [32]. The daily expression in clock genes (*Bmal1*, *Per1/2* and *Cry1/2*) was suppressed by Cd exposure. Interestingly, the addition of melatonin, a sleep inducer hormone with a circadian behavior, reversed the effects on LPO and oxidative stress enzymes. Cd exposure also disrupted the expression of redox enzymes and clock genes. Indeed, rats exposed to Cd through drinking water lost the 24-h pattern in the following genes: *nitric oxide synthase*, *heme oxygenase*, *Mn-superoxide dismutase*, *catalase* and *glutathione peroxidase/reductase* [33]. These changes occurred when some of the clock genes *(Per1/2* and *Cry2*) were suppressed or changed in phase. Cd could also influence some behavior in organisms associated with circadian rhythms. In the amphipod *Gammarus aequicauda*, 90% of the locomotor activity occurred during the night in organisms kept under a 12-h light and dark cycle [34]. Even after 10 days in the dark, the amphipods maintained their daily rhythm in locomotor activity, suggesting the presence of an endogenous control mechanism (i.e., not only related to light perception). Exposure to Cd (160–280 μg/L) did not change the nocturnal behavior of the amphipods, although nocturnal swimming activity decreased at 240 and 280 μg/L Cd, which are close to the Cd concentrations in the present study. This would suggest that increased activity in the dark hours would be associated with an increase in mitochondrial activity, such as peroxidase, NAD(P)H oxidase activities and NAD(P)H levels.

CONCLUSION

This study revealed that contaminants such as Cd could disrupt the oscillatory pattern in energy and redox homeostasis in aquatic organisms which represents a new aspect of toxicity in the environment. The production of MT, LPO and TrxR occurred early in the day during low mitochondria activity, while peroxidase, NAD(P)H oxidase, NAD(P)H levels and NAD^+ kinase occurred later at > 18:00. Frequency analysis revealed that most of the

above endpoints displayed a frequency of change corresponding to the 24-h circadian cycle, with the exception of NAD^+ kinase, which revealed a 12-14-h cycle corresponding to half of the circadian time period. Exposure to Cd generally disrupted these cycles with the exception of NAD(P)H oxidase and NAD(H)P levels which maintained their circadian oscillatory patterns. At the highest Cd concentration, the NAD(P)H oxidase activity maintained its intensity but was out of phase by 3 h compared with the controls (i.e., the acrophase from 21:00 to 18:00). A frequency analysis revealed that Cd generally decreased the amplitude of changes at 24 h, but also produced changes at higher frequencies. This study revealed that contaminants such as Cd could disrupt the oscillatory pattern in energy and redox homeostasis in aquatic organisms which could represent novel biomakers of toxicity.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest with the publication process.

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