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Bioenergetic responses of freshwater mussels *Unio tumidus* to the combined effects of nano-ZnO and temperature regime

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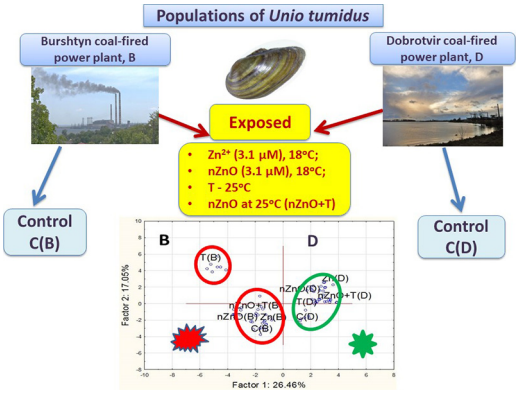
HIGHLIGHTS

- Bivalves from two cooling ponds were subjected to Zn^{2+}, nZnO and warming.
- The stress-induced depletion of energy reserves and pyruvate was population-dependent.
- Cathepsin D activity and/or efflux were up-regulated.
- Exposure to nZnO did not cause ATP decrease and anaerobic shift.
- Warming diminished the effect of nZnO in co-exposures.

GRAPHICAL ABSTRACT



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ABSTRACT

Bivalves from the cooling reservoirs of electrical power plants (PP) are exposed to the chronic heating and chemical pollution making them a suitable model to study the combined effects of these stressors. We investigated the effect of in situ exposures to chemical and thermal pollution in the PP cooling ponds on the metabolic responses of unionid bivalves (*Unio tumidus*) to a novel widespread pollutant, ZnO nanoparticles (nZnO). Male *U. tumidus* from the reservoirs of Dobrotvir and Burshtyn PPs (DPP and BPP) were maintained in clean water at 18 °C, or exposed for 14 days to one of the following conditions: nZnO (3.1 μM) or Zn^{2+} (3.1 μM, a positive control for Zn impacts) at 18 °C, elevated temperature (T, 25 °C), or nZnO at 25 °C (nZnO + T). Baseline levels of glycogen, lipids and ATP were similar in the two studied populations, whereas the levels of proteins, lactate/pyruvate ratio (L/P) and extralysosomal cathepsin D level were higher in the tissues of BPP mussels. The levels of glycogen and glucose declined in most experimental exposures indicating elevated energy demand except for a slight increase in the digestive gland of warming-exposed BPP mussels and in the gills of the nZnO + T-exposed DPP-mussels. Experimental exposures stimulated cathepsin D activity likely reflecting onset of autophagic processes to compensate for stress-induced energy demand. No depletion of ATP in Zn-containing exposures was observed indicating that the cellular metabolic adjustments were sufficient for such compensation. Unexpectedly, experimental warming mitigated most metabolic responses to nZnO in co-exposures. Our data thus indicate that metabolic effects of nZnO strongly depend on the environmental context of the mussels (such as temperature and

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acclimation history) which must be taken into account for the molecular and cellular biomarker-based assessment of the nanoparticle effects in the field.

1. Introduction

Engineered nanoparticles used in the industry, medicine and household goods have become an inalienable feature of contemporary life. They have physical and chemical properties that provide unique benefits for many applications compared to the traditional bulk substances (Pacheco-Torgal and Jalali, 2011). However, the rapid growth of nanotechnologies makes engineered nanomaterials an emerging threat to the environment due to the unintentional release of nanoparticles. Nanoparticles of zinc oxide (nZnO) are among the most commonly used nanomaterials due to their desirable chemical and physical properties, as well as putative biocompatibility and biodegradability (Kołodziejczak-Radzimska and Jesionowski, 2014). nZnO is commonly found in the industrial and municipal effluents and accumulates in surface waters and sediments reaching concentrations up into the micromolar range (Dumont et al., 2015). High Zn content, large surface area to mass ratio, as well as high surface reactivity of nZnO make these nanoparticles potentially harmful to aquatic organisms (review in: Kahru and Dubourguier, 2010; Kołodziejczak-Radzimska and Jesionowski, 2014). However, the impacts and the toxic mechanisms of low environmentally relevant concentrations of nZnO are not yet well understood in aquatic organisms. Furthermore, the bioavailability and biological effects of nanomaterials such as nZnO could be modulated by other abiotic factors such as temperature regime, salinity, UV exposure, or presence of other pollutants in the native environment (Heinlaan et al., 2016; Holmstrup et al., 2010).

Temperature is one of the most important environmental drivers that can modulate the impacts of pollutants and other stressors due to its direct effects on the rates of all physiological and biochemical processes. The impacts of temperature are especially important for ectotherms, as the body temperature of these organisms is directly dependent on the ambient temperature. Elevated temperatures may reduce toxicity of some xenobiotics (notably, organic pollutants) due to the temperature-enhanced rates of detoxification and excretion, but often increases the metal toxicity (Holmstrup et al., 2010; Sokolova and Lannig, 2008). Therefore, warming (such as occurs during seasonal warming and/or as a result of the global climate change) has been proposed to enhance the ecological impacts of toxic metals (Cherkasov et al., 2010; Hallman and Brooks, 2016; Nardi et al., 2017). For metal-containing nanoparticles, such as nZnO, little is known about the temperature-dependent effects on toxicity (Falfushynska et al., 2015b; Mos et al., 2017) which limits our ability to understand the physiological mechanisms and assess the potential ecological impacts of metal-containing nanoparticles on freshwater organisms and ecosystems.

Bivalve mollusks including unionid mussels are ecologically important species in freshwater ecosystems due to their key roles in freshwater food webs, benthic-pelagic coupling, bioturbation and nutrient cycling (Hoellein et al., 2017). Bivalves are potential targets for the engineered nanoparticle toxicity in the surface waters due to their filter-feeding behavior and the large surface area of the gills that accumulate, sort and transport the particles (Rocha et al., 2015). Freshwater bivalves might be especially prone to the impacts of nanoparticles due to the potentially greater bioavailability of nanoparticles entering the freshwater environments through the municipal effluents; unlike the marine environments, fresh water does not cause rapid agglomeration and precipitation of the nanoparticles allowing them to retain their nanosize-related properties and potential toxicity for longer (Li et al., 2011; Minetto et al., 2016). Earlier studies showed that freshwater bivalves can take up water-borne nanoparticles including nZnO and accumulate them in their tissues (Gagné et al., 2015, 2016). Moreover, unlike the vertebrates (fish and amphibians), mollusks appear less capable of decomposing the Zn-containing nanoparticles to release the free metal ions (Falfushynska et al., 2012, 2014, 2015a, 2016). Earlier studies have shown that the bioavailability of metal from the nanoparticles is associated with upregulation of and binding to the intracellular metal-binding proteins (metallothioneins), whereas the toxic effects that can be attributed to the nano-sized particles (and likely related to the mechanical damage of the membranes) are reflected in increased permeability of lysosomal membranes and reduced activity of ABC-transporters (Canesi and Corsi, 2016; Falfushynska et al., 2015b, 2018; Gnatyshyna et al., 2017; Kurelec et al., 2000; Wu et al., 2015). However, most molecular and biochemical stress markers for nZnO have been developed in single-stressor laboratory exposures, and their links to the organismal fitness under the more environmentally realistic conditions (particularly with regard to the thermal regime of the habitat) are not well understood. A recent study on a freshwater unionid showed that temperature may modulate the pollutant-specific effects of nanoparticles or ionic Zn (Falfushynska et al., 2018), yet the mechanisms and the potential fitness effects of such temperature-pollutant interactions have not been fully explored.

Bioenergetic markers are commonly used to assess the impacts of environmental stressors including temperature (Pörtner et al., 2017; Thomas et al., 2016) and pollutants (Erk et al., 2011; Ivanina et al., 2010; Knops et al., 2001; Muller et al., 2010; Smolders et al., 2004). Use of bioenergetics-related traits provides important advantages for environmental stress assessment as it permits integration of the physiological effects of multiple stressors with different mechanisms of action (including pollutants and their mixtures as well as natural abiotic stressors) and provides a direct link between the physiological change and the organism's fitness (Kooijman et al., 2009; Sokolova et al., 2012). Exposure to multiple stressors such as nZnO and elevated temperature may disrupt energy homeostasis of an organism and result in a trade-off between the energy demand for stress protection and damage repair, and other fitness–related functions such as growth and reproduction (Sokolova et al., 2012). Bioenergetic markers (including parameters of the whole-organism and cellular energy status) allow distinguishing between the moderate stress (where the organism's fitness may be reduced but the long-term survival and reproduction is possible) and bioenergetically unsustainable extreme stress resulting in time limited survival (Sokolova, 2013). Our earlier study showed that exposure to multiple stressors (such as chronic chemical pollution and elevated temperature) affects cellular stress protection mechanisms in unionid mussels *U. tumidus* inhabiting the cooling reservoirs of the thermal power plants (TPPs) (Falfushynska et al., 2018; Gnatyshyna et al., 2017). However, it is not known whether bioenergetic disturbances contribute to the limitation of the stress protection mechanisms during the combined exposures to nZnO and temperature stress.

This study aimed to determine the impacts of nZnO, elevated temperature and their combination on energy status of unionid mussels *Unio tumidus* from populations with the prior history of chemical and thermal pollution in the cooling ponds of the thermal power plants (TPP). We hypothesized that combined exposures to nanoparticle pollution and warming will disrupt energy balance of the unionid mussels acclimated to the chronically stressful environments of the TPPs' cooling ponds. We anticipated that chronic exposure to pollution and thermal stress in the cooling ponds will negatively affect energy balance of the mussels, and this energetic stress could be further exaggerated by

additional exposures to common (warming) and novel (nZnO) stressors. We also hypothesized that the negative effects of the nZnO and temperature stress on the energy balance would be more pronounced in a sensitive population from a more heavily polluted site. To test these hypotheses, we determined the effects of single and combined exposures to nZnO and elevated temperatures on tissue energy reserves (lipids, proteins and carbohydrates) and concentrations of a key glycolytic intermediate (pyruvate) and end product (lactate) in mussels from two cooling ponds of the TPPs – the moderately polluted Dobrotvir Power Plant cooling pond (DPP) and a more heavily polluted Burshtyn Power Plant cooling pond (BPP). We also assessed tissue levels of ATP as a proxy for the cellular energy status (Murphy, 2009) and activity of cathepsin D as a marker of autophagy induced by energy stress or starvation (Bursch et al., 2008; Man and Kanneganti, 2016). These data were analyzed in the context of the earlier findings about the expression levels of cellular protective mechanisms (including antioxidants, metal binding proteins, and molecular chaperones) in these two mussel populations (Falfushynska et al., 2018) to assess whether energy deficit contributes to the limitation of the cellular stress response in the chronically stressed mussels from human-modified habitats.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Merck (Synbias, Kyiv, Ukraine), and were of the analytical grade or higher. For experimental exposures, commercially produced nZnO particles were used (Sigma-Aldrich, catalog # 721077). The particle size range of nZnO suspensions in exposure water assessed by transmission electron microscopy was 50–100 nm (Supplementary Fig. 1), which corresponded to the manufacturer's specifications (average size of 92 nm). Dynamic Light Scattering analysis of nZnO suspensions yielded the following peak values for the hydrodynamic volumes: intensity-weighted volume of 441 ± 4.9 nm; volume-weighted volume of 187 ± 3.5 nm; and number-weighted volume of 132 ± 2.6 nm (N = 6). Zeta potential of nZnO was −1.39 ± 0.21 mV (N = 3). Temperature within the studied range (18–25 °C) had no significant effect on nZnO properties.

2.2. Sampling and experimental exposures

Sampling and exposures of experimental mussels are described in details elsewhere (Falfushynska et al., 2018). Briefly, adult *Unio tumidus* (*Unionidae*) were collected in early autumn of 2014 in the cooling ponds of two thermal power plants, Dobrotvir TPP (DPP) and Burshtyn TPP (BPP), the main energy producers in the west part of Ukraine.

In the laboratory, mollusks were placed in the aerated, softened tap water (17 ± 1 °C, pH 7.3 ± 0.2, $CaCO_3$ 86.8 ± 1.0 mg·L^{-1}, dissolved oxygen level 8.67 ± 0.51 mg·L^{-1}, ammonia (NH_3/NH_4^+) and nitrite levels below 0.1 mg·L^{-1}) and pre-acclimated for seven days. After the preliminary acclimation, mollusks from each site were randomly divided into five groups. One group from each site was maintained under the same conditions as during the preliminary acclimation and was considered control (C-DPP and C-BPP, respectively). Other groups were exposed to one of the following conditions: 1) 3.1 μM nZnO at 18 °C (nZnO); 2) 3.1 μM Zn^{2+} at 18 °C as a positive control for Zn exposure (Zn); 3) elevated temperature of 25 °C (T); and 4) a combination of 3.1 μM nZnO and elevated temperature of 25 °C (nZnO + T). No mortality of the mussels occurred during the experimental exposures.

After 14 days of exposures, mollusks were dissected on ice. Sex was examined microscopically, and males were selected for further analyses. Tissues were shock-frozen and stored at −40 °C/−80 °C until further analyses.

2.3. Tissue energy reserves, ATP and metabolite concentrations

Concentrations of carbohydrates, their metabolites and ATP were measured in the protein-free extracts of the digestive gland and gill tissues. Briefly, digestive gland and gills tissues of control and exposed mussels were quickly excised and immediately shock-frozen in liquid nitrogen. Tissues were powdered under the liquid nitrogen and homogenized with five volumes of ice-cold 0.6 M perchloric acid (PCA) with 150 mM EDTA for the maximum recovery of tissue ATP (Sokolova et al., 2000). For glycogen determination, PCA extracts were subjected to acid hydrolysis of glycogen to D-glucose by glucoamylase as described elsewhere (Keppler and Decker, 1984). Precipitated protein was removed from the PCA extracts by centrifugation. The extract was neutralized to pH 7.2–7.5 with 5 M potassium hydroxide. Precipitated potassium perchloride was removed by a second centrifugation. Extracts were stored at −80 °C. Concentrations of all metabolites were measured in neutralized PCA extracts spectrophotometrically at 340 nm using standard NAD(P)H-linked enzymatic tests. Briefly, D-glucose was assayed by an increase of NADPH absorbance (340 nM) in the assay media containing 38.5 mM triethanolamine buffer, pH 7.6, 0.04 mM NADP, 1 mM ATP, 7 mM $MgCl_2 \times 6H_2O$, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1} hexokinase. Glucose levels in the PCA extract prior to glucoamylase treatment was used to calculate the tissue levels of free D-glucose, and the glycogen concentrations were determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Lactate was assayed spectrophotmetrically following NAD^+ reduction during the enzymatic oxidation of lactate to pyruvate by bacterial D-Lactate dehydrogenase (D-LDH, EC 1.1.1.28) from *Lactobacillus leichmannii* (Gawehn, 1988). Pyruvate was measured by a reciprocal assay based on NADH-dependent conversion of pyruvic acid to D-lactic acid by D-LDH (Lamprecht and Heinz, 1988). ATP levels were measured by NADPH production in an assay media containing 38.5 mM triethanolamine buffer, pH 7.6, 0.04 mM NADP, 7 mM $MgCl_2 \times 6H_2O$, 50 mM glucose, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1} hexokinase. Tissue glycogen and metabolite concentrations were expressed as μmol g^{-1} wet tissue mass.

Total lipid content was measured using a standard gravimetric method (Folch et al., 1957; Iverson et al., 2001). The digestive gland and gills tissues were homogenized in a chloroform/methanol mixture (2:1 v/v) using a tissue to chlorophorm/methanol ratio of 1:20 (w/v). Samples were sonicated for 1 min, vortexed for 2 min and centrifuged for 5 min at 13,000 × g. The supernatant was transferred into a new tube and the chloroform/methanol extraction was repeated on the tissue pellet. The supernatants of two extractions were pooled, mixed with water (25% of the total volume of supernatant) and centrifuged for 5 min at 13,000 × g. The lower phase (chloroform) was transferred to a pre-weighed tube and the chloroform was evaporated to determine the mass of the extracted lipids. Lipid concentrations were expressed in mg g^{-1} wet tissue mass.

Soluble protein concentration in tissue homogenates in 0.25 M sucrose was measured by the method of Lowry et al. (1951) using bovine serum albumin as a protein standard.

2.4. Cathepsin D activity

Cathepsin D (EC 3.4.23.5) activity as a marker of autophagy was determined at 280 nm using 1% acid-denatured hemoglobin as a substrate as described by Dingle et al. (1971). Free cathepsin D activity was assessed in tissue homogenate without detergent addition, whereas the total cathepsin D activity was measured after Triton X100 treatment to release cathepsin from the lysosomes. Lysosomal cathepsin D activity was calculated as a difference of the total and free activities. Activities were determined using a standard curve with tyrosine, and expressed as nmol tyrosine min^{-1} mg^{-1} of soluble extracted protein.

2.5. Statistical analysis

Data were tested for normality and homogeneity of variance by Kolmogorov-Smirnoff and Levene's tests, respectively, and normalized as needed by Box-Cox common transforming method. For the data that were not normally distributed after the transformation, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney *U* test) were performed. Pearson's correlation test was used to assess correlations between the studied traits. Normalized, Box-Cox transformed data were subjected to the principal component analysis (PCA) to reduce the dimensionality of the data set and identify the potential biomarker signatures of different experimental exposures. The classification tree based on all studied traits was built using Classification and Regression Tree (CART) software using raw (non-transformed) data. To determine the possible associations between bioenergetics shifts and cellular toxicity in the mussels, the data of the present study were combined with the metadata from an earlier study on the same populations (Falfushynska et al., 2018) for the PCA and CART analyses. All statistical calculations were performed with Statistica v. 12.0 and Excel for Windows-2013. Differences were considered significant if the probability of Type I error was <0.05. The data are presented as means ± standard deviation (SD) unless indicated otherwise. Sample size (N) was 8 for all experimental groups.

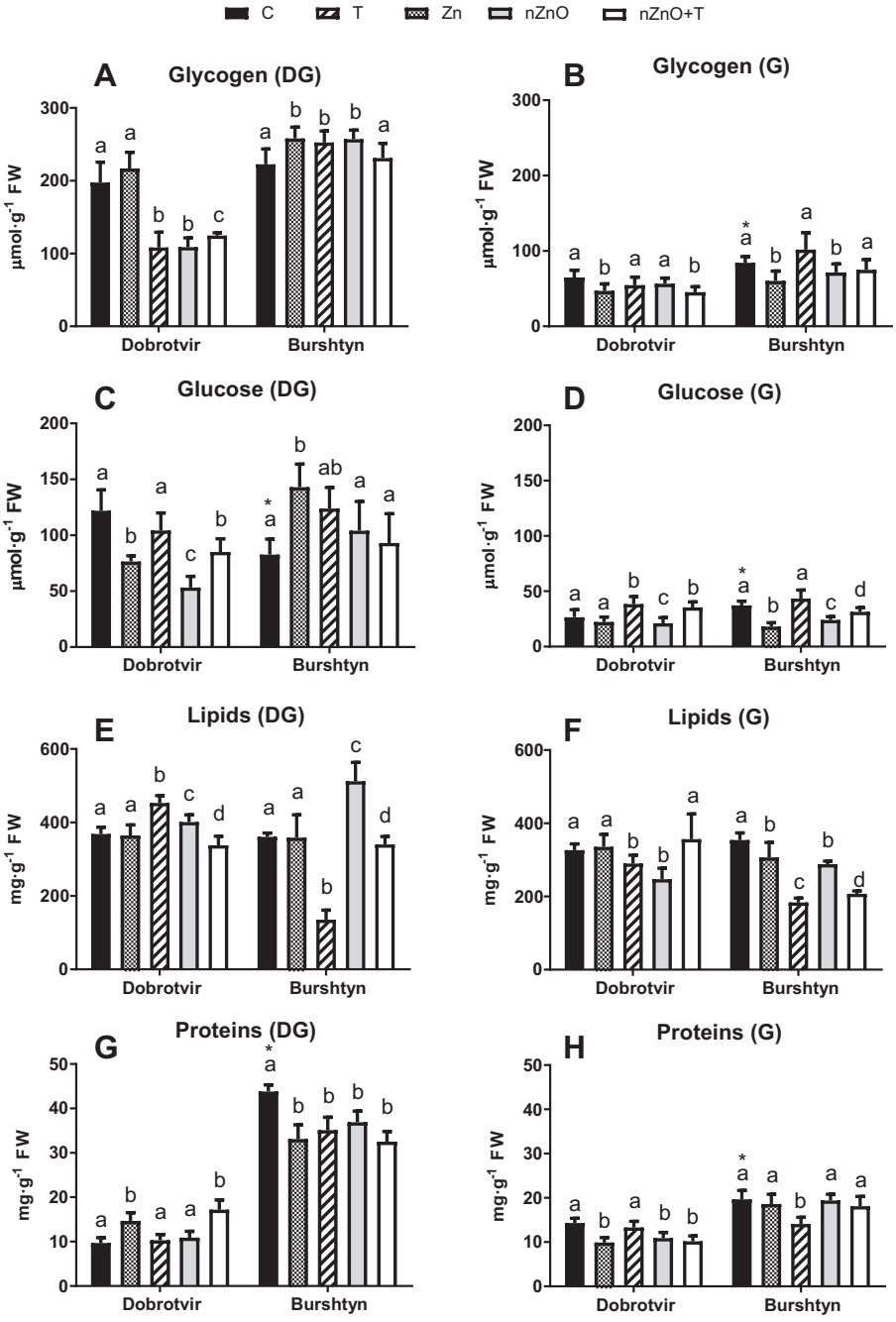


Fig. 1. Effects of experimental exposures to ionic zinc (Zn), elevated temperature (T), nanosized ZnO (nZnO), and the combination of nZnO + T on the concentration of glycogen (A, B); glucose (C,D); lipids (E, F) and proteins (G, H) in the digestive gland (A, C, E, G) and gills (B, D, F, H) of *Unio tumidus*. N = 8. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$).

3. Results

3.1. Tissue energy reserves

The glycogen and free glucose content was higher in the digestive gland compared with the gill of the mussels from both studied populations (Fig. 1). Control mussels from two TPPs had similar basal levels of glycogen. The basal levels of free glucose differed between the studies populations but the direction of the difference depended on the tissues (lower in the digestive gland but higher in the gills of the BPP compared with the TPP mussels). Exposures to Zn^{2+} or nZnO led to a decrease of the glycogen content in the digestive gland and gills of DPP mussels and in the gills of BPP mussels (Fig. 1A, B). In contrast, Zn^{2+} and nZnO exposures led to an increase of the glycogen content in the digestive gland of the BPP mussels. Similarly, elevated temperature led to a significant decrease of glycogen content in the digestive gland of the TPP mussels, but an increase in those of the BPP mussels (Fig. 1A). No effect of elevated temperature on the glycogen content was found in the gill of the DPP or BPP mussels (Fig. 1B). Combined nZnO and warming exposure led to the glycogen depletion in the tissues of the DPP but not BPP mussels (Fig. 1A,B).

The effects of Zn-containing substances and warming on the levels of free glucose were site- and tissue dependent. In the digestive gland of the DPP mussels and in the gills of BPP-mussels all Zn-containing exposures caused a decrease of the glucose levels. In contrast, the levels of free glucose increased in the digestive gland of Zn- and T-treated BPP-groups and in the gills of nZnO + T- and T-treated DPP-groups (Fig. 1C,D).

The basal tissue levels of lipids were similar in the mussels from the two studied populations, and did not considerably differ between the digestive gland and the gills (Fig. 1E, F). The lipid content increased in the digestive gland and decreased in the gills of nZnO-exposed mussels from both studied sites (Fig. 1E,F). The elevated temperature had a prominent negative effect on the lipid stores in the digestive gland and the gills of the BPP mussels (with a ~2.7 and 1.9-fold decrease, respectively) and in the gills of the DPP mussels (~1.1-fold decrease). The combined nZnO + T exposures also generally led to a decrease in the tissue lipid content, although the effect was weaker than during the exposure to the elevated temperature alone (Fig. 1E,F).

The basal protein content of the tissue was higher in the BPP mussels compared with their TPP counterparts (Fig. 1G, H). Protein content of the digestive gland decreased in response to all experimental exposures of the BPP mussels, whereas in the "BPP musels" digestive gland Zn^{2+} and nZnO + T exposures resulted in a slight but significant increase of the protein content. In the gills, the protein content declined in all but one experimental exposures of the TPP mussels and in the nZnO-exposed BPP mussels (Fig. 1G,H).

3.2. Glycolytic intermediates and ATP levels

Under the control conditions, tissue levels of pyruvate were higher and those of lactate – lower in the DPP mussels compared to their BPP counterparts, resulting in the higher lactate/pyruvate (and thus NADH/NAD+) ratios in the BPP mussels (Fig. 2). In the digestive gland, all exposures caused a decrease of the pyruvate concentration in mussels from both studied sites, with the strongest effects (a ~2.7–3.5-fold decrease) observed during warming. In the gills, warming was the only treatment that significantly affected the pyruvate levels, leading to a decrease in the pyruvate content in the DPP mussels and an increase in the DPP group (Fig. 2). The levels of lactate varied between the studied tissues and populations without a clear exposure-dependent pattern (Fig. 2). Overall, lactate to pyruvate ratio in the digestive gland increased in all experimental treatments in both studied populations (except during the nZnO exposure of the DPP mussels), with the strongest increase (~3.3–3.4 fold) in the mussels exposed to warming (Fig. 2E). In the gills, the lactate to pyruvate ratio varied less in response to the experimental treatments, except for a slight but significant decrease in the warm-exposed DPP mussels and in the Zn^{2+}, temperature-, and nZnO-exposed BPP mussels (Fig. 2F).

The basal level of ATP was higher in the digestive gland compared with the gills and did not differ between the mussels from the two studied populations (Fig. 3A,B). Notably, exposure to Zn^{2+} or nZnO led to an increase of ATP levels in the digestive gland and the gills of the BPP mussels and in the digestive gland of the DPP mussels, but a decrease in the gills of the DPP mussels (Fig. 3). Warming led to a decrease in the tissue levels of ATP, and this effect was alleviated by co-exposure to nZnO and warming (Fig. 3).

3.3. Cathepsin D activity

The baseline level of the cathepsin D activities was notably higher (by ~5.4-fold) in the digestive gland of the DPP mussels compared to their BPP counterparts (Fig. 3E,F). Experimental exposures had the strongest effect on the total activity of cathepsin D in the gills. In the gills of DPP mussels, cathepsin D activity increased in all exposures, and was accompanied by the cathepsin D efflux from the lysosomes in the warming- and nZnO-exposed groups. Similarly, warming (alone and in combination with nZnO) caused cathepsin D activation and its release from the lysosomes in the gills of the BPP mussels, whereas other experimental exposures had no significant effect (Fig. 3D). In the digestive gland, the total activity of cathepsin D did not strongly change during the experimental exposures, yet the elevated efflux of cathepsin D from lysosomes was detected in all experimental groups except for nZnO + T-exposed DPP mussels (Fig. 3C). Correspondingly, the lysosomal activity of cathepsin D was reduced in response to Zn^{2+}, warming and nZnO in the digestive gland of DPP mussels and in response to nZnO in the digestive gland of the BPP mussels (Fig. 3C).

3.4. Data integration

The PCA analyses of the studied bioenergetics traits and the cellular toxicity and stress biomarkers reflect population-specific differences in the biomarker response profiles (Fig. 4A,B). In the digestive gland, the first two principal components explained 34% and 15% of the total variation of the biomarkers, respectively, and the groups from the two study sites were clearly separated along the PC1 axis (Fig. 4A). The 1st principal component had high positive loadings of the activities of superoxide dismutase (SOD), total, free and lysosomal cathepsin D, and well as levels of lipofuscin and chaperone HSP60 (Supplementary Table 1). Levels of tissue energy reserves (glycogen and proteins) and lactate, as well as lactate/pyruvate ratio and phenoloxidase activity in the digestive gland had high negative loadings on the PC1.

In the gill tissues, the PC1 and PC2 explained 26% and 17% of the biomarker variation, respectively, and the TPP and BPP populations were likewise separated along the PC1 axis (Fig. 4B). The 1st principal component had high positive loadings of the free cathepsin D activity, and tissue levels of GSH and lipofuscin, and high negative loadings of the P-glycoprotein activity, as well as the tissue levels of energy reserves (glycogen and protein), lactate and HSP72 (Supplementary Table 1). Notably, in both studied tissues the PC2 clearly separated the warming-exposed BPP group from the rest of the BPP groups, while all other experimental groups formed relatively tight clusters within each studied populations (Fig. 4A, B). In the digestive gland, the 2nd principal component had high positive loadings of DNA damage levels, lysosomal stability and frequency of micronucleated hemocytes, and high negative loadings of GSH and lipid levels. The CART analysis identified classification trees with 9 splits and 10 terminal nodes in the digestive gland and the gills of experimental mussels (Fig. 4C, D). Overall, the CART analysis supports an important role of the bioenergetics-related traits in separating the treatment groups of mussels from different populations. Thus, in the digestive gland and gill tissues, 6 and 5 (out of 9) splits, respectively, were associated with the bioenergetics-related markers (Fig. 4C, D). The

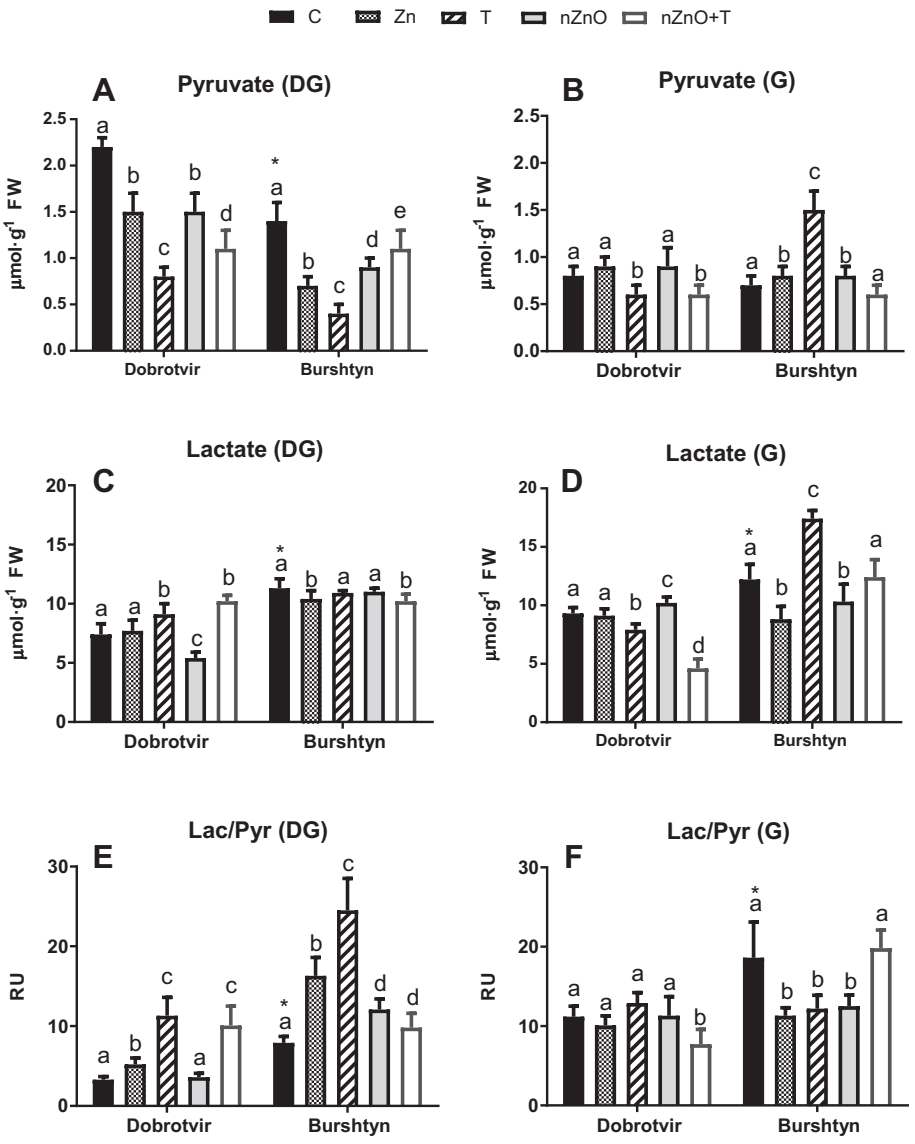


Fig. 2. Effects of experimental exposures to ionic zinc (Zn), elevated temperature (T), nanosized ZnO (nZnO), and the combination of nZnO + T on the concentration of pyruvate (A, B), lactate (C, D), and lactate/pyruvate concentration ratio (E, F) in the digestive gland (A, C, E) and gills (B, D, F) of *Unio tumidus*. N = 8. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

most important parameters discriminating the experimental groups include the frequency of the micronuclei, Lac/Pyr ratio, lactate concentration, lysosomal membrane stability, ATP concentration and cathepsin D activity in the digestive gland. In the gill tissue, the key discriminating indices were similar and included the frequency of the micronuclei, lactate and pyruvate concentration, as well as cathepsin D and caspase-3 activities. No classification mismatch was observed between the experimental groups in either of the two studied tissues.

4. Discussion

The effects of engineered nZnO nanoparticles on stress- and bioenergetics-related traits of *U. tumidus* strongly depend on the ambient temperature as well as on the population of origin of the mussels. Notably, the effect of the population of origin (encompassing both the potential genetic differences as well the prior acclimation history of the population) was the major driver of biomarker differentiation in *U. tumidus* from the two studied cooling ponds (Fig. 4). We did not investigate the genetic relatedness of the two studied population of *U. tumidus*, and therefore the potential contribution of the genetic differences to the observed variation in the bioenergetics parameters and stress responses cannot be assessed. However, regardless of whether the observed interpopulational differences in bioenergetics reflect the genetic differentiation or phenotypic plasticity, our study demonstrates that many studied bioenergetic traits show different magnitude and direction of change in response to the elevated temperature and nZnO exposure in different populations, so that no single trait can be reliably used as a biomarker of nZnO toxicity in the environmentally relevant context. Similar conclusion has been reached when classical cellular stress markers (such as the metal-binding proteins, antioxidants and molecular chaperones) were investigated in different *U. tumidus* populations from a pristine site as well as the cooling ponds of the power stations (Falfushynska et al., 2014, 2018; Gnatyshyna et al., 2017). Nevertheless, a common pattern of response to the experimental stressors demonstrates that exposure to the elevated temperature, Zn^{2+}, nZnO and nZnO + T causes bioenergetic stress and indicates

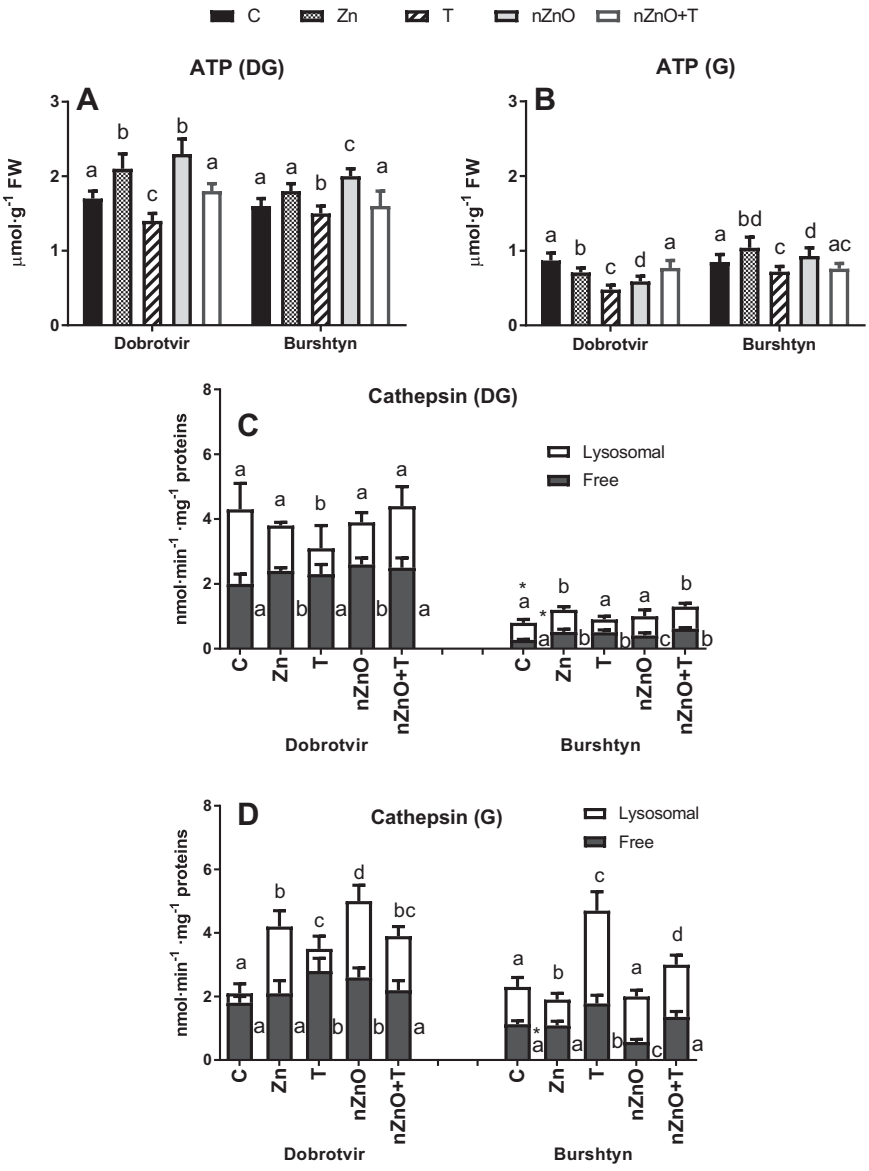
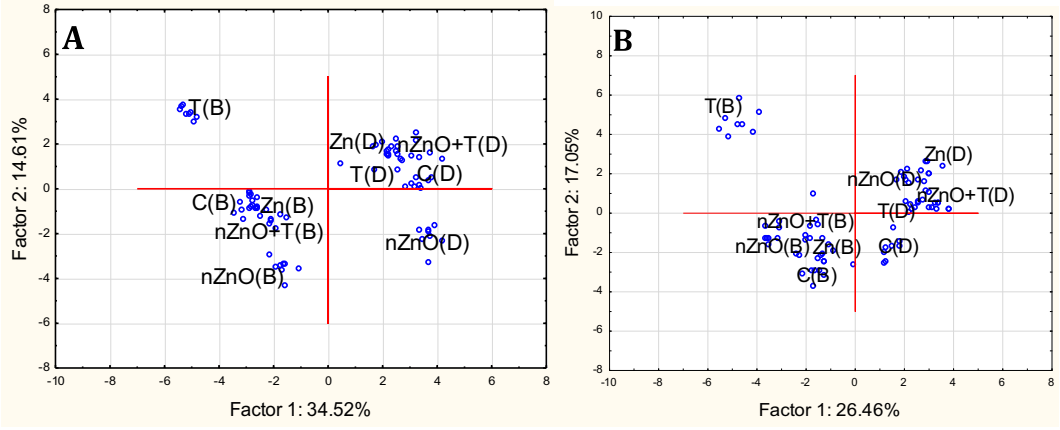


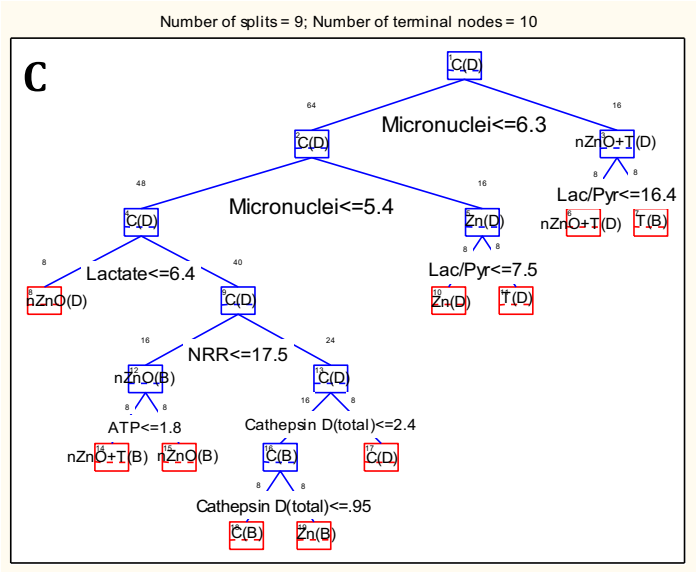
Fig. 3. Effects of experimental exposures to ionic zinc (Zn), elevated temperature (T), nanosized ZnO (nZnO) and the combination of nZnO + T on the concentration of ATP (A, B), and lysosomal and free cathepsin D activity (C, D) in the digestive gland (A, C) and gills (B, D) of *Unio tumidus*. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

that interpopulational differences in the sensitivity to the temperature- and nZnO-induced stress may depend on the baseline energy status of *U. tumidus*.

The gill appears a key target organ for nZnO toxicity in *U. tumidus* as shown by the greater bioenergetics shifts caused by nZnO-related exposures in this tissue. This may reflect the important role of the gills in particle filtration and uptake that makes the gill the first site for the potential exposure to nZnO particles (Trevisan et al., 2014). Thus, nZnO exposure led to a decrease in tissue energy reserves (including glycogen and lipids), depletion of the free glucose levels and (in the case of the DPP mussels) a decrease in the ATP content in the gills of *U. tumidus*. This indicates elevated energy demand during nZnO exposures in the gills, likely reflecting the costs of stress protection and damage repair (Sokolova et al., 2012). Furthermore, cathepsin D, an enzyme that plays a critical role in the terminal degradation of recycled proteins (Turk and Stoka, 2007), was activated in the gills in response to nZnO of the DPP mussels, indicating a strong energy deficiency that induces autophagy. It is worth noting that the metal-containing nanoparticles are sequestered by lysosomes (Jimeno-Romero et al., 2017; Rocha et al., 2015) that might thereby sustain mechanical damage to the membranes (Canesi and Corsi, 2016). In *U. tumidus* populations from the DPP and BPP study sites, the exposure to Zn^{2+} or nZnO decreased the lysosomal membrane stability to a critically low level (Falfushynska et al., 2018). This lysosomal membrane destabilization may contribute to the efflux of cathepsin D from lysosomes in the gills of nZnO-exposed mussels but is unlikely to be the only mechanism explaining autophagic activation, as the total cathepsin D activity also showed an increase in response to nZnO in the DPP mussels. The activation of autophagy in the gills of the DPP mussels goes hand-in-hand with a decrease in the protein content indicating enhanced protein breakdown in this tissue. In contrast, no activation of cathepsin D or protein loss was detected on the gills of the nZnO-exposed BPP mussels indicating that the nanoparticle exposures induced a relatively mild energetic stress in this population.

Exposures to nZnO also resulted in an increase in the lactate/pyruvate ratio in the gill of *U. tumidus*; however, this change reflected a decrease in the pyruvate levels rather than lactate accumulation. A decrease in the pyruvate content and the lack of lactate accumulation





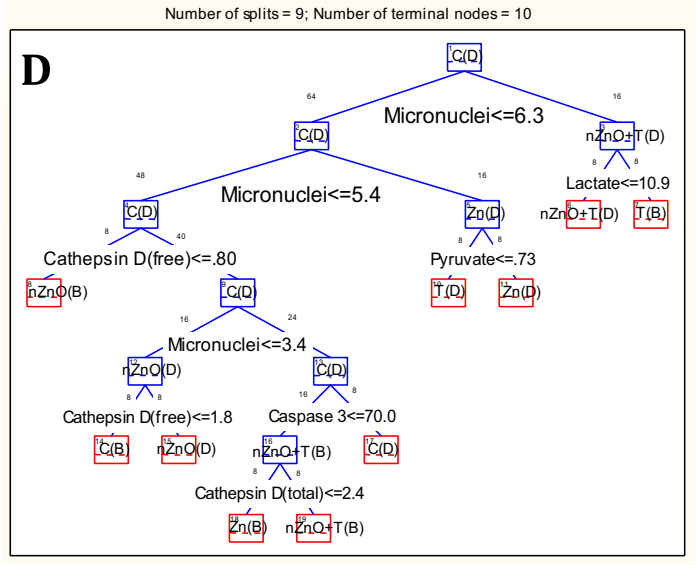


Fig. 4. Results of the principal component analysis (A, B) and classification tree models (C, D) including bioenergetics traits and cellular stress biomarkers in *U. tumidus* from cooling ponds of TPPs. A, C – digestive gland, B, D – gills. Mussels from Dobrotvir and Burshtyn power plants are marked by the letters D and B, respectively. Biomarker abbreviations: Lac/Pyr – lactate to pyruvate ratio; NRR – Neutral Red retention (index of the lysosomal membrane stability).

during the nZnO exposures indicates aerobic oxidation of pyruvate rather than the onset of the partial anaerobiosis in the gills of nZnO-exposed *U. tumidus* and suggests that the elevated energy demand of this tissue can be fully covered with the aerobic ATP supply. This finding is consistent with the results of earlier studies of bivalves and fish exposed to toxic metals (such as Cd^{2+} or Zn^{2+}) or nZnO particles showing

that while metal-containing exposures might reduce the aerobic scope of an organism, they rarely induce anaerobiosis in the absence of other stressors (Bagwe et al., 2015; Callaghan et al., 2016; Lannig et al., 2006; Lannig et al., 2008).

Notably, gills were also an important target organ for nZnO toxicity in other aquatic organisms including fish *Catostomus commersonii* (Bessemer et al., 2015). Exposure of *C. commersonii* to 1 mg L^{-1} (~16 μM) nZnO led to protein and membrane damage in gill epithelia, suppressed oxygen uptake and elevated energy demand of the gill by stimulating the activity of Na^+K^+ ATPase (Bessemer et al., 2015). Similar proteotoxicity of nZnO in the gills (indicated by upregulation of molecular chaperones) was found in *U. tumidus* from the DPP and BPP cooling ponds exposed to 3.1 μM nZnO (~250 μg L^{-1}) (Falfushynska et al., 2018). The decrease of the glycogen reserves associated with the elevated energy expenditure was also observed in the clams *Ruditapes philippinarum* exposed to the multi-walled carbon nanotubes for 28 days (De Marchi et al., 2017). In contrast, no damage to proteins, lipids or DNA was found in *R. philippinarum* subjected to the environmentally relevant nZnO concentrations (1 and 10 μg L^{-1}, correspondent to 12.3 and 123 nM) for 7 days (Marisa et al., 2016). This may reflect a short exposure time insufficient to trigger a stress response in the latter study and emphasizes the need for more investigations of the long-term exposures to engineered nanoparticles to assess their biological and toxic impacts.

Unlike in the gills, the bioenergetics shifts caused by the nZnO stress were less pronounced and more variable in the digestive gland of *U. tumidus*, indicating lower sensitivity of this tissue to the nanoparticle-induced stress. Thus, exposures to nZnO and ionic Zn resulted in the depletion of the glucose and glycogen reserves in the DPP (but not BPP) mussels, whereas the lipid content was not affected in either of the studied populations. No increase of cathepsin D activity was found in the digestive gland of the DPP mussels in response to nZnO exposures indicating that autophagic processes are not significantly upregulated. Notably, ATP content in the digestive gland of *U. tumidus* increased after two weeks of nZnO exposure. This indicates that energy demand caused by the nanoparticle exposures in the digestive gland of *U. tumidus* is fully compensated by the aerobic oxidation of energy fuels such as glycogen/glucose and pyruvate.

Elevated temperature was an important stressor for the studied populations *of U. tumidus*, especially those collected from the more polluted BPP site. Exposure to the elevated temperature (25 °C) resulted in the depletion of tissue lipids and (in the case of DPP mussels) glycogen, stimulation of autophagy indicated by the efflux of the free cathepsin D from lysosomes, and onset of anaerobiosis indicated by accumulation of lactate in the gill (BPP) and digestive gland (BPP mussels). Onset of partial anaerobiosis is an indicator of extreme physiological stress in mollusks where only time-limited survival is possible (Pörtner et al., 2017; Sokolova et al., 2011); therefore, 25 °C can be considered the upper critical temperature for *U. tumidus* likely close to the ecological tolerance limit. This notion is supported by the decrease in tissue ATP levels in warming-exposed *U. tumidus* observed in the present study, since tissue ATP levels are strongly buffered in molluscan cells and decrease only during extreme energy limitation (Sokolova et al., 2011; Sokolova, 2013). Notably, co-exposure to nZnO and elevated temperature did not result in the synergistic energy stress in *U. tumidus*. In fact, energy stress indices (such as depletion of glycogen or lipids, activation of cathepsin D or decrease in the tissue ATP levels) in nZnO + T exposed mussels were similar to those exposed to nZnO alone and in some cases, lower than during exposure to the elevated temperature alone, when compared within the respective populations and tissues. Paradoxically, this indicates that exposure to low sublethal nZnO levels may alleviate temperature-induced energetic stress in some tissues of *U. tumidus* via as yet unknown mechanisms. Earlier studies on the toxicity of dissolved metals such as Zn^{2+}, Hg^{2+} or Cd^{2+} showed that elevated temperatures increases ionic metal toxicity by enhancing metal accumulation, inducing energy deficiency, depleting the cellular protective systems and increasing metal-induced damage to intracellular organelles such as mitochondria and lysosomes (Sokolova and Lannig, 2008; Sokolova et al., 2012). Our present study indicate that the mechanisms and biological effects of temperature interactions with the metal nanoparticle toxicity might differ from those of free ionic metals and warrant further investigation.

The assessments of the health status of aquatic animals inhabiting the reservoirs of fuel PPs are uncommon and typically report impaired health status of the cooling reservoirs populations compared with the pristine sites (Falfushynska et al., 2015b, 2018; Javed et al., 2015, 2016; Javed and Usmani, 2015). However, our present study demonstrates a better bioenergetic status of the mussels from the more polluted BPP site compared to their DPP counterparts as indicated by the higher amount of tissue energy reserves (especially proteins and glycogen) and less active autophagy processes reflected in the lower activity of cathepsin D in the BPP mussels. Notably, an earlier study of *U. tumidus* populations from the same two cooling ponds showed lower baseline activity of detoxification and stress protection systems in the BPP mussels (Falfushynska et al., 2018). This could indicate that better energy status is associated with the lower background stress levels of the BPP mussels, despite the greater chemical pollution load in the BPP habitat (List of Top-100 companies in Ukraine responsible for pollution, 2017). Higher stress tolerance of the mussels from a polluted estuary compared with a pristine site was earlier reported for the blue mussels *Mytilus galloprovincialis* and was attributed to the better feeding conditions at the polluted sites leading to higher energy reserves and better bioenergetic status (Marigómez et al., 2017). High fitness (measured as the rate of the population growth) found in a metal-tolerant clone of *Daphnia longispina* in copper-polluted as well as non-polluted environments was linked with to the bioenergetic efficiency and associated with the ability of the tolerant clone to maintain high feeding rates across all experimental conditions (Agra et al., 2011). In contrast, in the metal-sensitive *D. longispina* clones, copper exposure led to a mismatch between energy demand (measured as respiration) and supply (assessed by feeding rates) and resulted in the suppressed growth and reproduction rates (Agra et al., 2011). Similarly, populations of a ground beetle *Pterostichus oblongopunctatus* inhabiting highly metal-polluted sites were able to maintain high levels of body energy reserves, despite having to cope with elevated metal burdens (Bednarska et al., 2013). Taken together, these data indicate that successful acclimation and/or adaptation to highly polluted environments may be associated with the physiological adjustments that maximize energy uptake and/or storage and may have implications for tolerance to additional stressors (Sokolova et al., 2012; Sokolova, 2013).

5. Conclusions

Our present study shows that the cellular and physiological effects of metal-containing nanoparticles strongly depend on the environmental context (such as the temperature or food abundance) and population history of aquatic sentinel organisms such as unionid mussels so that the molecular and cellular biomarker-based assessment of the nanoparticle effects may be hindered by this variability. Experimental exposures to nZnO and elevated temperatures resulted in the marked disturbances of energy metabolism; however, no synergy was detected between these two stressors during the combined exposures. Our data indicate that the mussels' ability to mount an appropriate stress response and compensate for stress-induced energy disturbance depends on the baseline bioenergetics status implying that the populations from habitats with abundant food supply (e.g. where the primary production is stimulated by eutrophication and/or warming) might be better able to cope with pollution and thermal stress.

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