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Original Paper

Bivalves Transmissible Neoplasia: Biochemical Aspects of Contagious Cancer in a Clam *Macoma Balthica*

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Key Words
Leukemia-like neoplasia • Energy homeostasis • FAA • ROS • Mitochondrial respiration • *Macoma balthica*

Abstract
Background/Aims: Occurring in marine invertebrates infectious haemic neoplasia (bivalves transmissible neoplasia, BTN) arises from genome instabilities leading to multilevel malfunctions and unregulated cell division of presumably haemocyte precursors. As its biochemical characterisation remains unknown, we here present the first data describing selected aspects of the physiology and biochemistry of the disease a in model clam *Macoma balthica*. We chose free amino acids (FAA) composition, mitochondrial respiration and enzymatic activity, oxidative stress enzymes activities and corticosteroids profile as markers of this contagious cancer. ***Methods:*** Selected markers were measured in neoplastic and healthy clams and two tissue types, haemolymph and solid tissue. FAA composition was assessed in the haemolymph samples using high performance liquid chromatography-mass spectrometry (LC/MS). Mitochondrial respiration analysis was performed on haemocytes using oxygen electrodes integrated system Seahorse XFp. Mitochondrial enzymes activities were measured using spectrophotometry (cytochrome oxidase, COX) and commercial kit (succinate dehydrogenase, SDH). Total Antioxidant Capacity (TAC), Acetylocholinesterase (AChE), Protein Carbonyl Content (CBO) and Malondialdehyde (MDA) levels were measured in the solid tissue using analytical kits, and glutathione (GSH) was measured spectrophotometrically. Corticosteroids profile, measured in the solid tissue, was obtained with Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC–ESI-MS/MS) technique. ***Results:*** In both clam groups nine FAAs were detected with Asp, Glu, Pro, Ser constituting over 90% of total FAA content. Significantly higher Gln level was detected in BTN positive clams. In neoplastic clams, an impairment of mitochondrial metabolism was observed as a decrease in mitochondrial oxygen consumption and lower cytochrome c oxidase activity. In the neoplastic clams significantly

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higher concentration of low molecular weight antioxidants was found. Finally, we report high level of corticosterone and lower levels of dehydrocorticosterone, cortisol and cortisone in healthy clams and elevated cortisol level in BTN individuals. ***Conclusion:*** Neoplastic clams are characterized by altered mitochondrial metabolism, with a potential key role of glutamine (Gln) in cancer cells energy production. Despite low aerobic respiration, BTN cells have efficient antioxidative response to elevated concentration of ROS. Elevated cortisol level in BTN-positive clams may indicate an important role of this corticosteroid in cancer biochemistry. Thus, we here provide the first results of selected physiological and biochemical aspects of BTN, making an important step in studying cancer epidemiology in wildlife.

Introduction

Neoplasia is considered a prominent disease affecting various species of commercial and ecological importance [1–3]. Distinctive cell morphology (cellular and nuclear polymorphism, hypertrophy and anaplasia), high mitotic activity, and a high level of aneuploidy are typical for the disease [4, 5]. Bivalves disseminated neoplasia (DN), as it originates from unregulated cell division presumably of haemocyte precursors, is also called haemic neoplasia or leukaemia-like cancer [3]. Some characteristics of DN (immunosuppression, altered respiration pathways and energetic metabolism) were found to be similar to those typical for leukaemia cancers present in vertebrates. DN in marine bivalves have been documented since the 1960's in around 30 species [1–3, 6]. Historically, a role of pollutants in genome destabilization and induction of the disease was suggested, but its aetiology remains unclear [7]. Most recent data suggest that DN may belong to diseases of infectious nature, possibly transmissible by cohabitation of healthy and neoplastic animals in large bivalve assemblages [8, 9]. Indeed, various molecular analysis revealed that some DN's have been identified as transmissible cancers in several bivalve species thus this type of a disease is called bivalves transmissible neoplasia (BTN). Although ten BTN lineages have been described to date [10–16], much investigation is still needed to know how many and which DN have a transmissible origin.

The disease was also found to occur in the Baltic clam *Macoma balthica* (previously known as *Limecola balthica*), and was diagnosed in various clam populations inhabiting different parts of the Northern Europe, e.g.: Wadden Sea [17], Finnish Baltic coast [18] and the most comprehensive set of research concern Southern Baltic populations from Polish coastal zone [5, 19, 20]. The possible transmission of independent cancer lineages was recently studied as a factor responsible for the high frequency of neoplasia in *M. balthica* from the Gulf of Gdańsk [16]. In cases of BTN, genotypes of neoplastic cells are nearly identical and they do not match those of the solid tissue of the host animal with different alleles dominating in normal and neoplastic cells. Indeed, published data on mitochondrial cytochrome c oxidase I (*mtCOI*) sequences and nuclear elongation factor 1α (*EF1α*) highlighted unique and divergent genotype of neoplastic cells and confirmed high similarity between DN cases obtained from different *M. balthica* clams. Thus, the contagious nature of the disease and the occurrence of BTN in *M. balthica* inhabiting the Gulf of Gdańsk were confirmed [16].

Do to the relatively often occurrence of the disease in the southern Baltic Sea population of the species, *M. balthica* appears as a perfect model for studying various, still unknown, physiological and biochemical aspects of BTN. Researching the disease using clams population suffering from BTN in natural conditions opens a new avenue not only for studying cancer epidemiology in wildlife, but also for using BTN as a model in cancer research. That includes cancer pathogenicity, energy metabolism or the ability to affect the new host.

In neoplastic disorders, the Warburg effect is a commonly accepted theory explaining cancer energy metabolism. The Warburg effect shifts the metabolic pathway of ATP generation in cancer cells from mitochondrial oxidative phosphorylation to anaerobic glycolysis and production of lactate. Hence, mitochondrial energetic functions of those cells are suppressed, and, as a consequence, decreasing activities of electron transport complexes

and lowered oxygen consumption by mitochondria occur [21]. Indeed, according to Galuzzi et al. [22] formation of ATP in cancer cells is coupled to comparatively low levels of oxidative phosphorylation. Recent findings highlighted that in some leukemic disorders leukemic cells lose their capacity for mitochondrial respiration at the level of complex I (NADH-coenzyme Q reductase) and complex II (succinate-coenzyme Q reductase), the major sites for entry of electrons into the respiratory chain [23]. However, despite being an extremely important factor potentially boosting cancer pathogenicity, energy metabolism in DN, and in particular BTN, is unknown. Other important end products, substrates and/or intermediates of anaerobic energy metabolism supporting crucial life functions are free amino acids (FAA) [24]. It is a common knowledge that FAA levels in the tissues of aquatic organisms change not only seasonally, but also as a response to environmental stress or worsening physiological conditions [25, 26]. Yet, there is no data about FAA levels in haemolymph of bivalves suffering from either BTN or DN. Due to high mitotic and metabolic activities of DN/BTN cells, the working hypothesis here was that changes in FAA content, in particular in glutamine (Gln) level, point to the presence of additional source of energy for use and storage at the cellular level able helping to satisfy their elevated energy requirements.

Steroids are naturally made by a variety of living organisms as they help controlling many functions including the immune system, reproduction, inflammatory response and/or metabolism. Corticosteroids are steroid hormones that regulate metabolism of proteins, carbohydrates and fats, which is of crucial importance for the body's energy homeostasis. They are also known for their anti-inflammatory and immunosuppressive abilities [27]. The final metabolites of the corticosteroid synthesis pathway include dehydrocorticosterone (D-CRT), corticosterone (CRT), cortisone (CN) and cortisol (HCN). However, synthesis and hormonal activity of HCN has so far been documented mainly in Primates, while CRT is known to occur in other animal species. In marine bivalves, the levels, the role and the origin of corticosteroids (glucocorticoids in particular) are not well understood, particularly in neoplastic disorders. Also, altered steroids levels may indicate an important role of this hormones in cancer biochemistry and their possible role in immunosuppression of the host.

As the occurrence of BTN in *M. balthica* population studied here was confirmed [16], we present the first data describing the physiology and biochemistry of BTN contrasted with those measured in healthy clams. Selected markers included glycolytic capacity, ROS production, FAA levels and enzymatic activity. As the BTN cells, after entering the host, must bypass its immune system, we studied if there are changes in signalling molecules such as corticosteroids (also as they have the ability to reduce inflammatory processes) in DN positive and negative clams. As BTN cells are characterised by elevated mitotic and metabolic activities and a high potential to metastasise, we looked at cellular respiration processes to highlight potential changes in DN cells able to satisfy their elevated energy requirements. Thus, the purpose of our work was to analyse physiological and biochemical aspects of DN/BTN in the Baltic clam *M. balthica* that can increase our knowledge about environmental carcinogenesis.

Materials and Methods

M. balthica was collected onboard of *R/V Oceanograf* using a benthic sledge in the Gulf of Gdańsk (Baltic Sea, Poland) at a sampling location (18°50.5'E 54°36.2'N) where BTN has recently been confirmed to occur in the clams' inhabiting the area [16]. Thus, despite the fact that in this work we used physiological diagnostic tools to distinguish between DN and healthy clams, DN occurring in studied here clam population was proven to have a transmissible origin, and will be further called DN-BTN or BTN. After transportation to the laboratory, clams were opened, and haemolymph samples were collected from the pericardium or adductor muscle using microliter glass syringes. Preliminary neoplasia diagnosis was performed under light microscope based on haemolymph subsample stained with 0,5% methylene blue. For further analyses 46 healthy and all (in total 40) with advanced DN-BTN clams were selected. The latter ones were characterised by strong domination of neoplastic over normal haemocytes in the haemolymph sample

with over 90% of abnormal haemocytes present in the haemolymph sample. Individuals at early DN-BTN stages were not taken into consideration in this study. Selected clams were treated according to three main protocols as described below. The first protocol included measurement of FAA levels (section 2.1) in the haemolymph. After collecting haemolymph sample, remaining soft tissue was stored in Davidson fixative for 48 h, transferred to 4% buffered formaldehyde solution, dehydrated, impregnated in xylene, embedded in paraffin and sectioned using a semi-automatic microtome on 3µm thick sections. Such prepared semi-thin sections were re-hydrated and stained with H&E using basic histology protocols. This set of samples (12 healthy and 11 neoplastic individuals) was used for final diagnosis of DN-BTN and for assessing the accuracy of preliminary diagnosis based on haemolymph subsample (Fig. 1A-E). Second protocol included measurement of mitochondrial respiration in 18 healthy and 13 neoplastic individuals. Here, mitochondria were isolated from the haemolymph sample while the soft tissue was homogenized and further processed as described in the sections 2.2.1 and 2.2.2 Third protocol (section 2.3.) included an assessment of the antioxidative response and measurement of biomarkers of physiological stress. For that purpose, after diagnosis, the remaining soft tissue was immediately frozen in liquid nitrogen and further stored in -80 °C until described in the section 2.1 analyses were performed. Here, 6 healthy and 6 neoplastic clams were selected. Final protocol was designed for analyses of corticosteroids levels performed based on 10 healthy and 10 neoplastic clams (section 2.4). A table showing the numbers of individuals used for each analysis is presented in the Supplementary Material (Supplementary Table S1 – for all supplementary material see www.cellphysiolbiochem.com).

Free Amino Acids (FAA) in haemolymph samples

FAA concentrations were determined according to Olkowicz et al. [28]. 10 µL of the internal standard solution (2-chloroadenosine) was added to 50 µL of haemolymph (without haemocytes), and the mix was extracted with 120 µL of acetonitrile. Samples were precipitated for 30 minutes in ice, centrifuged 14 000 x g at 4 °C for 20 min., the supernatant was transferred into new tubes and freeze dried. The samples were

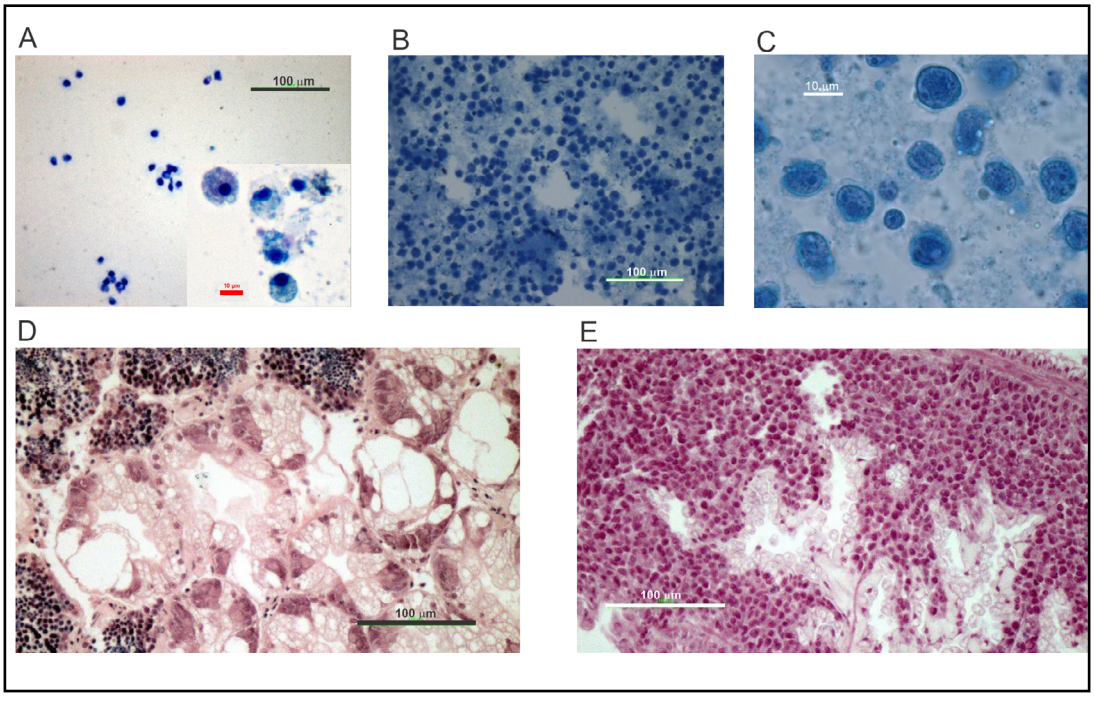

Fig. 1. Diagnosis DN-BTN in *M. balthica*; A) normal haemolymph sample, B-C) haemolymph sample with high number of neoplastic cells (different magnifications), D) histological cross-section through normal *M. balthica* showing connective tissue of the digestive gland and male gonads and E) histological cross-section through highly neoplastic individual (corresponding to leukaemia-like case shown in the Fig. 1B-C) with neoplastic cells infiltrating the connective tissue around the digestive gland. Staining: A-C) methylene-blue, D-E) basic hematoxylin and eosin.

further dissolved in 100 µL water, centrifuged for 15 min at 14 000 x g, 4°C and analysed using high performance liquid chromatography-mass spectrometry (LC/MS) on a Surveyor HPLC system coupled with a TSQ Vantage Triple-Stage Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Heated electrospray ionization in positive mode was used. Chromatographic separation was achieved with a 50 x 2 mm Synergi Hydro-RP 100 column with a 2.5 µm particle size (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water with 5 mM nonafluoropentanoic acid (Buffer A) and acetonitrile with 0.1% formic acid (Buffer B). 2 µL aliquots of samples were injected into a column eluted with a mobile phase at a flow rate of 0.2 mL/min. Based on the retention times of individual amino acid standards, the identities of amino acids in the samples were determined and compared to the peaks of corresponding standards. The procedure was followed by calculation of quantities of individual amino acids.

Measurement of mitochondrial respiration and enzymatic activities

Used reagents include substrates: succinic acid, malic acid, pyruvic acid, ADP, inhibitors: rotenone (complex I inhibitor), antimycin A (complex III inhibitor), oligomycin (ATPase inhibitor), and ascorbate with N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine (TMPD) as electron acceptor, carbonylcyanide-3-chlorophenylhydra-zone (CCCP) as ionophore (uncoupler).

Mitochondrial isolation. Mitochondria were isolated according to Schnaitman and Greenawalt [29] with some modifications. Single clam soft tissue was homogenised in 1 ml of MSHE buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA and 0.5% BSA (fatty acid free), pH 7.2) at 4°C using glass homogenizer. Homogenate was centrifuged at 800 x g for 10 minutes at 4°C, obtained supernatant was centrifuged at 8000 x g for 15 minutes at 4°C and the pellet was resuspended in MSHE buffer. Total mitochondrial protein (mg/mL) was determined using the Bradford Assay reagent (Bio-Rad) and Bradford [30] method.

Mitochondrial respiration. Mitochondrial respiration was measured by the Agilent Seahorse XFp analyzer (Agilent Technologies, MA, USA). For analysis of mitochondrial coupling efficiency, the plate containing 25 µg mitochondria per well in 25 µL mitochondrial assay buffer (MAS; 220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1mM EGTA and 0.2% BSA (fatty acid free), pH 7.2) was centrifuged (2000 x g, 15 min, 4°C) and 155 µl MAS containing 10 mM succinate pre-warmed to 20 °C was used. After calibration, the mitochondria plate was inserted. After 10 min, two cycles (1 min mixing, 3 min waiting time) were followed by three cycles (1 min mixing, 1 min waiting, 3 min measurement). The protocol applied before each injection included 2 min mixing and 1 min waiting, addition of the indicated compound, 1 min waiting and a final measurement (3 min). At the indicated time points, 4 mM ADP, 2.5 µg/mL oligomycin A, 4 µM CCCP and 4 µM antimycin A were added.

For analysis of the electron transport chain (ETC) complexes activities the plate containing 25 µg mitochondria per well in 25 µL MAS buffer was centrifuged (2000 x g, 15 min, 4°C) and 155 µl MAS buffer containing 10 mM pyruvate, 2 mM malate and 4 µM CCCP prewarmed to 20 °C were used. In the same time sequence as in mitochondrial coupling assay, at the indicated time points, final concentrations of 2 µM rotenone, 10 mM succinate, 4 µM antimycin A and 10mM ascorbate plus 100 µM TMPD were added. Oxygen consumption rates (OCR; pmol/min) were calculated by the Wave software (Seahorse Bioscience).

Mitochondrial enzymes activities assay. The succinate dehydrogenase (SDH, EC 1.3.5.1) activity was determined in isolated bivalve mitochondria using a commercially available test kit (MAK197, Sigma-Aldrich). SDH catalyzes the oxidation of succinate to fumarate and carries electrons from FADH to CoQ. SDH activity was determined by generating a product (commercial DCIP) of which absorbance at 600 nm was proportional to the observed enzymatic activity. SDH activity was expressed in nmol/min/mg of the mitochondrial protein.

The cytochrome c oxidase (COX, EC 1.9.3.1) activity was determined using a commercially available test kit (CYTOCOX1, Sigma-Aldrich). COX is the principal terminal oxidase of high affinity oxygen in the aerobic metabolism of all animals. The activity of COX was measured based on its decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. COX activity was expressed in nmol/min/mg of the mitochondrial protein.

Antioxidative and physiological stress biomarkers

Individual organisms were homogenized manually with a Teflon-pestle homogenizer in ice-cold buffer (to obtain 20% homogenate) containing 50 mM Tris-H_2SO_4, pH 7.6 with 0.1 mM EDTA, 1 mM PMSF, 2 mM DTT and 0.2% Triton X-100. The homogenates were centrifuged at 14 000 x g for 30 minutes at 4°C using

Centrifuge SIGMA 3K18. The supernatants were transferred into fresh tubes and used for analysis. Cytosolic protein was determined by Lowry method [31] with modification of Peterson [32].

The TAC (Total Antioxidant Capacity) assay was measured using Total Antioxidant Capacity Assay Kit (MAK187, Sigma-Aldrich). Concentrations of small molecule and protein antioxidants (e.g. tocopherols, carotenes, vitamin A, ubiquinols) were determined using Trolox, a water-soluble vitamin E analog serving as an antioxidant standard. The prepared samples were measured at 570 nm in Synergy 2 Multi-Mode Reader (BioTek). The amount of TAC was normalized to the protein content of the samples and expressed in nmol/mg of total protein concentration.

Acetylcholinesterase (ACHE; EC 3.1.1.7) activity was measured with AChE Activity Assay Kit (MAK119, Sigma-Aldrich). This assay is an optimized version of the Ellman method in which thiocholine, produced by AChE, reacts with 5,5'-dithiobis(2-nitrobenzoic acid) to form an colorimetric product, proportional to the AChE activity present. The absorbance was measured for 10 min at 412 nm in Synergy 2 Multi-Mode Reader (BioTek). AChE activity was expressed in nmol/min/mg of total protein concentration.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined spectrophotometrically (UV-VIS Spectrophotometer, Beckman Coulter) by the method of Habig et al. [33]. The reaction mixture contained 100 mM phosphate-buffered saline buffer (pH 6.5), 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) and the reaction started by adding supernatant and 100 mM glutathione as substrate. The absorbance was measured for 5 min at 340 nm. GST activity was expressed in nmol/min/mg of total protein concentration.

CBO level was detected using the Protein Carbonyl Content Assay Kit (MAK094, Sigma-Aldrich). Carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts that are proportional to the carbonyls present. Absorbance was measured at 375 nm in Synergy 2 Multi-Mode Reader (BioTek). The amount of CBO was normalized to the protein content of the samples and expressed in nmol/mg of total protein concentration.

Malondialdehyde (MDA) level was detected using the Lipid Peroxidation (MDA) Assay Kit (MAK085, Sigma-Aldrich) by the reaction of MDA with thiobarbituric acid (TBA) forming amount of colorimetric product proportional to MDA. Samples were pipetted on a 96 - well microplate and absorbance was measured at 532 nm in Synergy 2 Multi-Mode Reader (BioTek). The amount of MDA was normalized to the samples protein content and expressed in nmol/mg of total protein concentration.

Determination of corticosteroids in M. balthica tissues

Corticosteroids concentration was measured according to Jakubowska et al. [34]. Each clam was homogenized using 5 ml of buffer containing 8.5 mM $MgCl_2$, 3.13 mM KCl, 7.59 mM NaCl, 2.7 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.4) per tissue gram. Next, MTBE (tert-butyl methyl ether) was added, the sample was mixed for 1 min and the mixture was centrifuged at 3000 × g for 15 min at room temperature. The supernatant was transferred to a new glass vial and MTBE was allowed to evaporate to dryness at 45°C. Upon evaporation, the dry residue of the eluate was dissolved in 100 µl of methanol and 5 µl of this solution was injected into the HPLC–ESI-MS/MS system. The elution was performed under a 10-min gradient. Testosterone-2,3,4-$^{13}C_3$ and 4-Androstene-3,17-dione-2, 3,4-$^{13}C_3$ were used as internal standards. HPLC-ESI-MS/MS analysis was performed using a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific) equipped with an ion electrospray source (ESI) coupled with a HPLC system (Thermo Scientific) using selected reaction monitoring (SRM) for detection in positive ion mode. All analytes were detected in SRM. The columns Kinetex Phenyl-Hexyl 50 × 2.1 mm 1.7u and Kinetex Biphenyl 50 × 1 mm 1.7u (Phenomenex) were used. Buffers used for the HPLC gradient elution included buffor A (0.1% formic acid in deionized water) and B (0.1% formic acid in methanol). Column equilibration was performed for 10 min. The total analysis time was 10 min. Calibration curves were prepared based on matrix and plotted as the peak area ratio (standard/IS) versus the amount of analytes. Next, the quantity of selected corticosteroids was calculated.

Statistical analysis

Data were tested for normality with Shapiro–Wilk test. Statistical significance of differences between groups (healthy and DN-BTN) was verified with Kruskal-Wallis test (non-parametric ANOVA) for data with non-normal distribution. The differences were considered significant at $p<0.05$. The results are presented as means ± standard errors of the mean (S.E.M). Calculations were performed using STATISTICA 13 software, figures were prepared in Sigma-Plot 11 software.

Results

FAA concentration

In general, nine amino acids were determined in bivalve haemolymph (Table 1). Aspartate (Asp) was present at the highest concentration (about 2.7 mM) and taurine (Tau) was found at the lowest level (about 1.3 µM). Overall, the comparative analysis showed no differences between FAA levels in haemolymph of healthy and neoplastic clams, except for glutamine (Gln), which occurred at a significantly higher concentration in neoplastic (86.6 µM) when compared to healthy clams (22.2 µM).

Table 1. Concentrations of FAAs (µM) detected in haemolymph of healthy and neoplastic clams. Data are presented as mean ± S.E.M. Asterisk indicate significant differences between healthy (n=12) and neoplastic (n=11) clams (Kruskal Wallis ANOVA, $p < 0.05$)

FAAs concentration (µM)	healthy clams	neoplastic clams	p
Aspartate/Asp	2765.4 ± 653.86	2653.3 ± 395.94	0.643
Glutamate/Glu	306.7 ± 69.26	427.3 ± 54.51	0.136
Proline/Pro	345.6 ± 79.56	565.6 ± 82.83	0.063
Serine/Ser	330.4 ± 73.97	383.8 ± 64.39	0.470
Alanine/Ala	74.5 ± 16.41	80.9 ± 13.43	0.683
Glutamine/Gln*	22.2 ± 2.57	86.6 ± 15.16	< 0.001
Glycine/Gly	4.2 ± 0.99	5.3 ± 0.64	0.136
Taurine/Tau	1.3 ± 0.31	2.3 ± 0.51	0.088
5-Hydroxylysine/Hyl	15.3 ± 2.75	23.8 ± 4.02	0.105

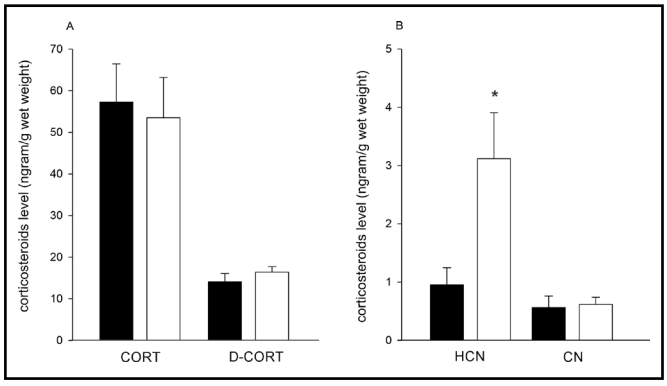

Fig. 2. Corticosteroids concentration in healthy (black bars) and neoplastic (white bars) *M. balthica*: A) corticosterone (CORT) and dehydrocorticosterone (D-CORT) level (n=10 in both groups); B) cortisol (HCN) and cortisone (CN) level (n=10 in both groups). Data presented as mean ± S.E.M. Asterisks indicate significant differences between healthy and neoplastic group (Kruskal Wallis ANOVA, $p < 0.05$).

Mitochondrial respiration and mitochondrial enzyme activities

Coupled mitochondria isolated from neoplastic bivalves were characterised by decreased respiration efficiency when compared to those isolated from healthy clams. Mitochondrial oxygen consumption in the presence of succinate, as well as reduced respiratory induction in the presence of ADP, were 3 and 2.5 times lower in neoplastic than in healthy clams, respectively. Additionally, mitochondria isolated from DN-BTN bivalves were found to be less sensitive to used inhibitors in comparison to healthy ones (Fig. 2A). Examination of individual components of the respiratory chain in uncoupled mitochondria showed similar activity of respiratory chain proteins in the mitochondria of healthy and DN-BTN bivalves, except for complex I, in which the oxygen consumption capacity was significantly higher in healthy clams (Fig. 2B). Sensitivity of respiratory chain complexes to inhibitors such as rotenone, antimycin A and oligomycin was observed in the mitochondria of healthy and neoplastic bivalves. Moreover, the transfer of electrons through ETC to the artificial acceptor ascorbate/TMPD was observed in both clam groups. Mitochondrial SDH activity was low (3.9 nmol/min/mg of protein) and comparable in both groups (Fig. 2C). Mitochondrial cytochrome c oxidase activity was significantly lower in BTN-positive individuals (9.1 ± 1.0) in comparison to the healthy ones (12.9 ± 0.7 nmol/min/mg) (Fig. 2C).

Antioxidative and physiological stress biomarkers

BTN-positive clams were characterised by significantly higher low-molecular antioxidant concentration then healthy ones (218.9 ±36.1 nmol/mg vs 171.3 ± 23.7 nmol/mg, p=0.028, Fig. 3A). AChE activity in tissues from healthy and BTN clams was at a similar level, 19.8 ± 2.4 and 22.8 ± 3.3 nmol/min/mg protein, respectively (Fig. 3B). Also, no statistically significant

differences between healthy and BTN clams in GST activity were observed (18.5 ± 2.4 nmol/min/mg protein in healthy and 16.9 ± 3.2 nmol/min/mg protein in BTN clams, Fig. 3B). No statistically significant differences in the level of accumulation of protein peroxidation (CBO) and lipid peroxidation (MDA) products between healthy and neoplastic bivalves were found. The amount of CBO in healthy clams was 3.3 ± 0.7 nmol/mg protein while in BTN clams 3.2 ± 0.8 nmol/mg protein. The amount of MDA in healthy clams was 1.7 ± 0.1 nmol/mg protein while in those affected by BTN 1.9 ± 0.3 nmol/mg protein (Fig. 3C).

Corticosteroids

Corticosterone was found at similar concentration in both healthy (57.2 ± 9.2 ng/g w.w.) and DN-BTN (53.4 ± 9.7 ng/g w.w.) clams. Levels of dehydrocorticosterone were lower and comparable between both clam groups (14.1 ± 2.0 ng/g w.w. and 16.4 ± 1.3 ng/g w.w. in healthy and BTN clams, respectively, Fig. 4A). Cortisol was found at a concentration of 0.95 ± 0.3 ng/gram w.w. in healthy clams, and its level was significantly higher in BTN-positive clams (3.1 ± 0.8 ng/g w.w., p=0.005) (Fig. 4B). Cortisone was at the lowest level of all corticosteroids measured, and its concentration was similar in both clam groups (0.6 ± 0.2 ng/gram w.w. in healthy and 0.6 ± 0.1 ng/gram w.w. in BTN clams) (Fig. 4B).

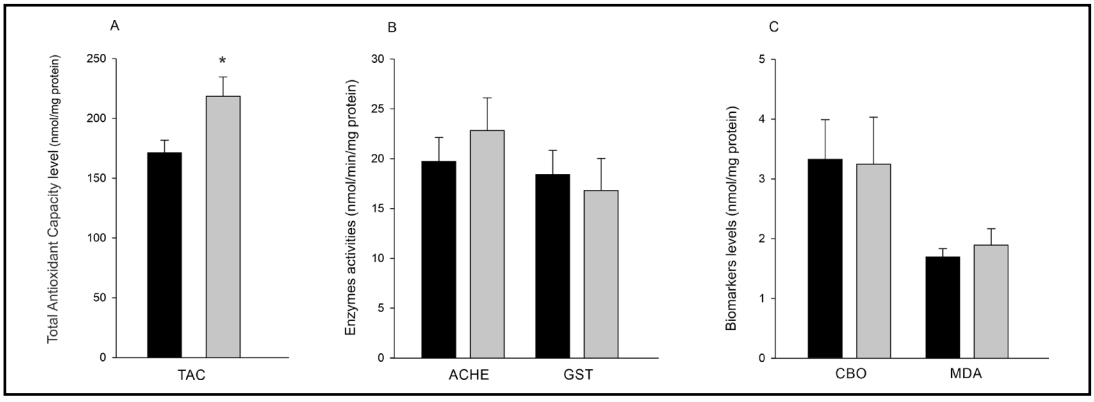

Fig. 3. Antioxidative and physiological stress biomarkers responses in *M. balthica* tissues in healthy (black bars) and neoplastic (grey bars) clams: A) TAC (Total Antioxidant Capacity) level (n=5 in both groups); B) AChE and GST activities, n=6 in both groups; C) protein carbonyl groups (CBO) level and lipid peroxidation products (MDA), n=6 in both groups. Data presented as mean ± S.E.M. Asterisks indicate significant differences between healthy and neoplastic clams (Kruskal Wallis ANOVA, p<0.05).

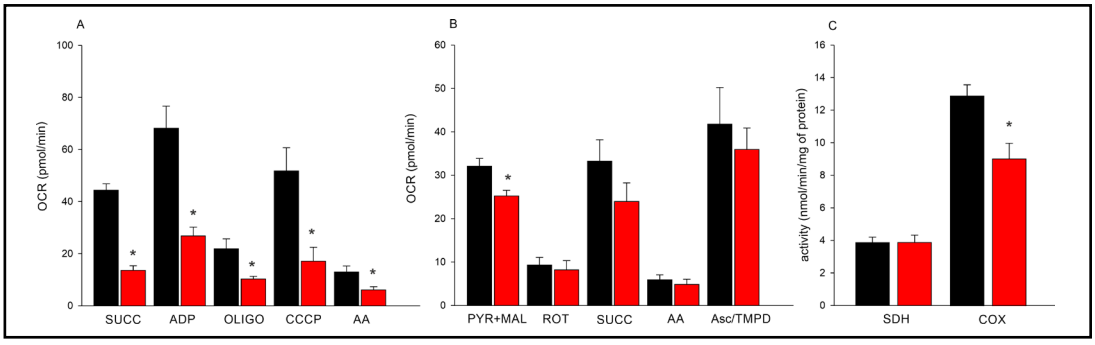

Fig. 4. Mitochondrial oxygen consumption rate (OCR) of A) coupled mitochondria isolated from healthy (n= 8, black bars) and neoplastic (n=5, red bars) clams in the presence of succinate (SUCC) and ADP as substrates, oligomycin (OLIGO) and antimycin A (AA) as inhibitors and CCCP as uncoupler; B) uncoupled mitochondria isolated from healthy (n=10, black bars) and neoplastic clams (n=7, red bars) in the presence of pyruvate, malate (PYR+MAL) and succinate as substrates, rotenone (ROT) and antimycin A as inhibitors and Asc/TMPD as artificial electron acceptor; C) activity of SDH and COX in mitochondria form healthy (n=15, black bars) and neoplastic (n=13, red bars) clams. Data presented as mean ± S.E.M. Asterisks indicate significant differences between healthy and neoplastic clams (Kruskal Wallis ANOVA, p<0.05).

Discussion

In this work we present the first data describing selected physiological and biochemical features of DN/BTN based on the model species *Macoma balthica*. What is more, some of these markers have the potential to distinguish DN-BTN cells from normal tissue cells.

Marine molluscs have a great ability to regulate haemolymph osmolarity with inorganic solutes and levels of free amino acids. The dominant amino acids in bivalve haemolymph are aspartic acid (or its ionic form aspartate), glutamic acid (or its ionic form glutamate, Glu), Ser, Pro, and Tau, which is typical for marine molluscs [24]. These amino acids may perform two of the most important functions determining physiological functions of osmoconformers such as marine bivalves: (i) provide ions necessary for the balance of internal cations; and (ii) provide extra solutes necessary for osmoregulation [24]. Additionally, FAAs act as substrates for energy metabolism and protein synthesis. In bivalves, FAA composition may differ at species, population or even at individual levels [26, 35, 36]. These differences are related to factors such as sex, gametogenesis stage, nutritional status [25, 26] or may be regarded as indicators of stress [36, 37]. FAA profile and concentration in clams was also proven to be tissue-dependent [38, 39]. Our results indicated the presence of nine free amino acids in *M. balthica* haemolymph, namely Asp, Glu, Pro, Ser, Ala, Gln, Gly, Tau and 5-hydroxylysine with the latter being an elementary part of collagen [40]. According to Sokołowski et al. [26], overall 14 amino acids were detected in *M. balthica* tissues (the whole body) with Ala, Gln, Arg, Gly and Orn composing over 80% of the total FAAs. In our study, Asp, Glu, Pro, Ser and Ala constituted of over 90% of total FAA in the haemolymph of both healthy and DN-BTN clams. Performed comparative analyses indicated no significant differences in the amount of FAAs between healthy and DN-BTN-positive clams except for Gln, which level was significantly higher in neoplastic individuals. Gln is a key amino acid as its metabolism contributes to energy production, macromolecular synthesis, and redox homeostasis, and is essential for survival of some cancer cells as they are highly dependent on Gln for glutaminolysis [41]. The major function of glutaminolysis is to supply intermediary metabolites in the TCA (Krebs) cycle for cell growth. In the haemolymph of DN-BTN bivalves, Gln may have a similar function as in vertebrate serum, as it acts as the main substrate for intensively proliferating cells such as haemocytes. Indeed, according to our results contagious type of DN is a condition affecting Gln level in the haemolymph, and its increased concentration in the DN-BTN clams is most likely related to the pathology of the disease (e.g. inhibition of glutaminase involved in Gln catabolism or its increased synthesis) [42, 43]. On the cellular level, however, as cancer cells need to adapt to increased biosynthetic activity, an increased Gln concentration plays an essential role in energy production, possibly via glutaminolysis [41, 44]. This result also finds elevated levels of Gln as a useful marker of DN/BTN.

The metabolic pathways of vertebrate cancer cells are examined at various levels but the knowledge about metabolic pathway of DN/BTN occurring in marine bivalves is lacking. Normal cells obtain energy through aerobic respiration but solid tumour cells most commonly obtain energy through anaerobic mechanisms. In various neoplastic disorders, cells convert glucose by fermentation into lactate even with sufficient oxygen availability. Only a small amount of pyruvate is used for oxygen respiration leading to the commonly known Warburg effect. The lactate in cancer cells is not only synthesized from glucose, but, as mentioned earlier, it may also be formed from Gln during glutaminolysis [45]. The Warburg effect may also result from mitochondrial dysfunction caused by mutations within the mtDNA [23, 46]. Yet, most of the available information on glucose metabolism in cancer cells comes from solid tumours. Changes in signal pathways and morphology occurring in leukemic cells may resemble activation of normal lymphocytes as higher levels of Gln were also detected in neoplastic *M. balthica* clams. Performed here functional analyses of mitochondria isolated from healthy and DN-BTN clams indicated for the first time that in coupled mitochondria, despite the availability of respiratory substrates such as pyruvate, malate and succinate, oxygen consumption was three times lower when compared to healthy clams. Furthermore, in uncoupled mitochondria lower oxygen consumption by respiratory complex I was observed in clams affected by DN/BTN.

As an occurring Warburg effect reduces effectiveness of mitochondrial respiration and switches on anaerobic metabolism, we, also for the first time, measured activities of succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) in healthy and neoplastic bivalves. SDH is a mitochondrial enzyme that catalyzes the oxidation of succinate to fumarate and carries electrons from FADH to CoQ in electron transport chain. It has a central function in the maintenance of cellular energy metabolism via the Krebs (tricarboxylic acid) cycle and the ETC. Our results indicate that the activity of mitochondrial SDH was low and there was no difference in SDH activity between healthy and DN-BTN-positive bivalves. Cytochrome C oxidase (COX) is the principal terminal oxidase of high affinity oxygen in the aerobic metabolism of all animals, plants, yeasts, and some bacteria. The enzyme is present in mitochondria and is probably unique in providing energy by coupling electron transport through the cytochrome chain with oxidative phosphorylation. According to our results, in the clams with DN-BTN activity of COX was significantly lower than in healthy molluscs. This may confirm the impaired mitochondrial respiration in clams suffering from BTN. Decreased COX activity may be a consequence of occurring mtDNA mutations in the BTN cells, but may also be a consequence of changes occurring in the non-cancer clam tissues as a response to progressing disease. Also, the structure and function of COX are affected in a wide variety of diseases and dysfunctional COX promotes oxidative stress induced by superoxide elevation and ATP depletion [47].

Invertebrate immunity is based on the innate immune system, in which haemocytes are the first line of defence against any of the external and internal assaults, playing a major role in both humoral and cellular immunity. Haemocytes as well as other immune cells present in haemolymph produce various cytokines such as IL-α and β, IL-2, IL-6-like and TNF-α-like molecules [48, 49]. Phagocytes, sometimes containing brown pigmentation (lipofuscin or melanin), are involved in the processes such as phagocytosis, melanisation and lysosomal digestion. Also, production of ROS by neutrophils and macrophages as a mechanism to kill tumour cells is well established. In these cells, a rapid burst of superoxide formation primarily mediated by NAPDH oxidase leads to subsequent production of hydrogen peroxide [50, 51]. Furthermore, during inflammatory reactions, activated macrophages also generate nitric oxide, which reacts with superoxide to produce peroxynitrite radicals that are similar in their activity to hydroxyl radicals and contribute to tumour cell apoptosis [52]. Thus, elevated rates of ROS have been detected in almost all cancers, where they promote many aspects of its development and progression. However, cancer cells also express increased levels of antioxidant proteins, suggesting that a delicate balance of intracellular ROS levels is required for cancer cell function, as increased levels of low-molecular antioxidants (tocopherols, carotenes, ubiquinols, glutathione and ascorbate) correspond to the rising ROS concentrations. Indeed, we observed significantly higher concentrations of antioxidants in DN-BTN-positive bivalves than in the healthy ones. However, an increased amount of oxidative stress products (MDA and CBO) was not found, suggesting an efficient antioxidant response in neoplastic clams. Also, GST activity plays a critical role in cellular detoxification and its response did not differ between both clam groups. Similarly, individuals suffering from BTN showed no signs of neurotoxicity, as AChE activity was at the same level as in healthy ones.

Finally, we for the first time, demonstrated the content of various corticosteroids in healthy and DN-BTN *M. balthica*. Our results highlighted high levels of corticosterone and lower amounts of dehydrocorticosterone, cortisol and cortisone present in normal bivalve tissues. In vertebrates, the brain and immune systems are linked by two primary pathways: (i) the sympathoadrenal system (SAS), via either direct neural innervation of lymphoid tissue or catecholamine (e.g. epinephrine, norepinephrine) release from the adrenal medulla and (ii) the hypothalamo-pituitary- adrenal (HPA) axis and subsequent release of glucocorticoids (e.g. cortisol, corticosterone) from the adrenal cortex [27]. Analogous neuro-endocrine-immune connections can also be found in invertebrates and multiple neuro-endocrine systems can modulate immune function [53]. In most species, cortisol regulates not only metabolism, but also immune response, and its release takes place during an organism

response to stress [54, 55]. In molluscs, the presence of cortisol has long been described [55, 56] but its origin and genomic mechanism of action is unknown, as no corticosteroid receptors have been found so far. The non-genomic effect of corticosteroids in vertebrates may affect the signalling pathways of MAPK kinases, adenylyl cyclase, protein kinase C (PKC), and G-proteins [57], and this interaction could be similar in bivalve molluscs. Our results highlighted a significant increase in cortisol level in the DN-BTN-positive individuals that may result from activation of the clams' immune systems, and in particular haemocyte activation. However, such an increase can also be a consequence of massive changes occurring in haemocytes at structural, morphological and genetic levels due to BTN spread. As a result, an increase in the ability of antigens to induce the increase of ACTH and CRH levels in haemolymph and the rise of synthesis of corticosteroids, mainly cortisol, can be expected. Nevertheless, there are no other available data linking the levels of corticosteroids with the progression of disseminated neoplasia in marine bivalves. An enhanced knowledge about the MoA (mechanism of action) of cortisol in molluscs would therefore allow a broader understanding of the function of steroid hormones in invertebrates in general and of cortisol in particular, and of its role in neoplastic disorders occurring in marine bivalves. Yet, bivalve endocrinology is deeply under researched and there are various gaps in the knowledge to be filled in order to fully understand the processes behind environmental carcinogenesis.

Conclusion

Disseminated neoplasia, and in particular bivalve transmissible neoplasia, is a disease of unknown biochemical characteristics occurring in various bivalve species. To increase our knowledge about this phenomenon, we described FAA levels in bivalve haemolymph, corticosteroids levels, oxidative and physiological stress biomarkers response in clam tissue and measured mitochondrial respiration in DN-positive and healthy cells. In both clam groups nine FAAs were found (Asp, Glu, Pro, Ser, Ala, Gln, Gly, Tau and 5-hydroxylysine) but in the neoplastic clams significantly higher content of Gln was observed when compared to the healthy ones. Such high levels of Gln suggest its essential role in energy production in leukaemia-like cancer and stresses a potential role of Gln as a marker of environmental carcinogenesis. Analyses of the respiratory pathway highlighted significantly lower oxygen consumption in coupled and uncoupled mitochondria (respiratory complex I) isolated from DN-positive clams that indicated altered mitochondrial function in BTN. As an occurring Warburg effect reduces effectiveness of mitochondrial respiration, a switch to anaerobic metabolism and glutaminolysis was expected. Indeed, significantly lower cytochrome c oxidase activity was found in the DN-BTN-positive clams, potentially confirming their impaired mitochondrial respiration, and detectable activity of succinate dehydrogenase suggested the occurrence of aerobic metabolism. Indeed, an increased level of Gln highlights glutaminolysis as a potential energy source for BTN cells. Also, a significantly higher concentration of low molecular weight antioxidants level was confirmed in DN-BTN clams when compared to the healthy ones, but no elevated oxidative stress markers (MDA and CBO levels and GST activity) were found, suggesting an efficient antioxidant response. Finally, high levels of corticosterone and lower amounts of dehydrocorticosterone, cortisol and cortisone were present in healthy bivalves, while in the DN-BTN ones significantly elevated level of cortisol was found. As bivalve haemocytes belong to the main neuro-endocrine secretion systems, these changes may be explained by haemocytic activation and/or massive changes occurring in BTN.

Finalising, based on our results we are able to select three biomarkers as potential candidates for standardized biochemical markers for DB-BTN, namely Gln level, OCR rate and cortisol concentration. Yet, our study is the first one describing various biochemical features of the disease, thus more studies are needed to provide more comparative data regarding this aspect of the disease. That is in particular important as some of the measured features, such as FAA level, are known to change in a response not only to the physiological state of an individual, but also in response to the external environmental conditions.

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Author Contributions

Anna Hallmann – conceptualisation, analysis, data acquiring and interpretation, writing the original draft.

Alicja Michnowska – field sampling, analysis, data acquiring and interpretation, manuscript reviewing and editing.

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Katarzyna Smolarz – funds provider, field sampling, analysis, data interpretation, manuscript writing, reviewing and editing.

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Statement of Ethics

The authors confirm that the study was conducted ethically. There are no regulations considering using lower invertebrates, such as clams, for scientific purposes. However, the authors made sure to provide as little harm as possible to the studied animals.

Disclosure Statement

The authors have no conflicts of interest to declare.

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