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Does age pay off? Effects of three-generational experiments of nanodiamond exposure and withdrawal in wild and longevity-selected model animals

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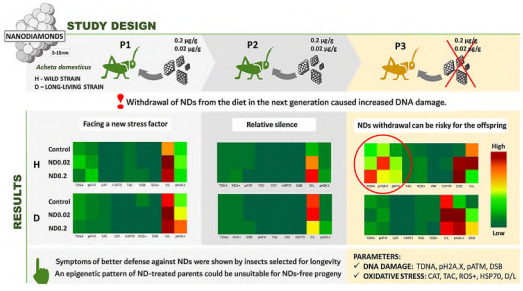
HIGHLIGHTS

- Exposure to low doses of nanodiamonds (NDs) in one generation seems to be safe.
- The offspring of ND-exposed parents reveal fairly efficient defense against NDs.
- Symptoms of better defense against NDs were shown by insects selected for longevity.
- Withdrawal of NDs from the diet in the next generation caused increased DNA damage.
- An epigenetic pattern of ND-treated parents could be unsuitable for ND-free progeny.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanodiamonds (NDs) are considered a material with low toxicity. However, no studies describe the effects of ND withdrawal after multigenerational exposure. The aim was to evaluate ND exposure (in the 1st and 2nd generations) effects at low concentrations (0.2 or 2 mg kg^{-1}) and withdrawal (in the 3rd generation) in the wild (H) and longevity-selected (D) model insect *Acheta domesticus*. We measured selected oxidative stress parameters, immunity, types of cell death, and DNA damage. Most of the results obtained in the 1st generation, e.g., catalase (CAT), total antioxidant capacity (TAC), heat shock proteins (HSP70), defensins, or apoptosis level, confirmed no significant toxicity of low doses of NDs. Interestingly, strain-specific differences were observed. D-strain crickets reduced autophagy, the number of ROS+ cells, and DNA damage. The effect can be a symptom of mobilization of the organism and stimulation of physiological defense mechanisms in long-living organisms. The 2nd-generation D-strain insects fed ND-spiked food at higher concentrations manifested a reduction in CAT, TAC, early apoptosis, and DNA damage, together with an increase in HSP70 and defensins. ROS+ cells and cells with reduced membrane potential and autophagy did not differ significantly from the control. H-strain insects revealed a higher number of ROS+ cells and cells with reduced membrane potential, decreased CAT activity, and

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early apoptosis. Elimination of NDs from the diet in the 3rd generation did not cause full recovery of the measured parameters. We noticed an increase in the concentration of HSP70 and defensins (H-strain) and a decrease in apoptosis (D-strain). However, the most visible increase was a significant increase in DNA damage, especially in H-strain individuals. The results suggest prolonged adverse effects of NDs on cellular functions, reaching beyond "contact time" with these particles. Unintentional and/or uncontrolled ND pollution of the environment poses a new challenge for all organisms inhabiting it, particularly during multigenerational exposure.

Author contribution statement

M.A.: Conceptualization, Formal analysis, Investigation, Validation, Writing–original draft, Visualization, Supervision, **M.D.**: Methodology, Investigation, Formal analysis, Visualization, Writing–original draft; **J. K.-K.**: Conceptualization, Formal analysis, Project administration, Funding acquisition, Writing – review & editing; **A.B., B.F., A.K., B.M., K.R., R.S.A., M.S., E.Ś., A.Ś., M.T., K.W., P.Z.**: Methodology, Investigation, Validation, Formal analysis, Writing – review & editing.

1. Introduction

Currently, many scientists studying carbon nanoparticles and nanomaterials are prone to consider nanodiamonds (NDs) as materials with high biocompatibility, unique and excellent properties, and low toxicity (Ma et al., 2021; Mi et al., 2021). Such viewpoints make NDs useful for designing electrochemical coatings, polymer composites, polishing materials, lubricants, biosensors, image sensors, implants, and drug carriers (Basso et al., 2020; Huang et al., 2008; Stursa et al., 2016; Yuan et al., 2010). NDs appear in everyday life, e.g., as ingredients of cosmetics, shampoos, soaps, toothpaste, and washing powders. Their use as flavor, color, and aroma enhancers of food or in the production of food and food packaging has also been described (Kazi, 2014; Ranjha et al., 2022). The inevitable increase in the production and consumption of NDs poses a potential risk for organisms. In a far-reaching vision, one can expect NDs to be released into the environment, consequently affecting nontarget organisms and, ultimately, the stability of ecosystems.

However, there are still discussions about different models of ND interactions with molecules in cells and on their surfaces. There are also reports on the toxicity mechanisms of these nanoparticles, including cellular ND uptake, reactive oxygen species (ROS) production, aggregation of particles, stimulation of capsaicin receptors, and covalent modifications of cell structures (Cid et al., 2015; Nel et al., 2006). Among them, ROS production and the generation of oxidative stress have attracted the most attention (Li et al., 2008). Most ND biocompatibility/toxicity studies were conducted on vertebrates or human cells and tissues (Dworak et al., 2014; Khanal et al., 2017; Mytych et al., 2015; Tasat et al., 2017; Yuan et al., 2010; Zhang et al., 2010). In these studies, the exposure time was usually short and limited to several to dozen hours or, rarely, several days or even a single injection (Table S1). NDs are suggested to be neutral to macrophages, keratinocytes, neuroblastoma, and PC-12 cells (Schrand et al., 2007). On the other hand, the adverse effect of NDs on lymphocytes and cervical cancer cells was reported (Dworak et al., 2014; Mytych et al., 2014). Additionally, intratracheally administered NDs have low pulmonary toxicity (Yuan et al., 2010), which contrasts with their ability to penetrate and redistribute into tissues/organs that have no direct contact with NDs (Yuan et al., 2009; Zhang et al., 2010).

Other studies have investigated the *in vivo* effect of NDs using insects as model organisms. Karpeta-Kaczmarek et al. (2016a) exposed adult *A. domesticus* to NDs at concentrations of 20 and 200 mg kg^{-1} in food for ten days. The results suggested that short-term exposure to NDs in the diet seems to be safe for the organism. However, long-term exposure to NDs at the same concentration indicated an unfavorable effect on *A. domesticus*, such as an increase in oxidative stress and DNA damage (Karpeta-Kaczmarek et al., 2016c), damage to the structure of gut epithelial cells (Karpeta-Kaczmarek et al., 2016a), and disturbances in the development and reproduction of insects (Karpeta-Kaczmarek et al., 2018). Irrespective of the complex interactions described above, ROS generation is perceived as the main effect of nanoparticle toxicity. Therefore, in line with the free radical theory of aging (le.Bourg, 2001; Muller et al., 2007; Sohal, 2002; Sohal and Orr, 2012), they may contribute to the changes in the lifespan of exposed animals. Conversely, long-living organisms may be able to take advantage of potentially better antioxidant mechanisms to restrain ND-induced stress.

At our institute, an original strain of long-living house cricket (*Acheta domesticus*) was obtained by multigenerational selection (over 20 years). The selected strain differs from the control in many aspects, such as survival, development, lifespan, or susceptibility to stress agents (Flasz et al., 2020, 2021a, 2021b, 2021a). One can suppose that the selection of organisms for longevity will favor individuals with more efficient mechanisms of removing free radicals. Therefore, there are reasons to believe that age-dependent selection improves features that contribute to handling additional stressors, which increase the production of ROS in cells. Assuming ROS-mediated toxicity of NDs, the long-living strain can be extremely useful in understanding the mechanism of the impact of NDs on organisms. Using it in research is a novel approach to the problem of nanoparticle toxicity.

The main aim of the study was to assess the effects of chronic, two-generation exposure of the model insect *Acheta domesticus* to low concentrations of NDs in food by examining selected parameters of oxidative stress, immunity, various types of cell death (autophagy, apoptosis), and the level of DNA damage. The other goal was to check if the response of the crickets to the ND-free diet in the so-called recovery experiment (third generation) differed from the exposed ones. The following hypotheses were tested:

H1.0. Low doses of nanodiamonds provided as food admixture over the long term do not show a significant effect on generating free radicals and oxidative stress. There were also no significant differences between long-living and wild insects in terms of ND-induced effects.

H1.1. The ND toxicity mechanism was based on increasing ROS generation. Both strains react to stress associated with NDs in the diet similarly. Selection favoring the longer lifespan of insects does not seem to be significantly related to better coping with oxidative stress.

H1.2. Long-living insects show better abilities to handle stress than individuals from the wild-type strain. Individuals from the long-living strain employ mechanisms of removing free radicals developed during multigenerational selection. They have more efficient enzymatic mechanisms responsible for removing ROS and/or repairing the damage they cause.

H2.0. Removing the stressor (NDs) brings all the parameters to the control level. The phenomenon of ND penetration through the gut barrier and subsequent accumulation in other organs (e.g., the ovaries) is insignificant. The transfer of NDs from mothers to eggs is negligible, and there are no specific mechanisms preparing offspring to live in increased oxidative stress.

H2.1. Despite ND removal from food, the organisms still reveal significant destabilization of the measured parameters, with values different from those typical for the control. Chronic exposure to low

concentrations of NDs in the parental generation results in disturbances in offspring consuming an ND-free diet. A detailed explanation should be sought in epigenetic research.

2. Materials and methods

2.1. Nanodiamonds

Single Digit NanoDiamonds (commercial name) were purchased from PlasmaChem GmnH, Berlin. According to the manufacturer, NDs were obtained by chemical disintegration (diamond crystallite size: 3.5–5.2 nm; particle size: 5–15 nm). The aqueous suspension was transparent, stable in water/polar organic solvents, and free of impurities. Before the study, ND were characterized by multiple microscopic and spectroscopic techniques presented in a previous paper (Karpeta--Kaczmarek et al., 2016c). Additional analyses confirmed that the tested material is a nanodiamond (3–5 nm) with an irregular shape and structural defects (Fig. S1). Additionally, before use in this experiment, the stability of the ND suspension was examined using a particle analyzer (Litesizer 500, Anton-Paar®). The negative and low zeta potential (−15.6 mV, 25 °C) indicated mild aggregation in the suspension and the presence of oxygen groups on the ND surface (Fig. S3).

2.2. Experimental design

The Acheta domesticus (Insecta, Orthoptera: Gryllidae) used in the experiment was derived from our laboratory population (Dziewięcka et al., 2020). The selection of long-living insects (strain D) was started by systematically postponing reproduction in subsequent generations twenty years ago. In strain D, individuals who have reached 35–42 days of age are allowed to reproduce. Such selection resulted in a reduction in larval mortality and prolonged adult life. In the second strain (wild: H), insects reproduce without delay, usually a few days after reaching the imago stage (Flasz et al., 2021b).

The experiment was conducted under controlled conditions, optimal for this species (28.7 ± 0.44 °C; L:D 12:12, RH: 43.80 ± 6.72%). One-week-old larvae from the two strains, D and H, were selected to create experimental groups: control (receiving food without ND admixture) and two ND-treated groups ND0.2 and ND2 (fed with ND-contaminated food at concentrations of 0.2 mg kg^{-1} or 2 mg kg^{-1}, respectively) for their whole life. The experiment lasted three generations. Insects from the 1st and 2nd generations received ND-contaminated food, and those from the 3rd generation (recovery) received ND-free food (the same as the control group). The food was prepared as described in our previous papers (Dziewięcka et al., 2018, 2020; Karpeta-Kaczmarek et al., 2016a, 2018). Five mature insects (three months old since hatching from an egg) were randomly selected in each generation and experimental group (Scheme 1). Insects were anesthetized on ice (5 min) and dissected, and tissues were prepared for analysis of the parameters described below.

2.3. Biochemical analysis

2.3.1. Catalase and total antioxidant capacity

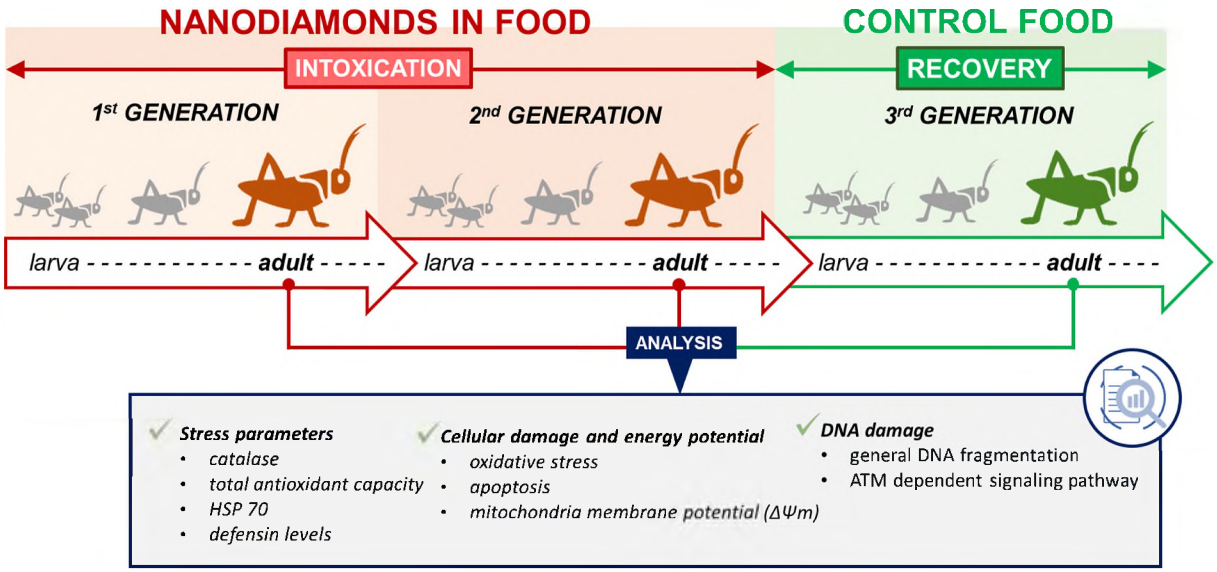
The protein level, catalase activity (CAT), and total antioxidant capacity (TAC) were measured in the gut submitochondrial fraction using a UV–vis spectrometer (TECAN Infinite M200, Austria). The gut was homogenized on ice (4 °C) with 0.1 M PBS (pH 7.4) and centrifuged (10 min, 4 °C, 15.000×*g*), and then the supernatant was transferred into new Eppendorf tubes.

The total protein concentration was measured according to the Bradford method (1976) using 1% BSA (bovine serum albumin) as a standard. Catalase (CAT, EC1.11.1.6) activity was assessed at a wavelength of 230 nm with 10 mM H_2O_2 according to Aebi (1984). The CAT activity was expressed in μmol H_2O_2 min^{-1} mg $protein^{-1}$.

The total antioxidant capacity was determined using 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Re et al., 1999). The decolorization of ABTS with 5 μL of the sample was tested at 734 nm. The results are presented in μmol Trolox mg protein $^{-1}$.

2.3.2. HSP70 and defensins

For HSP70 and defensin concentrations in the insect gut, an indirect ELISA method was used as described by Crowther (2009). First, 100 μL aliquots of gut supernatant were placed into the wells of 96-well transparent flat-bottom plates. After incubation (overnight at 4 °C) and removing the supernatants, all wells were washed (3 × PBS buffer with Tween-20 (0.2%) - PBST, pH 7.4). Next, 1% bovine serum albumin (Sigma; 1 h, 37 °C) was used to block potentially uncoated areas. After washing (2 × PBST), primary antibodies were added (mouse anti-heat shock protein monoclonal antibody; Sigma–Aldrich; 1:1000 or rabbit *anti*-B-defensin 3 antibody; Sigma–Aldrich, 1:1000) and incubated for 2 h at 37 °C. Next, the samples were rewashed (4 × PBST), incubated (1 h; 37 °C) with a secondary antibody (goat anti-mouse IgG polyclonal antibody, AP conjugate; Stressgen; 1:1000 or goat anti-rabbit IgG, Sigma–Aldrich; 1:1000), and then washed again (4 × PBST). An aliquot of



Scheme 1. Experiment design.

100 μL of a phosphatase substrate solution pNpp (*p*-nitrophenyl phosphate, Sigma–Aldrich) was added to the samples and kept at room temperature for 0.5 h. HSP70 and defensin concentrations were measured at 405 nm using a UV–Vis spectrometer (TECAN Infinite M200, Austria). The concentration of the proteins was expressed as absorbance values.

2.3.3. Cellular damage and energy potential

The effects of nanodiamonds on apoptosis, autophagy, oxidative stress, and mitochondrial membrane potential (ΔΨm) in gut cells were assessed via flow cytometry using the Muse® Annexin V & Dead Cell Kit, Muse® Autophagy Assay Kit, Muse® Oxidative Stress Assay Kit, and Muse® MitoPotential Assay Kit, respectively. All the diagnostic kits were purchased from Luminex Corporation (California, USA) and intended for a Muse® Cell Analyzer (Millipore, Billerica, MA, USA). Analyses were performed according to the manufacturer's protocols. The cell suspensions for Muse® analysis were prepared 0.1 M PBS (pH 7.4) using a Minilys homogenizer (Bertin Technologies). More details are described elsewhere (Augustyniak et al., 2016b; Dziewięcka et al., 2017, 2020, 2018; Flasz et al., 2020; Karpeta-Kaczmarek et al., 2018).

The Muse® Annexin V & Dead Cell Kit was used to detect early (early apo) and late (late apo) apoptotic cells in the suspension of gut cells. The Muse® Autophagy Assay Kit, utilizing a monoclonal antibody conjugated to Alexa Fluor® 555, allowed us to detect the mean autophagy intensity (MAI) by monitoring lipidated LC3-II in the samples. The Muse® oxidative stress assay distinguished two populations of cells with ROS (ROS+) and without ROS (ROS-) using dihydroethidium (DHE) dye.

The Muse® MitoPotential Assay allowed the measurement of the change in transmembrane potential (ΔΨm) that enables the cell to produce ATP. Apoptosis and cellular stress were associated with the loss of mitochondrial membrane potential (MMP). The method used the MitoPotential dye to measure changes in the MMP and 7-aminoactinomycin D (7-AAD) as markers of cell membrane structural integrity and cell death. During analysis, various subpopulations of gut cells were distinguished. Two of them were analyzed in this study: live cells with depolarized mitochondrial membranes [MitoPotential (−) and 7-AAD (−)] (Depolarized/Live) and dead cells with intact mitochondrial membranes [MitoPotential (−) and 7-AAD (+)] (Depolarized/Dead).

2.3.4. DNA damage

The genotoxic effect of NDs was tested in a hemolymph suspension of *A. domesticus* by the comet assay (SCGE; Single Cell Gel Electrophoresis assay) and in a gut cell suspension via the Muse® Multi-Color DNA Damage Kit. Briefly, the cell suspension was mixed with agarose, spread onto slides (one slide/one individual), and incubated in lysis buffer for 1.5 h at 4 °C. Horizontal electrophoresis was performed under alkaline conditions (pH > 13, 4 °C, 20 min at 0.3 A). Next, slides were visualized with DAPI and analyzed under a fluorescence microscope using a Komet 5.5 image analysis system (Kinetic Imaging, Liverpool, UK). DNA damage was described by tail DNA (TDNA), tail length (TL), and olive tail moment (OTM) parameters in 50 randomly selected nuclei on each of 5 slides per experimental group (Augustyniak et al., 2016a, 2016b; Dziewięcka et al., 2020; Flasz et al., 2020; Karpeta-Kaczmarek et al., 2016b).

The Muse® Multi-Color DNA Damage Kit is a test involving conjugated antibodies: a phospho-specific ATM (Ser 1981)-PE and a phospho-specific histone H2A.X PECy5. The test allows exploration of DNA damage through the ATM-dependent signaling pathway. ATM-dependent phosphorylation of H2A.X accompanies increased DNA damage. The ATM protein indicates DNA repair by mobilizing adequate enzymes. The analysis showed (percentage): ATM-activated cells (pATM), H2A.X activated cells (pH2A.X), and DNA double-strand breaks (DSB). Similar to the above methods dedicated to the Muse® Cell Analyzer, the test kit was made following the manufacturer's instructions.

2.4. Statistical procedures

All parameters were measured with five replicates per group. The median for each parameter was calculated from 50 randomly selected nuclei in each slide (the comet assay). Then, the median values were used to calculate the mean for each experimental group and statistical tests. Before selecting the statistical tests, normality was assessed using the Kolmogorov–Smirnov and Lilliefors tests, and the homogeneity of variance was assessed using the Levene test. These tests provided a rationale for the use of parametric tests. Two-way ANOVA ($p < 0.05$) was used to diagnose the influence of individual independent variables and their interactions on dependent variables. MANOVA (Wilks' Lambda test; $p < 0.05$) was used to determine the effect of factors and their interactions on all dependent variables. The post hoc test, the Least Significant Difference test (ANOVA, LSD test, $p < 0.05$), was used to check the differences between the experimental groups. All parameters are expressed as the mean ± SD in the figures. Hierarchical clustering analysis was also performed to visualize relations among all parameters. Statistical analysis was performed using the Statistica 13.1 software package (StatSoft, Inc.).

3. Results

The MANOVA (Wilks' Lambda test; $p < 0.05$), including all examined parameters, revealed a significant influence of all the factors and their interactions, except for the 'strain' × 'treatment' interaction. However, the statistical test value for this interaction was $p = 0.055$ (Table S2). Clustering analysis revealed that CAT, defensins, HSP70, TAC, and apoptotic parameters were relatively stable among generations/strain/experimental groups. In contrast, Depolarized/Live cells and parameters of DNA damage were much more influenced by the analyzed factors (Fig. 1). Subsequently, two-way ANOVA was carried out to diagnose the effects of the factors and differences in each parameter separately.

3.1. Biochemical markers

The oxidative stress markers CAT and TAC were at the same level in both the H and D strains. The effect of NDs was found only in the P2 generation, where compared to the control, a higher concentration of NDs led to a significant reduction in CAT activity in both strains and a reduction in the TAC level in the D strain. Additionally, a significant decrease in CAT activity was observed in insects consuming ND-contaminated food (ND0.2 group) but only in the P2 generation of the H strain (Fig. 2). Two-way ANOVA revealed a significant influence of the 'generation' factor on CAT activity. A significant interaction effect of 'generation' × 'treatment' on TAC level was also detected (Table S3).

Stress protein (HSP70) and defensin levels were similar in both strains of the P1 generation and were unaffected by NDs. The D strain receiving food with a higher concentration of NDs revealed a significant increase in both parameters in the P2 generation. Interestingly, removing NDs from the diet (generation P3 – recovery) led to an increase in the concentration of HSP70 in the ND0.2 group compared to the control. Additionally, a significant increase in the concentration of defensins in the H strain individuals whose parents received NDs at higher concentrations was observed (Fig. 3). The 'generation' and 'treatment' factors and their combination had a significant impact on the level of HSP70. The factors 'treatment', 'generation' × 'strain' and 'generation' × 'strain' × 'treatment' had a significant effect on the level of defensins (Table S3).

3.2. Cellular damage and energy potential

In general, the D-strain insects showed a higher percentage of cells in the early apoptosis phase than the H-strain insects. However, this interstrain difference was significant only in the P1 and P3 control

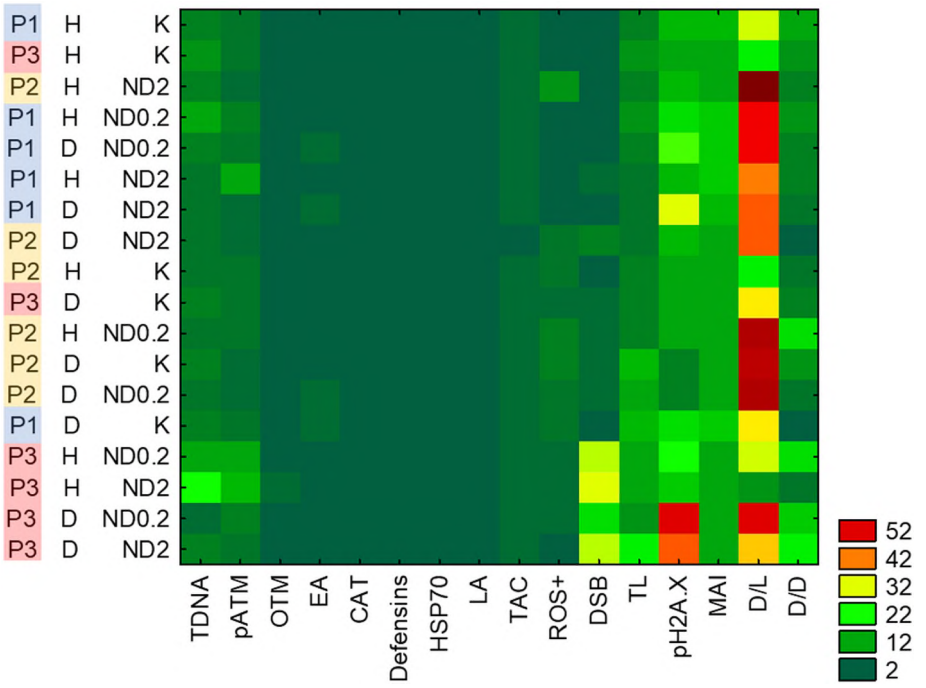


Fig. 1. Heatmap of all measured parameters in *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). Abbreviations: H – the wild strain of crickets; D – the long-living strain of crickets; control – insects fed with uncontaminated food, ND0.2; ND2 – insects receiving food contaminated with NDs in the concentration of 0.2 mg kg^{-1} or 2 mg kg^{-1}, respectively; TDNA – the amount of DNA in comet tail, pATM – activated ATM, OTM – olive tail moment, EA – early apoptotic cells, CAT – catalase, HSP70 – heat shock proteins, LA – late apoptotic cells, TAC – total antioxidant capacity, ROS+ - cells with increased oxidative stress, DBS – double-strand breaks, TL – tail length, pH2A.X – cells with activated histone H2A.X, MAI - mean autophagy intensity, D/L – Depolarized/Live - live cells with depolarized mitochondrial membrane, D/D – Depolarized/Dead. The colors in the heatmap indicated the values intensity of each parameter. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

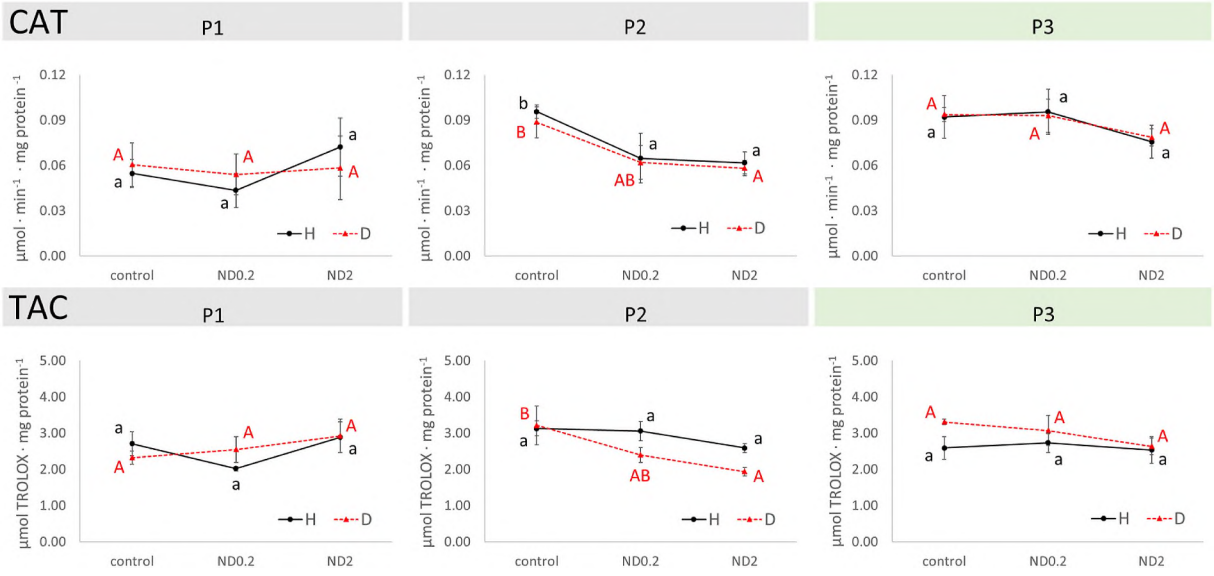


Fig. 2. Mean ± SD of catalase (CAT) activity and total antioxidant capacity (TAC) in the gut of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). Abbreviations: H – the wild strain of crickets; D – the long-living strain of crickets; control – insects fed with uncontaminated food, ND0.2; ND2 – insects receiving food contaminated with NDs in the concentration of 0.2 mg kg^{-1} or 2 mg kg^{-1}, respectively; the same lower case letters denotes homogenous groups within H strain; the same capital letters denote homogenous groups within D strain (ANOVA, LSD test, $p < 0.05$).

groups and the ND0.2 group in the P2 generation. With one exception (generation P2, strain D, group ND0.2), NDs reduced the percentage of cells in the early apoptosis phase, especially in strain D, where the effect was significant in the P2 generation in the ND2 group and the P3 generation in both ND groups. In the H strain, there was a significant reduction in early apoptosis only in the P2 generation in both groups receiving NDs (Fig. 4).

The number of cells in late apoptosis in the P1 generation was similar in both strains and all experimental groups. In the P2 generation, higher concentrations of NDs caused an increase in late apoptosis in both strains. In contrast, removing NDs from the diet (P3 generation) reduced the percentage of cells in late apoptosis, but only in the D-strain insects (Fig. 4). NDs had a low effect on the percentage of autophagic cells, which was limited to the first generation only. Interestingly, there was a decrease in strain D and an increase in strain H of autophagy in the ND2 group compared to the controls (Fig. 4). Both apoptosis and autophagy were significantly influenced by the 'generation' or 'strain' factors. The 'treatment' factor significantly influenced early apoptosis. Additionally, a significant interaction effect of 'generation' × 'treatment' was revealed for late apoptosis as well as 'strain' × 'treatment' and 'generation' × 'strain' × 'treatment' for the MAI parameter (Table S4).

Both concentrations of NDs decreased the percentage of ROS+ cells

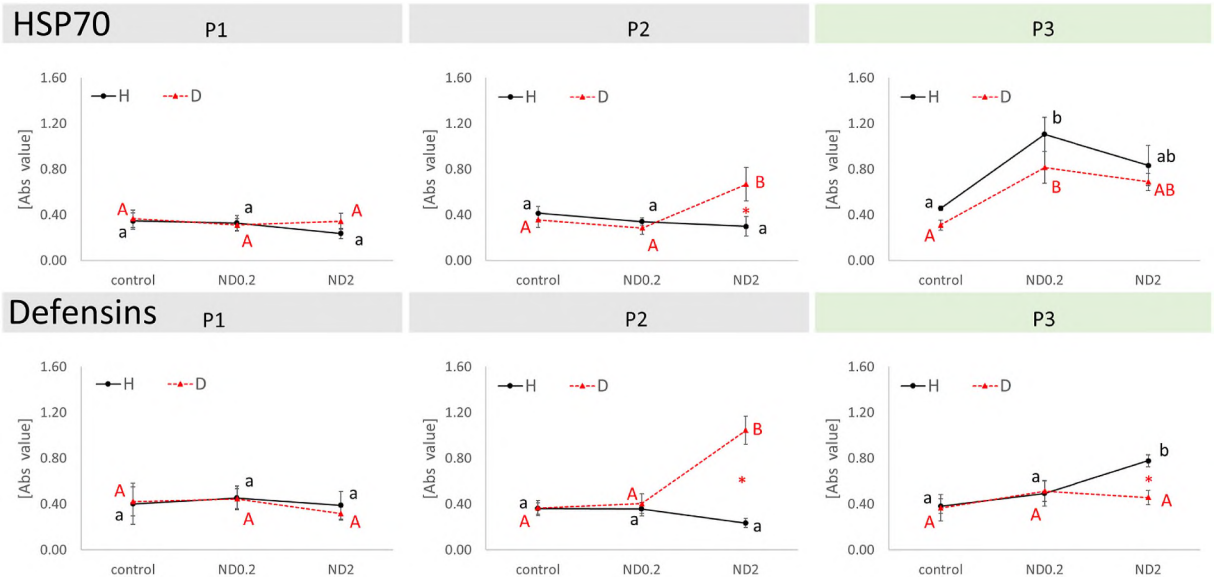


Fig. 3. Mean ± SD of HSP70 and defensins concentration in the gut of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). *Abbreviations: see* Fig. 1; *stars – significant differences between H and D strain in a given experimental group.*

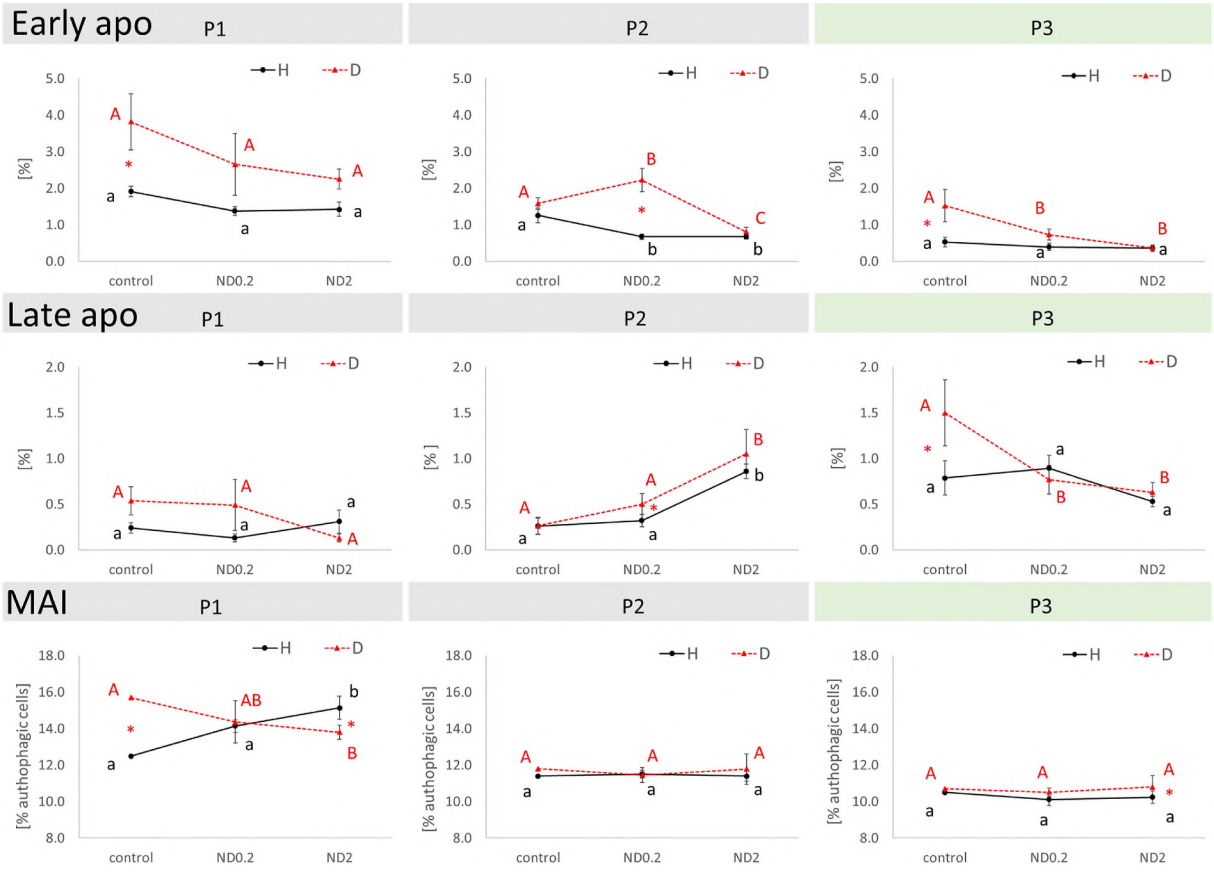


Fig. 4. Mean ± SD of early apoptosis (Early apo), late apoptosis (Late apo), and mean autophagy intensity (MAI) in the gut cells of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). *Abbreviations: see* Fig. 1 and 2.

in D-strain insects but only in the P1 generation. In contrast, strain H, the P2 generation, had significantly more ROS+ cells in the ND2 group than in the control (Fig. 5). The MMP was decreased in the ND-treated group, mainly in the P1 generation, manifested by an increased number of cells marked as Depolarized/Live (in the H strain at both tested concentrations, in the D strain only at the higher concentration). In the P2 generation, a significantly higher percentage of Depolarized/Live cells in the groups receiving NDs was found only in the H strain. Elimination of

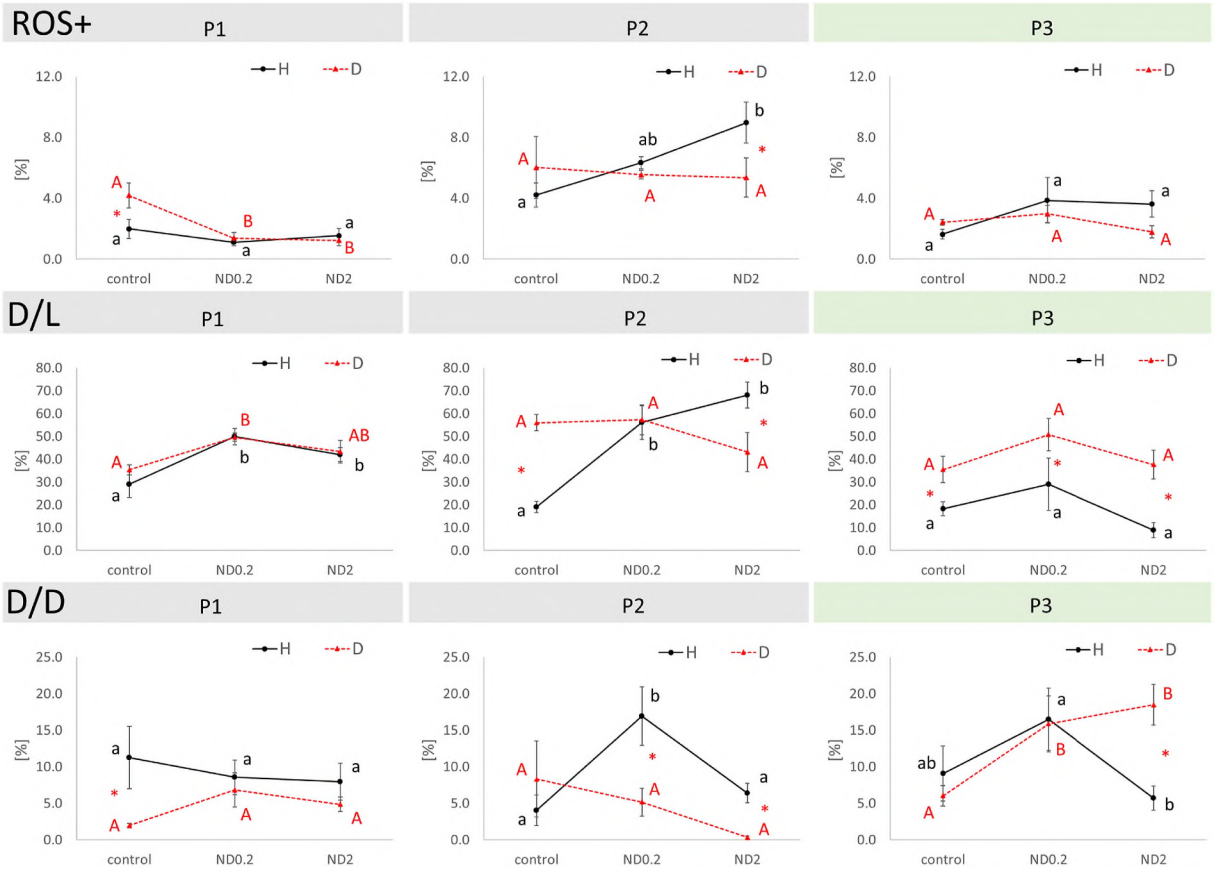


Fig. 5. Mean ± SD of populations of cells with ROS (ROS+), live cells with depolarized mitochondrial membrane (D/L – Depolarized/Live), and dead cells with intact mitochondrial membrane (D/D – Depolarized/Dead) in the gut of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). Abbreviations: see Fig. 1 and 2.

NDs from the diet (P3) returned this parameter to values similar to the control. However, strain D had a significantly higher percentage of Depolarized/Live cells than strain H in all experimental groups (Fig. 5).

NDs did not affect the number of depolarized/dead cells in either strain in the P1 generation. In the P2 generation, NDs at a lower concentration induced a significant increase in Depolarized/Dead cells compared to the control only in the H strain. The D-strain insects reacted more strongly than the H-strain in the recovery experiment (P3). In the D strain, the number of depolarized/dead cells was significantly higher than that in the control. ANOVA revealed that the 'generation' factor significantly modified the ROS+ and MMP parameters. Additionally, ROS+ was under the interactions of 'generation' × 'treatment' and 'strain' × 'treatment'. Almost all combinations of factors (except for 'generation' × 'treatment') affected the Depolarized/Live parameter. The variable Depolarized/Dead was significantly modified by 'generation' or 'treatment', 'generation' × 'treatment' or 'generation' × 'strain' × 'treatment' (Table S4).

3.3. *DNA damage*

In the D-strain insects, the amount of DNA in the comet tail (TDNA) measured by the comet assay was at the same level in all generations and experimental groups. Mean values were approximately 5% and were typical for this species. In the P1 and P2 generations of H-strain insects, NDs did not cause significant changes in the TDNA compared to the control. Surprisingly, after removing NDs (P3 generation), TDNA was significantly higher in the H strain receiving higher ND concentrations than in the control. However, the comet tail length (TL) of the H strain remained unchanged in all generations and experimental groups. This parameter manifested other tendencies in strain D. In the P1 and P2 generations, the TL in the ND2 group was significantly lower than that in the control. In the P3 generation, there was an opposite response. OTM generally confirmed the trends observed for TDNA and TL. The most visible was the decrease in OTM in the D line (P1 and P2 generations) and the increase in OTM in the H line (P3 generation) in the ND2 groups compared to the controls (Fig. 6). The different reactions to NDs of both insect strains were confirmed by significant interaction effects of 'strain' × 'treatment.' Additionally, the considerable influence of 'generation' × 'strain' factors was statistically confirmed for TDNA and OTM and 'generation' × 'treatment' for all three parameters (Table S5).

The above-described dependencies were supported by the analysis with the Muse® Multi-Color DNA Damage test. DSB was low and similar for both strains in the P1 and P2 generations and almost all experimental groups. However, after removing NDs from the diet (P3 generation), the percentage of cells with DSB increased markedly, especially in the H strain. ATM activation (pATM) was found only in H-strain insects in the P1 and P3 generations in the ND2 group compared to the control. The activation of histone H2A.X followed this tendency. In contrast, significantly higher (than in the control) histone activation in the D strain was found only in the ND2 group of the P2 generation (Fig. 7). The activation of histone H2A.X was influenced by all the factors and their interactions, except for the 'generation' × 'strain' × 'treatment' (Table S5).

4. Discussion

The obtained results can be discussed in the context of dose and/or time of exposure to NDs and/or predisposition to deal with oxidative stress by organisms selected for longevity. Possible phenomena in each generation are discussed below, as it seems that the NDs posed a slightly different challenge for each of them.

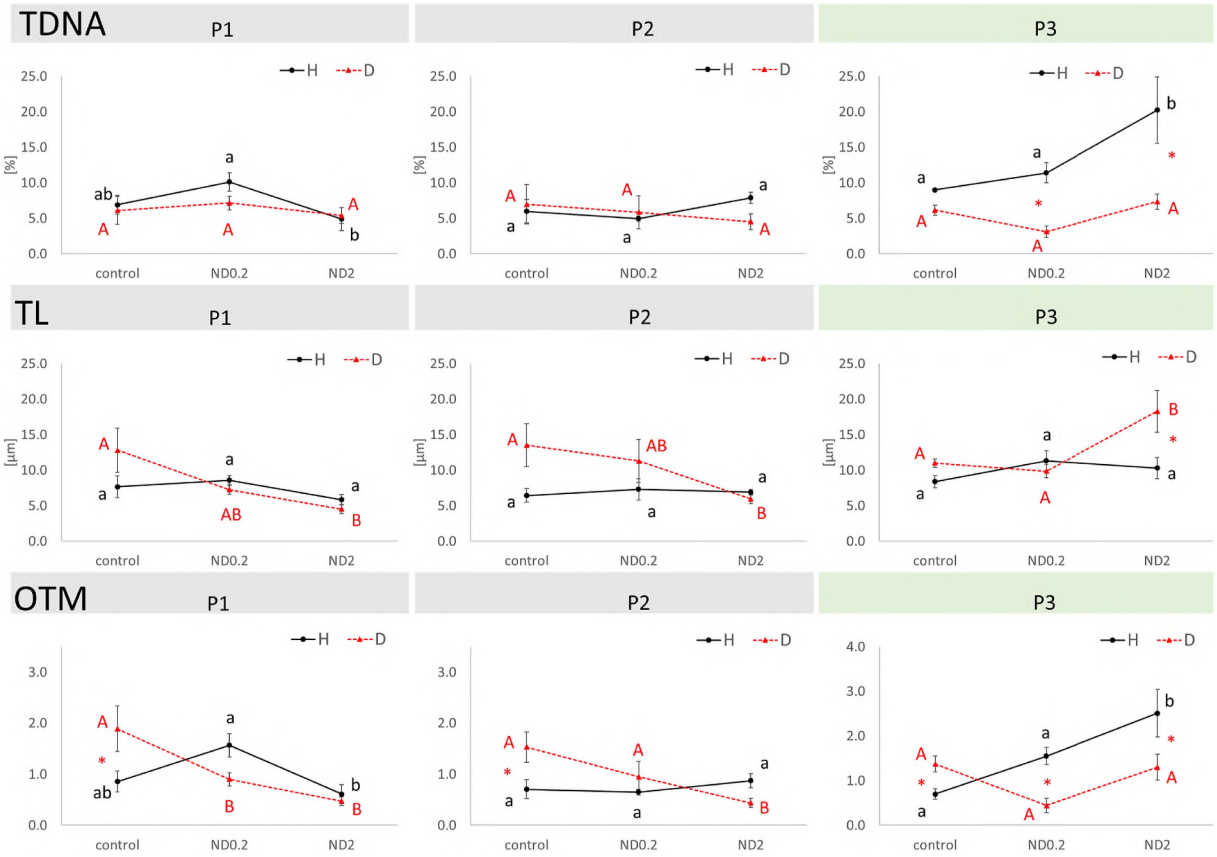


Fig. 6. Mean ± SD of the Comet assay parameters: tail DNA (TDNA), tail length (TL), and olive tail moment (OTM) in the hemolymph cells of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). Abbreviations: see Fig. 1 and 2.

4.1. *P1 generation – facing a new stress factor*

NDs for P1 insects represent a new stress factor. The results should then be considered as effects of chronic exposure to low doses of NDs. Such an approach does not engage multigenerational phenotype changes but directs the physiological effects/toxicity of the exposed individuals (Skinner, 2014). Our previous studies used ND concentrations of 20 and 200 mg kg^{-1} in food for 10 days (Karpeta-Kaczmarek et al., 2016a) or one generation (Karpeta-Kaczmarek et al., 2016b, 2016c, 2018, 2016b). In the present experiment, aimed at investigating the effects in subsequent generations and to 'mimic' predicted low environmental doses, we lowered the concentrations one hundredfold using 0.2 and 2 mg NDs kg^{-1} food.

Data on chronic exposure to NDs in invertebrates are limited. Cid et al. (2015) studied the effects of ND exposure in the freshwater clam *Corbicula fluminea* at concentrations of 0.01, 0.1, 1, and 10 mg L^{-1} for 14 days. There was an increase in oxidative stress parameters, enhancement of CAT and GST activity, intensification of lipid peroxidation, and changes in digestive gland cells (Cid et al., 2015). Other studies have assessed the effects of 24-h exposure to NDs of various sizes at concentrations of 1, 10, or 50 µg L^{-1} in *Daphnia magna*. Small (~5 nm) and large (~15 nm) NDs increased tissue ROS generation. Moreover, the 5 nm NDs stimulated the expression of HSP70 and GST (Domínguez et al., 2018). However, 21-day exposure to very high concentrations of NDs in water resulted in 100% mortality in *D. magna* (at a concentration of 12.5 mg L^{-1}), reproduction disturbances (at a concentration of 1.3 mg L^{-1}), accumulation of NDs in the gastrointestinal tract and sticking to the exoskeleton surface (Mendonça et al., 2011). In *Acheta domesticus*, which were exposed to the relatively high concentrations mentioned above for 6, 8, 10 days, or 1 generation (three months), increases in CAT activity and HSP70 concentration and intensification of DNA damage were described (Karpeta-Kaczmarek et al., 2016b, 2016c, 2018, 2016b). Additionally, a lack of side effects at low concentrations and a short exposure time were confirmed for fluorescent nanodiamonds (FND) in *Caenorhabditis elegans* exposed to ~3 ng per worm (~1 × 10^6 particles per worm) for 2–12 h (Mohan et al., 2010). Most of the results obtained in the present study, especially the frequently measured and standard stress parameters, such as CAT, TAC, HSP70, defensins, and apoptosis levels (Figs. 1–4), confirmed the lack of significant toxicity of low doses of NDs, even if the exposure time was as long as one generation. Indeed, it provides grounds for classifying NDs administered under these specific conditions as safe for the organism (Karpeta-Kaczmarek et al., 2016a; Ma et al., 2021; Mi et al., 2021).

Interestingly, however, strain-specific differences were noticed. D-strain crickets decreased autophagy (Fig. 4), the number of ROS+ cells (Fig. 5), and DNA damage (Fig. 6 – parameters TL and OTM). Such an effect may be a symptom of better mobilization of the organism and stimulation of physiological defense mechanisms in long-living strains. Perhaps chronic exposure to low doses of NDs can be beneficial. However, the percentage of cells with a depolarized mitochondrial membrane increased in groups of both strains exposed to NDs (Fig. 5). Depolarization of the mitochondrial membranes (causing loss of the mitochondrial inner transmembrane potential) is frequently seen as an early symptom and even the key event in the onset of apoptosis (Bourebaba et al., 2019; Li et al., 2003). The respiratory chain in the mitochondria is the most important cellular source of ROS (Herb et al., 2021; Knaus, 2021; Stowe and Camara, 2009). During the intensified respiratory process, the production of ROS in the mitochondria may be increased. The pessimistic scenario assumes the subsequent loss of cytochrome *c* (cyt *c*) contact with the inner mitochondrial membrane and

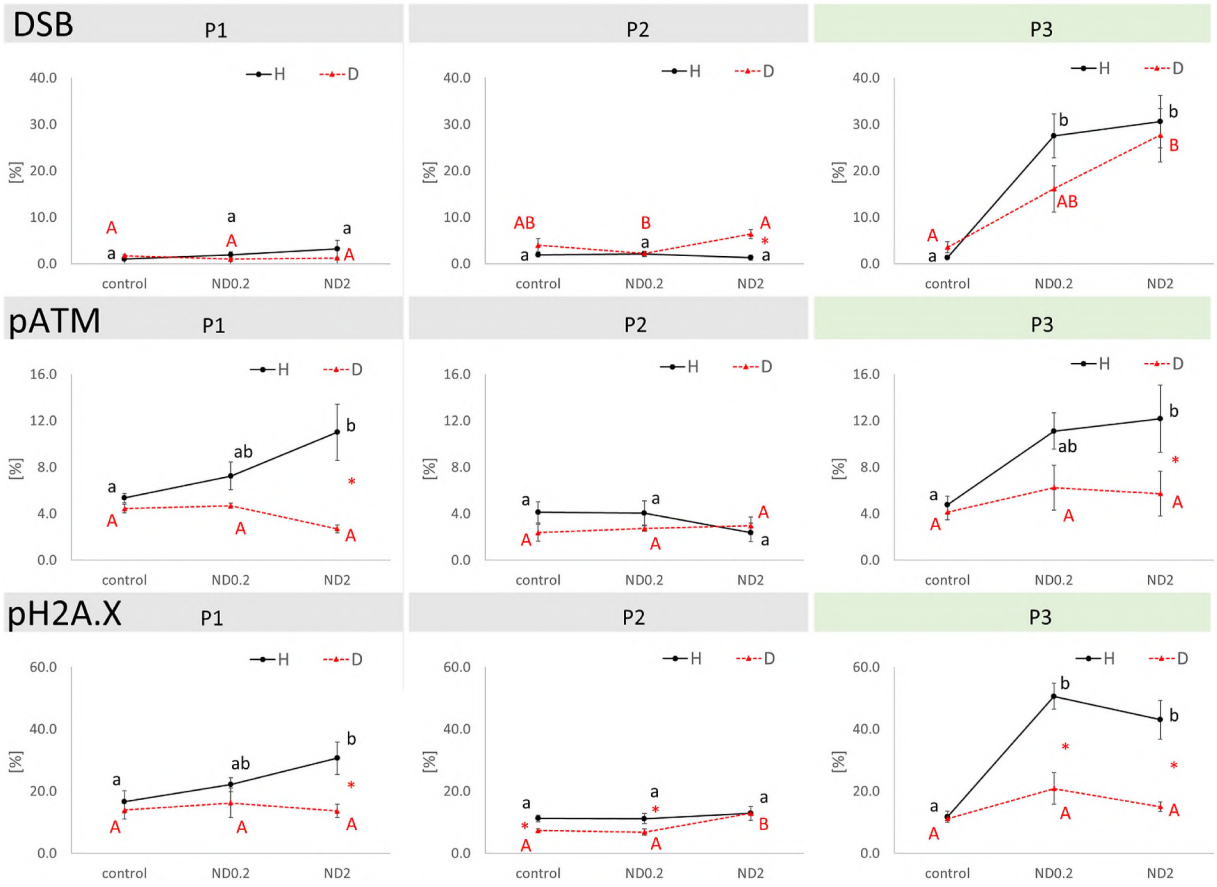


Fig. 7. Mean ± SD of parameters measured by Muse® Multi-Color DNA Damage: double-strand breaks (DSB), ATM activated cells (pATM), and histone H2A.X activated cells (pH2A.X) in the hemolymph cells of *Acheta domesticus* exposed to nanodiamonds (NDs) in food during the first (P1) and the second (P2) generation, then transferred on uncontaminated food during the third generation (Recov. – P3). Abbreviations: see Fig. 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

subsequent release of cyt *c* into the cytosol, which is an important step in early apoptosis but can be modified by mitochondrial uncoupling.

Therefore, increased production of ATP, which is necessary to support defense mechanisms, increases ROS production, which may lead to a decrease in the potential of mitochondrial membranes and impair ATP synthesis. The so-called "mild uncoupling" (MU) hypothesis, which explains the relationship between ROS and respiration and assumes that a slight decrease in ΔΨm reduces mitochondrial ROS production, may help explain this paradox (Cortassa et al., 2014; Skulachev, 1996). Therefore, according to the MU hypothesis, lowering the potential of mitochondrial membranes may protect against excessive oxidative stress. The most recent hypothesis explaining the relationship between mitochondrial respiration and ROS (redox-optimized ROS balance – R-ORB) assumes that the redox environment (RE) is the main regulating factor. When RE is highly reduced or highly oxidized, the production of ROS is the most intensive (Aon et al., 2010; Cortassa et al., 2014).

However, we did not observe increased apoptosis in the first generation (Fig. 4). It, therefore, seems that some membrane depolarization and reduction of ΔΨm is related to ROS reduction (Fig. 5), especially in the long-living strain, where selection favored individuals better able to cope with oxidative stress. Membrane depolarization had a slightly different effect in wild-type insects, where autophagy was intensified in the ND2 group. The increase in autophagy, observed, for example, in starvation, may indicate energy shortages (Lipovšek et al., 2018; Włodarczyk et al., 2019) and, understandably, may intensify in sudden intense exposure to a stressor (Babczyńska et al., 2020). Mitochondrial uncoupling together with autophagy activation has been shown in many studies on cell cultures and model animals (Demine et al., 2019). Additionally, activation of ATM, and therefore activation of histone H2A.X in wild-strain insects of the ND2 group (Fig. 7) indicated the initiation of DNA repair processes, especially DNA double-strand breaks (Celeste et al., 2003; Chen et al., 2021; Kim et al., 2019). The process seems efficient (no significant increase in DNA damage, including DSB), and genome integrity is preserved.

4.2. P2 generation – 'relative silence'

The second generation consisted of the offspring of the parents exposed to NDs and received dietary NDs throughout their development. Therefore, there is a considerable premise to believe that these individuals might have been better prepared to deal with stress, as they could benefit from their parents' experience.

The results for P2 insects from the D strain fed ND-spiked food at higher concentrations indicate sufficient effective suppression of oxidative stress. There was a reduction in catalase activity, TAC, early apoptosis, TL, and OTM levels (Figs. 2, 4 and 6). The number of ROS+ cells and cells with reduced membrane potential, the level of autophagy, TDNA and ATM activation did not differ significantly from the control insects (Figs. 4–6). Additionally, a subtle increase in the activation of histone H2A.X, with the simultaneous lack of DNA damage, can be interpreted as characteristic of an organism that tolerates exposure to NDs. Additionally, an increase in the concentration of HSP70 and defensins (Fig. 3) indicated an intensification of immune processes, protein remodeling, and regulation of their homeostasis (Ambrose and Chapman, 2021; Mayer and Bukau, 2005). As observed by others, NDs can enter the cell (Claveau et al., 2018; Hemelaar et al., 2017; Mi et al.,

2021) and interact with proteins and other molecules. Then, damaged ones must be utilized and replaced. The H-strain individuals reacted slightly differently from those from the D strain. A noticeable difference was the increase in ROS+ cells and cells with a depolarized mitochondrial membrane (Fig. 5) with the simultaneous lack of HSP70 and defensin stimulation (Fig. 3). In the D strain, longevity and the additional stress factor (NDs) overlap and seem to modify (and perhaps support) fitness. However, in the context of the results obtained for both strains, the issue of the fate of NDs in the organism/cell, especially after multigenerational exposure, is puzzling and creates new questions that can be answered after more advanced research.

There are no data in the available literature, apart from one article by our team, on the effects of multigenerational exposure to NDs (see Table S1). The interpretation given below is only our presumption - one of the possible scenarios that have to be explored in the future. Organisms exposed to chronic stress can use a variety of mechanisms to increase fitness. DNA methylation, histone modification, and the activity of noncoding RNAs are the most important processes underlying epigenetic inheritance, also in insects, and are essential for developmental plasticity, aging, and longevity (Glastad et al., 2019, 2014; Mukherjee et al., 2015). NDs are recognized as hypermethylating agents. In a study conducted on the HeLa cell line, epigenetic changes following exposure to low concentrations of NDs were found (Mytych et al., 2014). It is now known that stress-induced DNA methylation reprogramming can be passed from parents to offspring and can cause various diseases, cancer, and inflammation (Mukherjee et al., 2015; Skinner, 2014). However, in light of the new concepts of ecotoxicology, epigenetic changes may underlie stress resistance in the offspring, which may also apply to factors utterly different than those to which the parents were exposed (Vandegehuchte and Janssen, 2014). We did not thoroughly investigate the epigenetic mechanisms in our research. Therefore, the above information in interpreting our results is limited and should be seen as a suggestion and encouragement for further in-depth study.

4.3. P3 generation ND withdrawal can be risky for the offspring

After two generations of exposure, in the third generation, NDs were removed from the diet. Such an experimental model has not been studied thus far. It is unclear whether removing NDs may have reprogrammed the expression of crucial proteins (e.g., during embryonic development). However, if any changes that facilitate the offspring's life were passed on, they may be "out-of-date" in the P3 generation when the stress factor is missing (no NDs in the food).

In the P3 generation, NDs caused an increase in the concentrations of HSP70 and defensins (in the H strain) and a decrease in apoptosis (Figs. 3–4). Increased DNA damage, especially in the H strain (Figs. 6–5), was the visible regularity. These insects did not receive NDs in the diet, and ND transfer from the P2 females to eggs and then to embryos can be neglected. Therefore, one can exclude the DNA damage caused by electrons released from NDs, which can also interact with water, causing the production of secondary electrons and/or ROS generation (Mi et al., 2021). To attempt to explain the results, we have to look back to the previous generation and analyze possible scenarios of ND fate in parental cells. The NDs certainly entered the cells. We can assume this by analyzing data from research by other authors. In HeLa cells (usually after a short exposure time), they localized mainly in the cytoplasm and to a small extent in the nucleus (Barnard, 2009; Claveau et al., 2018; Hemelaar et al., 2017; Mi et al., 2021). However, under certain conditions, some particles may reach the nucleus and contact the DNA. Approximately 20% of the NDs were transferred to the nucleus in yeast cells. However, when linked to antibodies, as much as 70% of NDs entered the nucleus (Morita et al., 2020). Therefore, the interactions of NDs with DNA should be considered. Testing such interactions in an *in vivo* model is complex. Therefore, researchers use quantum-mechanical calculations or simplified models (Laptinskiy et al., 2021, 2018; Liu and Fyta, 2019). Laptinskiy et al. (2021) investigated the interaction of NDs with DNA using an aqueous suspension of NDs and an aqueous solution of DNA with concentrations of 0.5 mg mL^{-1} and 4 mg mL^{-1}, respectively. They confirmed that the surface groups of NDs interacted with DNA. However, no new chemical bonds were stated. Instead, electric (Coulomb) interactions between the ND surface and DNA were described. In an earlier simplified model study, the same team found adsorption of nucleic acid nitrogenous bases on nanodiamonds based on hydrogen bond formation. Cytosine was the most strongly adsorbed. Complementary pairs (A + T or C + G) adsorbed similarly by forming two strengthened hydrogen bonds between NDs and bases. Notably, there was no break of hydrogen bonds between the complementary pairs (Laptinskiy et al., 2018).

Is it possible, then, that *in vivo* hydrogen bond formation with bases occurs during chronic exposure to NDs? Could the consequence of such a phenomenon be a certain "stiffening" (increased integrity) of DNA? Given this concept, the relaxation of chromatin necessary for replication and transcription in the chain modified by NDs may be impeded. The consequence can be a compensatory modification of proteins that stabilize the genome, weakening their importance. Such modification could then be passed on to the offspring to modify proteins that stabilize and repair DNA damage. However, when offspring are not exposed to NDs (P3), there could be a deficiency in stabilizing proteins, possibly making DNA more susceptible to damage. It is supposed that NDs can mimic proteins including histones. Although NDs are smaller than histones (~5 nm vs. ~10 nm), oxidation or the formation of fine aggregates may increase their size. The appearance of artificial nucleosomes is therefore possible (Mochalin et al., 2012).

The bold concept presented above should undoubtedly be carefully tested, and a series of experiments should be planned to verify it. In another approach, one can perceive the mildly increased DNA damage in the P3 generation as an effect of the intended, programmed (and beneficial in consequence) decrease in genome stability for improving adaptation to stress factors in a changing environment (Augustyniak et al., 2020).

5. Conclusions

Parental experiences with NDs can shape the response of the offspring. This reaction, however, may be slightly different in the offspring of long-lived and wild crickets. The results obtained in the study allow us to accept hypothesis H1.2, which states that long-living insects have better abilities to handle stress than individuals of the wild strain. Elimination of NDs from the diet did not fully recover the measured parameters (especially DNA damage level), supporting the H.2.1 hypothesis that chronic exposure to low concentrations of NDs in the parental generation results in disturbances in offspring consuming an ND-free diet. Special care should be taken when using NDs. If their administration is necessary, e.g., during therapy, it should be limited to the lowest possible doses and the shortest possible time. A separate issue is unintentional and/or uncontrolled pollution of the environment by NDs, which poses a new challenge for all organisms inhabiting it. However, to fully understand the mechanism of the observed changes in subsequent generations, it is necessary to conduct further, more advanced studies, including the study of the methylation level, modification of histones, and some gene expression studies under ND exposure.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135129.

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