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Original research article

Influence of the antidiabetic drug metformin on the aquatic crustacean *Daphnia magna*

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Abstract
Background: The antidiabetic drug metformin has been repeatedly detected in surface waters worldwide. This study investigates the effects of the environmentally relevant concentration of metformin on a non-target aquatic organism – a freshwater crustacean, *Daphnia magna*, with an emphasis on the stress response of daphnids and the long-term effects on their consecutive generations.
Methods: The chronic toxicity test and the consecutive generations test were inspired by the OECD method. The total antioxidant capacity (Trolox equivalent – TEAC), superoxide dismutase (SOD) activity, and catalase (CAT) activity were related to the protein content in the tested daphnids.
Results: Elevated antioxidant activities were revealed in daphnids exposed to metformin in comparison to the control group (1.9 × for TEAC, 1.7 × for SOD; 1.3 × for CAT). Furthermore, diminished body sizes and malformations in the digestive system, spine and carapace were detected in newborn juveniles in the second and third generations exposed to metformin.
Conclusion: Long-term exposure to metformin in environmentally relevant concentrations led to a significant detrimental reaction in aquatic crustaceans.

Keywords: Daphnia; Effect on consecutive generations; Metformin

Highlights:
- Metformin affects daphnids in environmentally relevant concentrations.
- The impact is more pronounced in consecutive generations.
- The effect includes:
  - a stress reaction (elevated activities of enzymes with antioxidant activity);
  - diminished body size and malformations in consecutive generations.

Abbreviations:
ABTS – 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ADaM – Aachener Daphnien Medium, a special medium for Daphnia cultivation; BSA – Bovine Serum Albumin; CAT – Catalase; EC50 – half maximal effective concentration, it is a value representing the potency of a drug, operationalized as the concentration at which the drug exerts 50% of its maximal effect; LOD – Limit of Detection; LOQ – Limit of Quantification; OECD – Organisation for Economic Co-operation and Development; PBS – Phosphate Buffered Saline solution; ROS – Reactive Oxygen Species; SOD – Superoxide Dismutase; TEAC – Trolox Equivalent Antioxidant Capacity Assay

Introduction

Metformin 3-(diaminomethylidene)-1,1-dimethylguanidine has for a long time been the main pharmaceutical used to treat type 2 diabetes (Campbell et al., 1996; Kumari et al., 2025). Despite being successfully employed for many years, the mechanism underlying its therapeutic action is still not fully understood since metformin affects many target organs: it lowers glucose production in the liver, diminishes intestinal absorption, and alters the microbiome (Corcoran and Jacobs, 2023; Rena et al., 2017). At the molecular level, metformin inhibits the mitochondrial respiratory chain in the liver, which leads to activation of AMPK, enhances insulin sensitivity, and lowers cAMP, thus reducing the expression of gluconeogenic enzymes (Rena et al., 2017). Metformin also has AMPK independent effects on the liver that may include inhibition of fructose-1,6 -bisphosphatase by AMP (Rena et al., 2017). It seems that the mechanisms of action vary depending on the dose of metformin used and the duration of treatment (Foretz et al., 2023).

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Metformin exhibits several principal pharmaceutical advantages such as the ability to lower insulin resistance and addresses adult-onset hyperglycemia without weight gain or increased risk of hypoglycemia (Bailey, 2017); moreover, cardioprotective roles in atherosclerosis and myocardial injury have been described (Li and Li, 2023). Its easy oral administration is also considered advantageous (Corcoran and Jacobs, 2023). Thus, metformin has become the most prescribed anti-diabetic agent worldwide, taken by over 200 million people annually (Ragan, 2024). Besides diabetes, metformin is also used to treat polycystic ovary syndrome (Genazzani et al., 2010; Johnson, 2014) and has been tested as a cancer therapeutic agent in numerous trials (Dutta et al., 2023; Kasznicki et al., 2014; Lee et al., 2025).

In humans, metformin is not metabolized (Hardie, 2007) and is excreted in the urine, with a half-life of approximately 5 h (Graham et al., 2011). It has been detected in wastewater treatment plant effluents in many countries in concentrations of up to hundreds of μg/l (Hu et al., 2021, Yang et al., 2022) and in surface waters of up to ten of μg/l (Ambrosio-Albuquerque et al., 2021; Ussery et al., 2018). Metformin, among other pharmaceuticals used in human medicine, may exert adverse effects on aquatic biota. The review of Ambrosio-Albuaquerque et al. (2021) provides a survey of studies proving morphological and biochemical changes in aquatic organisms including bacteria, crustaceans, water plants, and fish after exposure to metformin. Comparing trophic levels, the authors concluded that of the tested organisms, crustaceans were the most sensitive to the toxicological effects of this drug.

Although the drug poses no extreme risk at the concentrations found in surface waters in short-term exposure (Caldwell et al., 2019; Parrott et al., 2021), it is important to evaluate the long-term effects. Daphnea (Crustacea: Cladocera), common filtre feeders in aquatic ecosystems, serve as a model organisms in ecotoxicological studies. Their ecological importance, given especially by their central position in the aquatic food web, is the reason for the research effort to investigate the impact of pharmaceuticals on daphnids.

In this study, a concept of long-term exposure of four consecutive generations of *D. magna* to metformin was adopted to cover the first time the outcomes in fertility, morphological changes, and stress responses.

Material and methods

Chemicals

For the preparation of all solutions, double-deionised water (Smart2Pure 6 UV/UF, Thermo Scientific) was used.

Metformin hydrochloride (Pharmaceutical Secondary Standard) was purchased from Sigma-Aldrich.

Chemicals for 1× phosphate buffered saline (pH = 7.4) and for K-phosphate buffer (pH = 7.0) were purchased from Sigma-Aldrich (Na_2HPO_4 p.a., KH_2PO_4 ≥99.5 %), Penta, CZ (NaCl p.a., KCl p.a.) or Lachema (K_2HPO_4 p.a.).

Bovine serum albumin (BSA, protein standard), potassium persulfate (p.a.), and the colorimetric detection kits (TEAC commercially available kit MAK334, SOD activity assay kit CS0009 and CAT assay kit CAT 100 – 1KT) were purchased from Sigma-Aldrich. Bicinchoninic acid disodium salt was obtained from Thermo Fisher, Germany, 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS, (p.a.) from VWR Life Science, Trolox (p.a.) from Acrõs organics and methanol (purity for HPLC) from Merck. $K_2Cr_2O_7$ (p.a), Na_2CO_3 (p.a.), NaOH (p.a.), $NaHCO_3$ (p.a.), $CuSO_4 \cdot 5\ H_2O$ (p.a.) and sodium tartrate (p.a.) were purchased from Penta (CZ), non-denatured ethanol was acquired in a pharmacy.

Chronic toxicity assays

Prior to all assays, the fitness of *D. magna* was tested using potassium dichromate as a reference substance according to the OECD 202 method (OECD, 2004). The calculated effective concentration for 50% of immobilised daphnids, EC50, was in all cases within the optimal range of 0.6–2.1 mg/l.

The chronic toxicity tests were based on the OECD 211 method (OECD, 2012). To evaluate the chronic effects of metformin, the stock solution of metformin was diluted using the Aachener Daphnien Medium, ADaM, a special medium for Daphnia cultivation (Klüttgen et al., 1994), to obtain two concentrations of metformin, 100 and 500 μg/l. As a control, pure ADaM solution was used. At the beginning of the experiment, female juveniles not older than 24 hours were individually placed into 50 ml of tested solutions, ten replicates for the control as well as for each metformin concentration. Daphnids were kept in an incubator (temperature 20.2 ± 0.2 °C and photoperiod 16 hours light and 8 hours dark) throughout the whole test lasting 21 days. The values of pH of all tested solutions were in the range of pH = 6.9–7.5, which was in the optimal range of pH = 6–9 (OECD 211, 2012) and the concentration of dissolved oxygen was higher than 6 mg/l during the whole test (recommended dissolved oxygen >3 mg/l). The daphnids were fed every second day with the green alga *Desmodesmus subspicatus* (inoculum obtained from CCALA Třeboň, Czech Republic, https://ccala.butbn.cas.cz/en; strain number BRINKMANN 1953/SAG 86.81) so that the incubation solution contained biomass of algae at a concentration of 3 mg C/l (expressed as total organic carbon content, TOC); TOC correlates well with the absorbance of the algae culture at 684 nm, TOC was therefore adjusted by measuring the absorbance of the algae culture and calculating the proper volume of algae culture for feeding the daphnids. The medium in which daphnids were incubated was changed every four days. The number of offspring was monitored every second day. At the end of the test, adult *D. magna* females were lyophilised in Labconco FreeZone 2.5 for 48 hours and then measured with Olympus CX41 microscope (magnification 4×).

Antioxidant activity measurements

The cultivation and extraction of the samples

For cultivation, the same procedure as for a chronic toxicity test described above (daphnids exposed to metformin at a concentration of 100 μg/l) was adopted. The duration of the test was 15 days, and the test was performed in 15 replicates. On the 15th day of exposure, the adult daphnids were transferred into the 2-ml centrifuge tubes (5 daphnids per tube), anesthetised using 60% ethanol (Rotllant et al., 2023), washed three times in 1.5 ml of double-deionised water, and stored at –80 °C.

For enzyme extraction, the tubes containing thawed daphnids were washed three times with PBS (pH = 7.4), and then 50 μl of K-phosphate buffer (pH = 7.0) was added into each tube. The samples were sonicated for 1 minute and centrifuged at 11 000 rpm for 5 minutes. The supernatant from three tubes was pipetted to a fresh 2-ml centrifuge tube. The extraction was repeated once more with the pellet in the original tubes. The supernatant gained from the second extraction was pipetted into the supernatant obtained in the first step; the final volume of supernatant was approx. 300 μl.

Protein analysis
The amount of protein in the samples was analysed by the bicinchoninic acid method inspired by Stoscheck (1990). Reagent A was prepared by dissolution of 0.50 g of bicinchoninic acid disodium salt, 1.00 g of Na_2CO_3, 0.080 g of disodium tartrate, 0.20 g of NaOH, and 0.475 g of $NaHCO_3$ in 50 ml. The pH was adjusted to 11.25 using 50% NaOH. The reagent B was 4% $CuSO_4 \cdot 5\ H_2O$. The final master mix consisted of reagents A and B at a ratio of 50:1. As a protein standard for the calibration curve, the 1 mg/ml BSA was used. The blank was K phosphate buffer (pH = 7.0) and the samples (supernatants gained in the previous step of extraction) were dissolved 10 times by the K phosphate buffer to obtain 100 µl of the dissolved sample. Then, 25-µl aliquots of the blank, protein standard and samples were pipetted in triplicates into a 96-well plate, then 200 µl of master mix was added into each well, and the plate was incubated at 37 °C for 30 minutes. After incubation, the absorbance at 562 nm was measured using the Synergy H1 microplate reader (BioTek).

The total antioxidant capacity measurement
For the evaluation of the total antioxidant capacity of the samples, the Trolox Equivalent Antioxidant Capacity Assay (TEAC), inspired by Re et al. (1999), Muñoz-Acevedo et al. (2011), and Kahoun et al. (2017), was used. The pre-formed radical monocation ABTS $^{\bullet+}$ was generated by the oxidation of ABTS with potassium persulfate. Approximately 16 hours before analysis, a 4.90 µmol/ml stock solution of potassium persulfate was prepared. Then 15.30–15.40 mg of ABTS was dissolved in 2 ml of water and 2 ml of the stock solution of potassium persulfate. This stock solution of ABTS $^{\bullet+}$ was kept in the dark until the next day, when approx. 600 µl of the ABTS $^{\bullet+}$ solution was dissolved using methanol in a 50-ml volumetric flask – the volume of stock solution of ABTS $^{\bullet+}$ was adjusted so that the absorbance of the dissolved solution at 744 nm was in the range of 0.700 ± 0.020. Further, 10-µl aliquots of supernatants originating from extraction were diluted 10 times using methanol; for a blank sample, 10 µl of K phosphate buffer (pH = 7.0) was used.

The calibration curve with five concentrations in the range of 0.02 to 0.20 µmol/ml of Trolox in methanol was prepared. Then, 20 µl of each sample was pipetted into the 96-well plate in triplicates, followed by adding 200 µl of working ABTS $^{\bullet+}$ solution to each well and 30 minutes incubation in the dark at 20 °C. The absorbance at 744 nm was read using the Synergy H1 microplate reader (BioTek). The decrease in absorbance in percentage, DA, was calculated according to the equation:

$$DA = 100\times \frac{(^{A}\text{ABTS} - {}^{A}\text{sample})}{{}^{A}\text{ABTS},}$$

as described in the study of Kahoun et al. (2017). The calculated concentration of Trolox expressed in µmol of Trolox/ml of the sample was divided by the amount of the proteins in mg/ml of the sample to obtain the TEAC/mg of the protein.

Validation of TEAC method
The TEAC method was validated according to the International Conference on Harmonisation (ICH) guidelines (EMA/CHMP/ICH/82072/2006) and Eurachem guideline (1998). The linearity of calibration curve expressed by the equation (y = 319.24 x ± 2.2587) constructed from 5 concentration levels (each concentration level in triplicate) was evaluated using two acceptability criteria: determination coefficient, $R^2 \geq 0.9800$ (measured $R^2 = 0.9935$) and quality coefficient, QC, ≤ 5.0% (calculated QC = 3.62%). Accuracy was determined as the relative error (%) and was expressed as recovery: [(measured concentration – nominal concentration)/nominal concentration × 100] with its value being in the optimal range of 90–107% (measured 97%). The nominal concentration was obtained by the TEAC commercially available kit MAK334, Sigma-Aldrich. The precision was determined as the coefficient of variation (%) and was expressed as repeatability: (standard deviation/mean concentration measured) × 100 with the value of 6.5 % (optimal value ≤ 7.3 %). Values of limit of detection, LOD, and limit of quantification, LOQ, were calculated as follows: LOD = [(3.3 × standard deviation of measured blank absorbance)/calibration curve slope], its value was 0.00012 µmol/ml; LOQ = [(10 × standard deviation of measured blank absorbance)/calibration curve slope], its value was 0.00037 µmol/ml. Based on the values of above validation parameters, the range for this method was 0.02–0.20 µmol/ml.

Superoxide dismutase (SOD) activity measurement
To analyse the SOD activity, the commercially available CS0009 (Sigma-Aldrich) superoxide dismutase activity assay kit was used. Before analysis, the samples and the blank sample (K phosphate buffer, pH = 7.0) were diluted in the dilution buffer (50 mM Tris HCl, pH = 8.0), which was part of the kit – the dilution factor was adjusted experimentally to fall into the kit's calibration curve range. Then, the whole procedure and the calculations were done according to the kit guideline, the SOD activity was expressed in units of SOD/mg sample protein. The absorbance at 450 nm was read using the Synergy H1 microplate reader (BioTek).

Catalase (CAT) activity measurement
To measure the CAT activity of the samples, the commercially available CAT 100 – 1KT (Sigma-Aldrich) catalase assay kit was used and the whole procedure (the preparation of the solutions, the standard calibration curve, and the setup) was performed according to its guidelines with some experimental adjustments in the dilution factors and incubation time. The final absorbance measurement was performed in a microcuvette at 520 nm using the JENWAY 6305 UV/VIS spectrophotometer. The CAT activity was expressed in µmol/min/mg sample protein.

Monitoring of consecutive generations of D. magna
Based on the chronic toxicity assay results, the concentration of 100 µg/l of metformin was chosen for further study of the influence of metformin on successive generations of *D. magna*. All treatments were performed in 15 replicates. At the beginning of the test (parental generation – generation 0, G0), one female juvenile not older than 24 hours was introduced into 50 ml of ADaM (control) or ADaM with metformin. Then, 15 newly-born females from both the control and metformin solutions were randomly chosen on the 15th day and treated in the same way (generation 1, G1). To evaluate the effects on the second (G2) and the third (G3) generations the procedure was repeated analogously as for G1. The conditions in the treatment were the same as in the chronic toxicity assay described above. The measurement of body sizes was performed on seven randomly chosen juveniles from each generation using an Olympus BX51 microscope equipped with an Olympus DP74 camera with the SellSens Standard software. The body sizes of 15 adult daphnids of each generation were measured using the Olympus DSX 1000 microscope (objective 3×) equipped with an Olympus DP74 camera with Olympus DSX software.

Statistical analysis

To compare the metformin treatments (100 µg/l and 500 µg/l) with the control group in the first chronic toxicity assay, the non-parametric Kruskal–Wallis test followed by the *post-hoc* Dunn's multiple comparisons test was used. The antioxidant measurements (TEAC, SOD, CAT) were evaluated using an unpaired two-tailed *t*-test, and the chronic toxicity assay for consecutive generations was evaluated using non-parametric two-tailed Mann–Whitney test. All statistical analyses were performed in GraphPad Prism software version 10.4.1 (https://www.graphpad.com/). In all analyses, *p*-values less than 0.05 were considered significant.

Results and discussion

The results of the chronic toxicity test in which the number of juveniles per female, number of juveniles per clutch, and the body size of adult females at the end of the treatment were monitored under two concentrations, are summarised in Fig. 1. Both the number of juveniles per female and the number of juveniles per clutch reached significantly higher values in metformin solutions in comparison to the control with an even more pronounced increase in the higher concentration of metformin. In the adult daphnids' body size, the exposure to metformin also caused an increase, but there was no such pronounced effect with increasing metformin concentration. These effects (larger daphnids producing more juveniles) may be associated with the hormetic effect, a biphasic dose response to an environmental agent characterised by a low dose with stimulating or beneficial effect and a high dose with inhibitory or toxic effect (Mattson, 2008). The toxic effect of high concentrations of metformin (non-environmental concentrations of tens of mg/l or more) on daphnids is well documented: immobilisation of *D. magna* after 48 h exposure (Cleuvers, 2003; Lee, 2017) and decreased feeding rate (Giannouli et al., 2023).

In this study, the total (Trolox equivalent, TEAC) antioxidant capacity and the activity of two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were investigated. Both individual enzyme activities as well as the total antioxidant capacity were significantly elevated after the long-term exposure of daphnids to metformin at a concentration of 100 µg/l (Fig. 2). The increase in the number of juveniles per female and per clutch demonstrated in Fig. 1 is therefore probably not a hormetic effect but a response to stressful conditions; the production of more offspring under distressing condition may represent an adaptive potential in daphnids (Messiaen et al., 2012). Living in a polluted environment is a stressful factor for organisms, which results in the production of the so-called reactive oxygen species (ROS); their production in the case of short-term or low-concentration pollutant exposure subsequently leads to an increase in the levels of antioxidant activity enzymes destroying ROS to protect the organism (Anetor et al., 2022; Mustafa et al., 2024; Yuan and Zhang, 2025; Zhao et al., 2024).

Oxidative stress caused by metformin was studied on fish embryos (Elizalde-Velázquez et al., 2021) and on fish (Lee et al, 2019; Sibiya et al., 2023) with the use of several enzymatic markers including SOD and CAT activity, however there is an absence of publication aiming at the oxidative stress caused by this drug in daphnids.

The negative effect of metformin (100 µg/l) was even more clearly shown in consecutive generations of *D. magna*. For this examination, the 15-day treatment was chosen to achieve a sufficiently long exposure to affect the animals and not too long an exposure to enable their adaptation. The body size of adult daphnids was larger in metformin treatment than in the control in the G0 generation (Fig. 3). There was no significant difference between treatments in the G1 generation but a steep decline in body size occurred in the G2 and G3 generations. Similar results with a decrease in body size were reported by Grzesiuk et al. (2024) from the multi-omics study of the effect of the antibiotic chloramphenicol on daphnids.

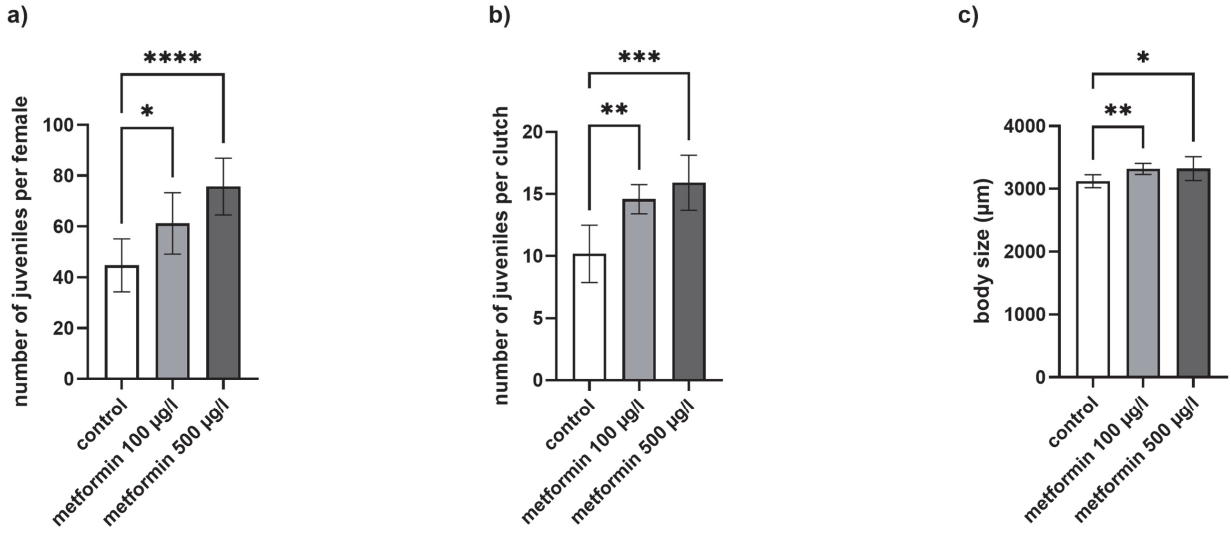

Fig. 1. The effect of two metformin concentrations (100 and 500 µg/l) on selected life history parameters derived from the chronic toxicity test lasting 21 days: (**a**) number of juveniles per female (Kruskal–Wallis test: H = 18.26, df = 2, *P* = 0.0001); (**b**) number of juveniles per clutch (Kruskal–Wallis test: H = 16.03, df = 2, *P* = 0.0003); (**c**) body size of adult *D. magna* females (Kruskal–Wallis test: H = 12.27, df = 2, *P* = 0.0022). Data represent the mean ± standard deviation (SD), *N* = 10, asterisks denote significant differences between treatments based on Dunn's test comparing two metformin concentrations from the control: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

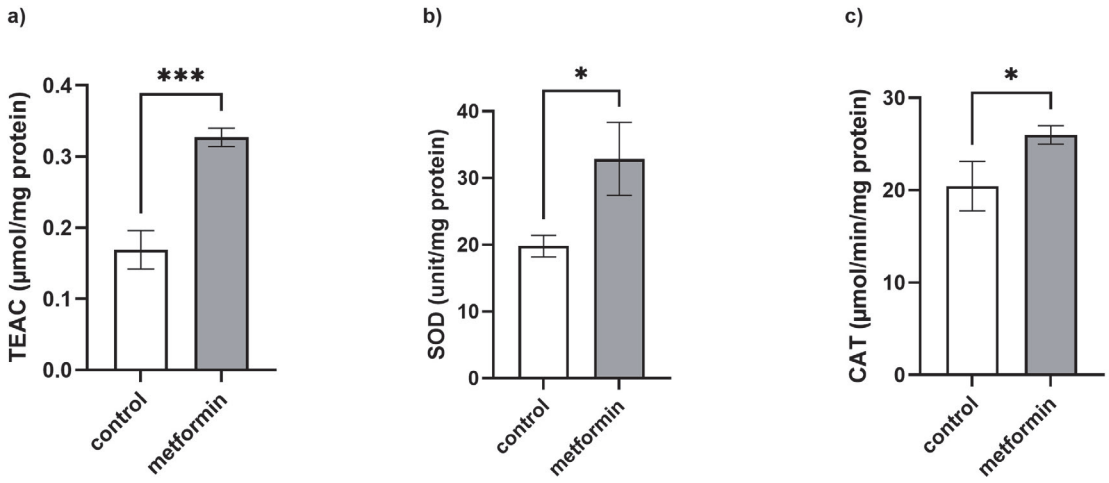

Fig. 2. The effect of metformin (100 µg/l) on antioxidant activities of *D. magna* after 15 days of exposure: (**a**) TEAC (the total antioxidant capacity per mg of protein, unpaired two-tailed *t*-test: $T = 9.127$; df = 4; $P < 0.001$); (**b**) SOD (superoxide dismutase activity per mg of protein, unpaired two-tailed *t*-test: $T = 3.966$; df = 4; $P = 0.012$); (**c**) CAT (catalase activity per mg of protein, test: $T = 3.350$; df = 4; $P = 0.029$). Data represent mean ± SD, $N = 3$ (five daphnids in each replicate). Asterisk denote significant differences between treatments based on Dunn's test: * $P < 0.05$, *** $P < 0.001$.

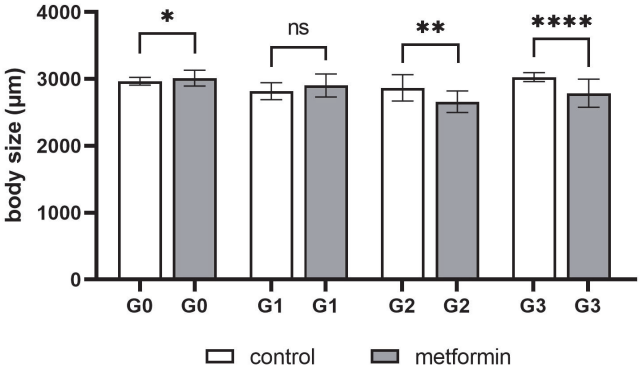

Fig. 3. The effect of metformin (100 µg/l) on the body size of adult *D. magna* in the chronic toxicity tests of consecutive generations (G0 – G3) lasting 21 days. Data represent mean ± SD, $N = 15$. Generation G0: Mann–Whitney test $U = 65$, $P = 0.0474$, generation G1: Mann–Whitney test: $U = 72$, $P = 0.0949$, generation G2: Mann–Whitney test: $U = 40.50$, $P = 0.0038$, generation G3: Mann–Whitney test: $U = 12$, $P < 0.0001$. Asterisks denote significant difference between treatments based on Dunn's test: ns – not significant, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

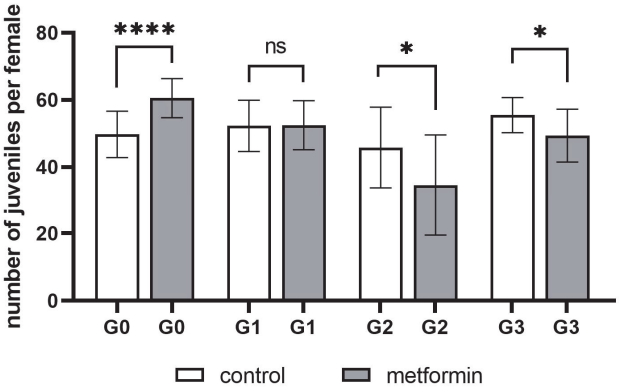

Fig. 4. The effect of metformin (100 µg/l) on the number of juveniles per female in the chronic toxicity tests of consecutive generations (G0–G3) lasting 21 days. Data represent mean ± SD, $N = 15$. Generation G0: Mann–Whitney test: $U = 25$, $P = 0.0001$, generation G1: Mann–Whitney test: $U = 110$, $P = 0.9266$, generation G2: Mann–Whitney test: $U = 60$, $P = 0.0285$, generation G3: Mann–Whitney test: $U = 54$, $P = 0.0248$. Asterisks denote significant differences between treatment based on Dunn's test: ns – not significant, * $P < 0.05$, **** $P < 0.0001$.

More juveniles per female were produced in the metformin treatment compared to the control group only in the G0 generation (Fig. 4). In the G1 generation, the number of produced juveniles was not statistically different from the control group. The juvenile numbers in the G2 and G3 generations were lower than in the control, but the value was similar in both the G2 and G3 generations. A similar response of daphnids to stressful conditions by increasing the number of offspring was observed by Agatz et al. (2013), Ellis et al. (2020) and Grzesiuk et al. (2024). Reproduction is crucial for maintaining populations; otherwise, survival of the affected organisms is at potential risk. To prevent such a consequence, an animal will make considerable physiological sacrifices to ensure reproductive success (Moberg, 1985). Nevertheless, the organisms may adopt this strategy only for some time – in this case in the G0 generation, while the subsequent generations lose the potential for such a strategy.

The body size of the produced juveniles in metformin treatment in the last generation (G3) was notably diminished in comparison to the control group (Fig. 5).

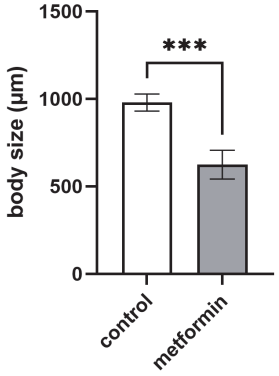

Fig. 5. The effect of metformin (100 µg/l) on body size (mean ± SD, $N = 7$) of G3 juveniles produced on the 15th day of the chronic toxicity assay (Mann–Whitney test: $U = 0$, *** $P < 0.001$)

Moreover, juveniles suffered from a variety of malformations (Fig. 6) such as extrusion of the digestive ceca, a bent spine, a noticeably more robust carapace which is probably associated with the change in chemical composition of the carapace, an elevated amount of α-chitin, and proteins with embedded amorphous calcium carbonate (Rabus et al., 2013; Ritschar et al., 2020a, b). The malformations observed in this study occurred in up to 45% of juveniles in the G2 and G3 generations exposed to metformin, while no malformations occurred in the control treatment. The similar deformations as observed in this study were also reported by Grzesiuk et al. (2020) who studied daphnids exposed to nonsteroidal anti-inflammatory pharmaceutical ibuprofen in consecutive generations of daphnids.

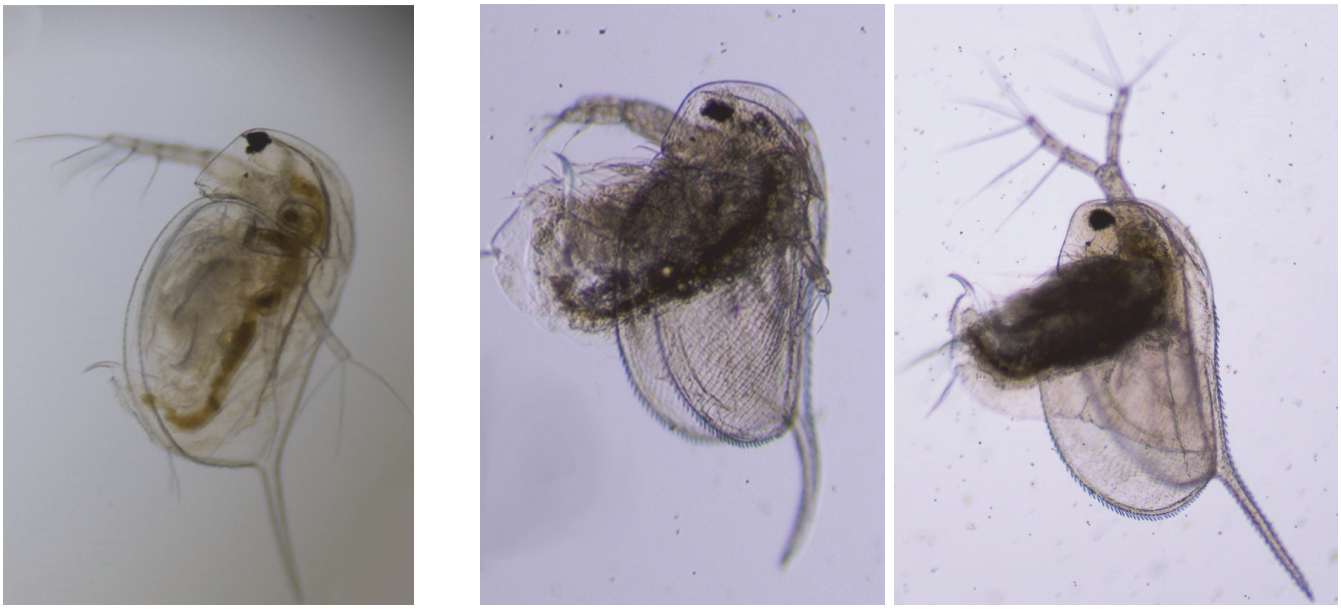

Fig. 6. Malformations in *D. magna* juveniles observed as the effect of metformin exposure. (**a**) a healthy and well-developed juvenile in the control group; (**b**) malformed juveniles from metformin (100 µg/l) treatment – extrusion of the digestive ceca, bent spine, and apparently more chitinous carapace.

Even though only healthy-looking juveniles were carefully chosen for any of the consecutive generations trials, some females exposed to metformin gained a milky white appearance during the experiment. Specifically, two individuals in the G2 (13%) and five individuals in the G3 (33%) generation exposed to metformin were affected in this way, while no females with this appearance were found in the control. The affected females produced significantly lower numbers of juveniles. An impaired adult daphnid is displayed in Fig. 7; the daphnids have a more robust carapace, an almost empty brood pouch, and a half-empty midgut, which may suggest that metformin may cause changes in food intake observed in humans (Lee and Morley, 1998; Lv et al., 2012) in daphnids as well.

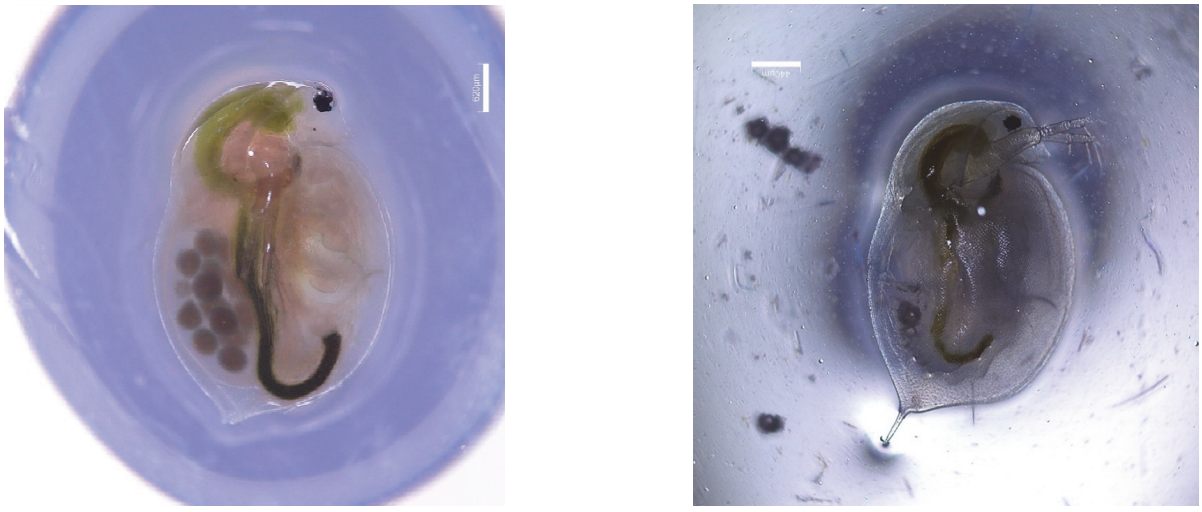

Fig. 7. The effect of metformin on the generation of malformations in adult females of *D. magna* raised as G3: (**a**) healthy control female; (**b**) female with robust carapace, almost empty brood pouch, and half-empty midgut from metformin (100 µg/l) treatment.

Conclusion

Pharmaceutical products contaminating freshwater ecosystems can alter the fitness of aquatic organisms, even at low concentrations, particularly under long-term exposure. This study represents the first investigation to demonstrate the multi-generational impact of the antidiabetic drug metformin on the aquatic crustacean *D. magna*.

Our main findings indicate several significant effects: a reduction in the body sizes of both newborn and adult daphnids in consecutive generations, a diminished number of juveniles produced per female in consecutive generations, and the occurrence of severe malformations in both newborn juveniles and adult females.

Furthermore, the observed significant increase in total antioxidant capacity (Trolox equivalent) and elevated activities of superoxide dismutase and catalase enzymes suggest the induction of notable stress in the organisms.

Future research, including the analysis of more biochemical parameters, including such as the proteomic profiling, could provide further and deeper insight into the effects of this and other pharmaceuticals on daphnids, an important part of the aquatic food web.

Contributions of authors

Martina Poncarová – conduction and interpretation of experiments, preparation of a draft of the manuscript. *Šárka Klementová* – planning and supervising the workflow, helping with manuscript preparation. *Michal Šorf* – guidance with implementation of OECD techniques for daphnids, manuscript inspection

Ethics statement

No specific permits were required for the studies described herein. In this study, there was no activity involving endangered or protected species.

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Conflict of 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 article.

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