Grandmother's pesticide exposure revealed bi-generational effects in Daphnia magna

Poulsen, Rikke; De Fine Licht, Henrik H.; Hansen, Martin; Cedergreen, Nina

Published in: Aquatic Toxicology

DOI: 10.1016/j.aquatox.2021.105861

Publication date: 2021

Document version: Publisher's PDF, also known as Version of record

Document license: CC BY

1. Introduction

The present age has been named the Anthropocene as humans are significantly impacting the Earth's eco- and geosphere with loss of biodiversity, contamination of waterbodies, air pollution and deterioration of ecosystems as the result (Barnosky et al., 2011; Waters et al., 2016). Man-made chemicals are a significant contributor to these effects (Hayes and Hansen, 2017). Many countries are increasingly monitoring and regulating the dispersion of pollutants (Gehen et al., 2019; Krewski et al., 2020). Progressing from the initial focus on acute environmental effects resulting from lethal dosages, the focus of the last decades has increasingly been on long-term effects such as endocrine disruption and effects on reproduction (Hayes and Gilbert, 2009; World Health Organization and United Nations Environment Programme, 2013). However, increasing scientific evidence of multigenerational effects suggests that the time perspective has to be extended even further (Alfonso et al., 2019; Dao et al., 2018; Giraudo et al., 2017; Hu and Yu, 2019; Jaspere et al., 2018; Jeremias et al., 2018; Mo et al., 2020; Nikinmaa et al., 2019),

Currently, the only legal requirement in, for example, pesticide risk assessments is to investigate one generation, sometimes with the inclusion of quantifying reproduction as number of offspring (The European Parliament and the Council of the European Union, 2009). The potential for multigenerational effects challenges the aptness of this status quo. If, for instance, effects of a chemical spill persist in several generations after the exposure has taken place, it will lead to a time gap and a misperception of correlations. Effectiveness of remediation may even be left unnoticed as intergenerational harmful effects continue after pollutants are removed from the ecosystem. Finally, if prenatal life stages are sensitive to chemical exposure, it means that current risk assessments are missing a critical time point for evaluating effects of chemical exposure, as analysis of for example offspring fertility is not included in any current guidelines. To address this concern, this study considers an extended time perspective of toxicity. Applying Daphnia magna as the model organism, we examine the intergenerational effects of the azole fungicide prochloraz over three generations.

Daphnia magna (Crustacea: Branchiopoda: Cladocera) is a much-favored model organism. They are ubiquitous in aquatic environments and are used as the favored model organism. They are ubiquitous in aquatic environments and are a key phenotypic effects, such as reproduction, were monitored. Acclimation to prochloraz was found after three generations of Daphnia magna. We studied both the effects of continuous exposure over all generations and the effects of first-generation (F0) exposure on two subsequent generations. Effects at different levels of biological organization from genome-wide gene expression, whole organism metabolite levels, CYP enzyme activity and key phenotypic effects, such as reproduction, were monitored. Acclimation to prochloraz was found after continuous exposure. Following F0-exposure, embryonically exposed F1-offspring showed no significant effects. However, in the potentially germline exposed F2 animals, several parameters differed significantly from controls. A direct association between these F2 effects and the toxic mode of action of prochloraz was found, showing that chemicals can be harmful not only to the directly exposed generation, but also to prenatally exposed generations and in that way effects may even appear to skip a generation. This implies that current risk assessment practices are neglecting an important aspect of toxicity, such as delayed effects across generations due to a time gap between chemical exposure and emergence of effects.

Man-made chemicals are a significant contributor to the ongoing deterioration of numerous ecosystems. Currently, risk assessment of these chemicals is based on observations in a single generation of animals, despite potential adverse intergenerational effects. Here, we investigate the effect of the fungicide prochloraz across three generations of Daphnia magna. We studied both the effects of continuous exposure over all generations and the effects of first-generation (F0) exposure on two subsequent generations. Effects at different levels of biological organization from genome-wide gene expression, whole organism metabolite levels, CYP enzyme activity and key phenotypic effects, such as reproduction, were monitored. Acclimation to prochloraz was found after continuous exposure. Following F0-exposure, embryonically exposed F1-offspring showed no significant effects. However, in the potentially germline exposed F2 animals, several parameters differed significantly from controls. A direct association between these F2 effects and the toxic mode of action of prochloraz was found, showing that chemicals can be harmful not only to the directly exposed generation, but also to prenatally exposed generations and in that way effects may even appear to skip a generation. This implies that current risk assessment practices are neglecting an important aspect of toxicity, such as delayed effects across generations due to a time gap between chemical exposure and emergence of effects.

Grandmother’s pesticide exposure revealed bi-generational effects in Daphnia magna

Rikke Poulsen a, b, *, Henrik H. De Fine Licht a, Martin Hansen b, Nina Cedergreen a

* Corresponding author.
E-mail address: au615379@uni.au.dk (R. Poulsen).

https://doi.org/10.1016/j.aquatox.2021.105861

Received 1 January 2021; Received in revised form 2 May 2021; Accepted 4 May 2021

Available online 11 May 2021

0166-445X/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
where they serve as an important link in the food chain (Ebert, 2005). Furthermore, they reproduce by cyclic parthenogenesis, which provides an opportunity to separate genetic and environmental components of phenotypic plasticity (Bell and Stein, 2017; Hales et al., 2017). In *Daphnia* anthropogenic impacts such as gamma irradiation, salinity- and thermal stress as well as exposure to the flame retardant tris (2-butoxyethyl) phosphate and the antidepressant sertraline has already been shown to result in effects that last over multiple generations (Giraudo et al., 2017; Im et al., 2020; Jeremias et al., 2018; Minguez et al., 2015; Trijau et al., 2018). Here we expand on this knowledge by investigating effects at different levels of biological organization, combining genome-wide gene expression (using transcriptomics), whole organism metabolite levels (using metabolomics), and measurements of CYP enzyme activity as well as key phenotypic life-history effects, such as growth and reproduction. Using non-target analysis methods i.e., transcriptomics and metabolomics, it is possible to follow the biological fingerprint over the generations and connect this mechanistic information to cell level enzyme activity and the apical endpoints of life history traits. We, furthermore, investigate a chemical with a highly relevant mode of action as it is characteristic of one of the most important groups of antifungal agents used worldwide (Verweij et al., 2020). Prochloraz, more specifically, is approved for use in several EU countries (European Commission, 2016), and have been found in the aquatic environment in concentrations up to 0.1 μg/L (Belenguer et al., 2014).

Prochloraz is known to both block and induce cytochrome P450 monoxygenase (CYP) enzymes (Bach and Snegaroff, 1989), leading to endocrine disruption (Andersen et al., 2002; Baumann et al., 2015) and synergistic interaction with other toxicants (Cedergreen, 2014; Kreuschmann et al., 2015; Rösch et al., 2017). Following the adverse outcome pathway (AOP) framework, prochloraz toxicity in fish has been shown to result in the adverse outcome of reduced cumulative fecundity and decreased population trajectory (Villeneuve, 2019), with effect

![Fig. 1. Intergenerational phenotypic and enzymatic effects in prochloraz-exposed D. magna for the three treatment scenarios: control (blue), continuously exposed (red) and F0-exposed (orange) (A) Experimental scheme. (B-D) Reproduction quantified as cumulative live offspring per surviving female in generation F0 (B), F1 (C) and F2 (D). Error bars show standard error of the mean (SEM) (n = 10 replicates), * indicate significant difference (Tukey’s post hoc T-test p<0.05). (E) Offspring length (mm) ± SEM (n = 10 replicates for each treatment and N = 4–19 animals per replicate) in the three treatment groups and for the three generations F0-F2. * indicate significant difference between groups in the same generation (Tukey’s post hoc T-test p<0.05). (F) Offspring size in the three treatment groups, plotted in the sequence that they occur in the PCA plot for gene expression (Fig. 2F). The horizontal line indicates the average length of the control animals and the dotted line SEM. Letters above the error bar show the statistical grouping (p-value < 0.05, N = 30–61 animals per replicate). Only the 5 lineages for which we had RNAseq data are included. (G) Cytochrome P450 ECOX activity normalized to length of animals (pmol/min/mm) ± SEM (n = 10 replicates for each treatment and N = 10 animals per replicate) in control (blue), continuously exposed (red) and F0-exposed animals (orange) and for generations F1 and F2. * indicate significant difference (Tukey’s post hoc T-test p<0.05) from control group in the same generation.
concentrations in the range 30–100 μg/L (Ankley et al., 2005; Zhang et al., 2008). The key mechanisms behind these effects are the reduced concentration of 17β-estradiol and reduced vitellogenin synthesis linked to the molecular initiating event of CYP19 inhibition. In Daphnia, prochloraz is known to be a potent inhibitor of non-specific CYP enzyme activity in vivo (Gottardi et al., 2016), decrease reproduction, affect molting following long-term exposure (Hassold and Backhaus, 2009), and lead to developmental abnormalities in offspring (Hassold and Backhaus, 2009). However, wider generational effects of prochloraz, or any other chemical with the same mode of action, have not been investigated beyond F1 offspring abnormalities. Here, we studied intergenerational effects from prochloraz exposure, addressing both adverse effects as well as acclimation effects during and beyond F0 exposure. Animals were followed through two exposure scenarios: 1) continuous exposure of all three generations (F0, F1 and F2) and 2) direct exposure of only the first generation (F0), which, however, also result in embryonic exposure of F1 and potentially germine exposure of F2 (Fig. 1A).

2. Materials and methods

2.1. Study design

Fig. 1A illustrates the experimental design. At study initiation, offspring (~24 h) from 30 4-week-old mothers were separated, pooled together and randomly chosen individuals were assigned to 60 mL clear glass vials closed with a white plastic lid with an aeration hole stopped with a pipette filter tip. Conditions remained the same as culturing (see Supplementary Material) except for food amount, which was modified to age (Table S1).

With the general setup based on the OECD guideline for Daphnia magna reproduction test (OECD/OCDE, 2008), animals were followed for 3 generations. There were three treatment groups. In one group all 3 generations were continuously exposed to prochloraz (Exposed). In another only the first generation was directly exposed (F0-exposed), conveying embryonic exposure for F1 and potentially germine exposure for F2. Finally, there was a non-exposed control group (Control). For each treatment in each generation there were 10 replicate glasses all containing one mother. The next generation was started with neonates from the 3rd clutch produced by the mother of the same lineage, resulting in 10 3-generation lineages per treatment. Unfortunately, blinding was not possible for the study. In generation F0, the continuously exposed lineages (Fig. 1A, Treatment “Exposed”) and the F0-exposed (Fig. 1A, Treatment “F0-exposed”) experienced the same exposure scenario and the number of replicates is therefore 20. Sample size was not determined based on statistical methods.

D. magna produce offspring in distinct clutches and the different clutches were used in different analyses (Fig. 1A). Hence, we made the assumption that clonal animals of the same lineage were so similar that they could be used as proxy for each other. The first clutch was only counted and added to total number of offspring. The second clutch was used for length measurements, and subsequent clutches (3–5) were used for molecular endpoint measurements (Fig. 1A). In order to achieve sufficient biomass and make the setup comparable with the enzyme assay protocol, measurements of all the molecular endpoints (i.e. ECOD, transcriptomics and metabolomics) were performed on 5-day old juveniles. 10 offspring from the same replica and line were reared to day 5 in a 250 mL blue cap bottle containing 200 mL of a test solution comparable to the one that the following generation was reared in (i.e. juveniles of the treatment group “F0-exposed” were transferred to pure M7 medium). They were fed ad libitum and treatment and medium was not changed during the 5 days.

2.2. Exposure

The D. magna mothers were kept individually in 60 mL vials containing 50 mL exposure medium until they had produced their 5th clutch (total exposure period between 28 and 32 days). The concentration of prochloraz was set to 100 μg/L based on a pilot study of the dose-response relationship (Figure S1). The selected dose affected length of offspring but not the number of offspring and it strongly inhibited cytochrome P450 ECOD activity. Reported environmental concentrations of prochloraz are below 1 μg/L (Belenguer et al., 2014), which is a factor of 100 lower than the targeted exposure in the current study. The environmental relevance of the reported effects is therefore not the main goal of this paper. The test solutions were mixed in blue cap flasks just prior to exposure renewal by adding 8 mL stock solution (6.25 mg/L prochloraz in MilliQ water), algae suspension (see Supplementary Material for feeding scheme), followed by M7 medium to a total volume of 500 mL. Test solutions were renewed every two days. Please see Supplementary Material for chemicals and regents.

2.3. Exposure verification by UPLC-MS/MS

In order to monitor actual exposure concentrations, water samples were collected from three random vials within each treatment group before change and from each of the new test solutions before distribution into test vials. Prochloraz concentrations were quantified using UPLC-MS/MS with deuterated prochloraz (d4-prz) as internal standard. The average start concentration was 84±12 μg/L and this decreased with 8% to an average of 78±12 μg/L during the 48 h that passed between treatment changes. Please see Supplementary Material for sample preparation, instrumental parameters, method validation and all results.

2.4. Magna reproduction and length measurements

When treatments were changed the mother was moved with a glass pipette to a new vial and offspring collected and counted. Every 2nd day the following physiological endpoints were recorded: time to first eggs in pouch, time to first clutch, viability of offspring, offspring per clutch, length of offspring in the 2nd clutch, total offspring and total clutches per mother until 5th clutch or until natural death if 5th clutch was not reached. The second clutch was transferred to a transparent slide, which was scanned (Canon CanoScan LiDE 220, 1200dpi, 48 bit color) and animal length measured according to the method by Agatz and Preuss (2015).

2.5. Cytochrome P450 ECOD activity

In vivo 7-ethoxy-9-carboxy-o-dealkylation (ECOD) activity was used as a measurement of CYP biotransformation capacity of the animals. The procedure followed the method by Gottardi et al. (2016). Please see Supplementary Material for details.

2.6. Metabolomics

An untargeted metabolomics analysis workflow was applied using two platforms: a nano-liquid chromatography (LC)-Orbitrap HRMS/MS system and an ion exchange chromatography (IC)-orbitrap system. 5–15 animals from the 5th clutch of each replicate mother, were reared to day 5 in a 250 mL blue cap bottle. Subsequently the animals were collected (sieve) and transferred to a cryovial, and immediately submerged in liquid nitrogen and stored at −80 °C until extraction. Metabolites were extracted in two fractions for subsequent analysis on the two platforms. Procedural blanks were included from the beginning. Please see Supplementary Material for sample preparation and instrumental parameters.

Compound Discoverer software version 3.1.305 (Thermo Scientific) was used for data processing (Bhattacharya, 2019). The workflow combined the search for unknown metabolites with a search for expected compounds resulting from prochloraz i.e., transformation products and mother compound (see Supplementary Material for software for comparison).
settings). The final number of replicate samples in the analysis were for F1: 6 controls, 6 continuously exposed and 6 F0-exposed and for F2: 9 controls, 8 continuously exposed and 8 F0-exposed.

Quality and validity of the chemical analysis was confirmed by principal component analysis (PCA) showing that the composite quality control (QC) samples were centrally located in the plot (Fig. 2A). The statistical analyses were performed using R studio v1.2.1335 (R Core Team 2020) and the package DESeq2 v1.22.2 (Love et al., 2014) to identify statistically significant differences between treatments in relative metabolite concentrations. Metabolite identification was based on the 4 levels of confidence described in Viant et al. (2019) and the functional analysis was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2012). Please see Supplementary Material for details on the data analysis.

### 2.7. RNA Sequencing

RNA sequencing was only performed for generation F2. Total RNA was extracted from 8 animals from the 4th clutch of each replicate line using TRIzol reagent (ThermoFisher Scientific, USA). The protocol followed manufacturers instruction with modifications as in Campos et al., 2018 (see Supplementary Material for details). All library constructions and sequencing were performed at Beijing Genomics Institute (BGI, HongKong).

RNA quality was assessed using an Agilent 2100 bioanalyzer and 19 samples (Exposed: n = 5, F0-exposed: n = 7, control: n = 6 replicates, N = 8 animals per replica) were of sufficient quality to be sequenced using the BGISEQ-500RS sequencing platform. Quality filtering and adaptor removal was performed using Trimmmomatic v0.38 (Bolger et al., 2014) and quality of reads was evaluated using FastQC (v0.11.5, Babraham bioinformatics). Trimmed reads were mapped to the D. magna genome (NCBI database; Assembly: ASM399081v1, BioProject: PRJNA490418), using the alignment software STAR with default settings (Dobin et al., 2013). The 123 Mbp D. magna genome published by Lee et al. (2019) consists of 4192 scaffolds and 16,817 contigs, with 15,721 annotated genes. The publication paid specific attention to defense related genes such as CYPs (Lee et al., 2019). The raw counts of the number of mapped transcripts were obtained with FeatureCounts (Liao et al., 2014), and the coverage uniformity and transcript integrity was assessed using the RSeQC package (Wang et al., 2012). This revealed a substantial 3’ bias (Figure S3), and consequently 4 replicate samples that showed considerable degradation were removed. Normalization of the remaining samples was performed using the transcript integrity number (TIN) as described in (Wang et al., 2016). Finally the R-package DESeq2 v1.22.2 (Love et al., 2014), R Core Team 2020), was used to identify differentially expressed genes. During data analysis it became evident that the physical length of D. magna was a key predicting variable of variation in gene expression between replicate samples. It was not possible to measure the length of the animals used for RNA extraction as the increased stress of the measuring procedure would almost certainly have affected sample transcription levels. Average length measurements of daphnids from the ECOD analysis of the previous clutch was therefore used as a proxy for the length of animals in the transcriptomic analysis. Unfortunately, one length-measurement was missing. Hence the final number of replicate samples in the analysis after filtering and normalization were: 5 controls, 4 continuously exposed and 5 F0-exposed, which each consisted of RNA-extracts from 8 five-day old D. magna juveniles. After the differential analysis we performed a manual curation of the annotation of all key differentially expressed genes using the blastx function of the D. magna release on the database wfleabase.org (Colbourne et al., 2005).

### 2.8. Statistics

Data for time to first clutch and ECOD-activity were modelled by analysis of covariance (ANCOVA) with generation and treatment as explanatory variables. In the analysis of length, number and mortality of offspring a linear mixed model of the R-package “lme4” (Bates et al., 2015) was applied. In all cases the initial model was an additive model consisting of the two explanatory variables. Subsequently, interactions between the explanatory variables were tested and if interactions were found, the co-variates were combined into one explanatory variable, which was used in the final model. An interaction between treatment and generation was found for all models. The linear mixed models for

---

**Fig. 2.** Intergenerational changes in genome-wide gene expression and metabolite levels in D. magna for the three treatment scenarios: control (blue), continuously exposed (red) and F0-exposed (orange). (A) Principal component analysis (PCA) for global metabolome analysis in generation F1 (upper) and F2 (lower) for controls (blue), continuously exposed (red) and F0-exposed animals (orange). Dots indicate individual replicates, each consisting of metabolite extracts from 2 to 15 5-day old juvenile D. magna. Black dots indicate 7 replicates of the composite quality control sample. (B-E) Volcano plots showing down- and upregulated metabolites in 5-day old D. magna in continuously exposed F1 (B) and F2 (D) animals and in F0-exposed F1 (C) and F2 (E) animals. The x-axis represents positive and negative log2-fold changes, respectively. Metabolites highlighted in red/orange differ significantly in their concentration when compared to control animals (p.adj < 0.05). (F) PCA for gene expression in generation F2 for controls (blue), continuously exposed (red) and F0-exposed (orange) animals. Dots with letters indicate individual replicates, each consisting of RNA-extract from 5 to 10 5-day old juvenile D. magna (G-H) Volcano plot of F2-RNaseq data showing up- and down-regulated genes in 5-day old D. magna in continuously exposed F2 and F0-exposed F2 animals compared to control animals of the same generation. The x-axis represents positive and negative fold changes, respectively. Genes highlighted in red/orange are significantly differentially transcribed compared to control animals (p.adj < 0.05).
neonate length included the replicate as a random variable. The linear mixed model for neonate number and mortality was adjusted for the day of measurement and the clutch. Model checking was performed by visual assessment of QQ-plots and residual plots and if okay, a post-hoc pair-wise comparison by t-tests (Tukey contrasts) was performed (Hot-horn et al., 2008). For each analysis a pre-specified significance level of 5% was used. All analyses were carried out using R v3.6.3 and R studio v1.1.383 (R Core Team, 2020).

3. Results and discussion

3.1. Acclimation after continuous prochloraz exposure

To investigate acclimation and compensatory responses over generations, we compared Daphnia lineages continuously exposed to prochloraz for three generations with control animals (Fig. 1A, Treatment “Exposed” vs “Control”).

3.1.1. Effects on reproduction during continuous exposure

Both offspring size (Fig. 1E) and cumulative number of offspring (Fig. 1B-D) provides information about the energy investment that the mother puts into reproduction. In the first generation (F0), exposure to 100 μg/L prochloraz significantly decreased offspring length with an average of 6% when compared to controls (Tukey’s post hoc T-test \( p = 0.002 \)) (Fig. 1E), while no difference was found in offspring number (Fig. 1B) (Tukey’s post hoc T-test \( p = 0.7 \)). This is consistent with our pilot study, where a 100 μg/L exposure corresponded to \( < EC \) for cumulative offspring size but \( > EC_{90} \) for length (Figure S1A-C, Table S1). It is therefore evident that without pre-exposure the fungicide decreases length of offspring. Furthermore, it should be noted that there was significant offspring mortality of 0.21 ± 0.04 in F0 of the exposed animals when compared to control lineages of the same generation where the mortality was 0.10 ± 0.05 (Figure S5) (Tukey’s post hoc T-test \( p = 0.001 \)). No significant offspring mortality was observed in F1 and F2.

With continued exposure into generation F1 and F2, the average length of offspring was first significantly larger in F1 with an average change of 19% (Tukey’s post hoc T-test \( p = 0.0001 \)) but then similar to controls in F2 (Tukey’s post hoc T-test \( p = 0.1 \)) (Fig. 1E). The size of offspring is important as larger offspring have better energy reserves to withstand environmental stress (Perrin et al., 1990). The increase in size in F1 is therefore a likely compensatory response to continuous exposure to prochloraz, although larger size also comes with a higher risk of predation (Reynolds, 2011). Such changes in life history traits have usually been found to correlate with a change in offspring investment (i. e. fewer but larger, or more but smaller offspring) (Glazier, 1992). However, we observed a comparable number of offspring across treatments in F1 (Fig. 1C). Such a lack of trade-off in D. magna reproduction was also observed for another azole fungicide, epoxiconazole, where both increased length and increased number of offspring were observed after low-dose exposure (Gottardi et al., 2017). In addition, increased offspring production without effects on length was observed in response to two serotonin re-uptake inhibitors (Campos et al., 2012b). The latter was later correlated with an increased aerobic catabolism and a higher sensitivity to anoxic conditions (Campos et al., 2012a), showing trade-offs on other fitness traits than offspring number and size.

While cumulative reproduction remained unaffected in F1 (Fig. 1C), it was significantly higher than controls in F2 (Fig. 1D) (Tukey’s post hoc T-test \( p = 0.7 \) and \( p = 0.01 \), respectively). Within generation F2, these continuously exposed animals, furthermore, had their first clutch earlier compared to controls (Tukey’s post hoc T-test \( p = 0.003 \)) (Figure S5). At concentrations 5 times higher than those tested here, prochloraz has been observed to delay development leading to delayed reproduction (Hassold and Backhaus, 2009). On the contrary, earlier onset of reproductive maturity was observed in response to compounds such as fish kairomones (Weider and Pijanowska, 1993) and fluvoxamine (Campos et al., 2012a).

It should also be noted that some variation between generations in terms of cumulative reproduction was observed in controls (see Supplementary Material).

3.1.2. Effects on overall CYP-enzyme activity during continuous exposure

In vivo 7-ethoxycoumarin-O-dealkylation (ECOD) activity was used as a measurement of cytochrome P450 monoxygenase (CYP) biotransformation capacity. The assay is based on a broad-spectrum substrate and measures overall CYP activity (Gottardi et al., 2016). The ECOD activity was significantly reduced for the continuously exposed animals in both F1 and F2 (Tukey’s post hoc T-test, \( p \)-value < 0.001) (Fig. 1G). There were no signs of acclimation as ECOD activity was equally low in the two generations. Prochloraz has earlier proved a potent inhibitor of Daphnia magna in vivo ECOD activity (Gottardi et al., 2016), so a decreased activity was expected. CYPs have key functions such as detoxification of xenobiotics (Rösch et al., 2016) and hormone regulation of molting (iga and Kataoka, 2012). The fungicide is known to interact with CYPs (Bach and Sægaphø, 1989) through coordination of its azole moiety lone pair to the heme iron that is present in the catalytic site of the enzyme (Correia and Monteflano, 2005). Hence, CYP-dependent pathways are likely targets for toxic mode of action of prochloraz in non-target organisms.

3.1.3. Effects on the Daphnia metabolome during continuous exposure

We used untargeted metabolome analysis to monitor changes of internal metabolites resulting from prochloraz exposure. In the untargeted metabolome analysis, a total of 2525 metabolites were detected that could be compared between treatments and generations (Fig. 2B-D). The highest number of differentiating metabolite concentrations was found in the continuously exposed F1-animals, which showed higher concentration of 421 compounds and less of 170 compounds compared to controls (Fig. 2B) (FDR-adjusted \( p < 0.05 \)). With continued exposure into generation F2, the number of differentiating concentrations decreased to a total of only 86 compounds (Fig. 2D) (30 up- and 56 down-concentrated, FDR-adjusted \( p < 0.05 \)). This corroborates the physiological- and life-history compensation and acclimation observed in life history traits and in CYP activity.

Annotation was possible for 73 of the up- and down-concentrated compounds. 44 of these were endogenous metabolites that could be assigned to metabolic pathways (Table S4 and S5). In generation F1 a general up-concentration of compounds in metabolic pathways involved in biosynthesis of amino acids, carbohydrate metabolism and lipid metabolism (Table S4 and S5) was observed. This is consistent with the observed larger reproductive output observed in the continuously exposed F1 mothers (Fig. 1E). Several compounds of differentiating concentration could also be annotated as tentative candidates belonging to metabolic pathways specifically connected to the interaction between prochloraz and several different heme-containing enzymes (Viant et al., 2019). For further discussion please see Supplementary Information. Among them were a tentatively annotated estriol, identified to level 2 with a 72% match to the spectral database mzCloud (https://www.mzcloud.org). In vertebrates estriol is a metabolite of 17β-estradiol, the enzymatic product of CYP19, and therefore a metabolite likely to be affected by prochloraz. The compound is however not currently known to play a biological role in daphnia (Litoff et al., 2014). The putative estriol was found slightly up-concentrated (logFC = 1.3, FDR-adjusted \( p < 0.05 \)) in continuously exposed F1 animals, indicating a compensatory response in the CYP-dependent metabolic pathways. Also, an important pathway in the invertebrate lipid metabolism, alpha-linoleic acid metabolism (Lee et al., 2018), was found to be affected in this treatment group. Alpha-linoleic acid as well as two possible CYP-substrates: (9Z, 11E,15Z)-(13S)-Hydroperoxyoctadec-9,11,15-trienoate and (10E,12Z,15Z)-(9S)-Hydroperoxyoctadeca-10,12,15-trienoic acid, were found in higher concentrations. A CYP-catalyzed reaction can convert the latter metabolite to (9S)-(10E,12Z,15Z)-9,10-Epoxyoctadeca-10,12,15-enoic acid and this compound was found in lower concentration in
exposed animals. This increased substrate concentration and decreased product concentration is another indication of CYP-enzyme inhibition. Effects were also observed on other compound concentrations that are controlled by heme-containing enzymes. This included compounds in the cutin, suberin and wax biosynthesis pathway, which for instance are involved in cuticle formation and metabolism of terpenoids involved in synthesis of insect hormones (Table S5).

The up- and down regulated metabolites add evidence to the molecular initiating event (MIE) of prochloraz toxicity in *Daphnia* being coordination of the lone pair of the azole moiety to the heme iron present in catalytic sites of enzymes. However, it also underlines that this can happen for several enzymes involved in many different pathways and not just for CYP19 with subsequent effects on the steroidogenesis.

Metabolites that were affected in F1 of continuously exposed animals were rarely affected in F2 or the effect was of opposite direction (Table S4 and S5). For instance, there was more of the steroid tetrahydrocortisol in F1 (log2 FC=−2.2, FDR-adjusted *p* < 0.05) but less in F2 (log2 FC=−1.8, FDR-adjusted *p* < 0.05). This is a further substantiation of the intergenerational compensatory response and acclimation in *Daphnia* continuously exposed to prochloraz.

Prochloraz and its metabolites were not separated from the endogenous metabolites in the data processing. Thirteen prochloraz metabolites were putatively annotated (Table S6) and transformations, such as partial loss of the imidazole ring with subsequent aldehyde formation corresponded well with previous studies of prochloraz metabolism in the freshwater invertebrate *Gammarus pulex* (Rösch et al., 2016).

### 3.1.4. Effects on the transcriptome in generation F2 after continuous exposure

Analysis of the transcriptome in F2 showed considerable inter-treatment variance (Fig. 2F), and notably the overall gene expression in continuously exposed lineages was very similar to control lineages (Fig. 2G). Three generations of continuous exposure to prochloraz gave only 10 up-regulated and 5 down-regulated genes (FDR-adjusted *p* < 0.05) (Fig. 2G). Among these were, however, an upregulation of a CYP gene (GeneID: Dapma7bEVm029432t1, log2 fold change = 33, padj=0.007), which confirms that this enzyme family plays a central role in prochloraz toxicity and acclimation in *Daphnia*, also after continuous exposure in three generations. Taken together, all our data is consistent with intergenerational compensatory response and acclimation as a result of constant prochloraz exposure in *Daphnia*. Such observed acclimatization processes are likely to result in ecologically relevant trade-offs and have consequences for the ability of the animals to handle other challenges such as predation, other xenobiotics and anoxic conditions.

### 3.2. Inter-generational effect of F0-exposure

After establishing that continuous prochloraz exposure has substantial physiological and metabolomic effects on *Daphnia*, we turned our focus to the F0-exposed treatment group to investigated whether toxic effects persist in generations beyond the directly exposed F0-individual. This was done by comparing generation F1, which was exposed during embryogenesis, and F2, which potentially was affected by germline exposure, with control lineages (Fig. 1A, Treatment “F0-exposed” vs “Control”).

#### 3.2.1. Effects on reproduction after F0-exposure

Following the direct exposure in F0, which resulted in a decreased offspring length (Fig. 1E), the animals in the subsequent F1- and F2-generations produced offspring of similar size to control animals. However, in F2 the animals had significantly more offspring compared to both controls and to continuously exposed animals (Tukey’s post hoc *T*-test *p* = 0.0001 and *p* = 0.004, respectively) (Fig. 1D). Equal to the increased offspring size observed in F1 of continuously exposed lineages, this increase in number of offspring supports a compensatory response in F2.

#### 3.2.2. Effects on overall CYP-enzyme activity after F0-exposure

The F0-exposed animals did not display any significant decrease in ECOD activity in the F1 generation, indicating full recovery from their embryonic exposure. However, when activity was measured in the F2 generation, it had decreased to a value intermediate between the control and continuously exposed animals (Tukey’s post hoc *T*-test *p* = 0.01 compared to controls of same generation) (Fig. 1G). These results suggest that direct exposure of grandmothers (F0) conveying embryonic exposure of F1 and potential exposure of primordial germcells (F2) leads to more pronounced effects on enzyme activity in granddaughters (F2) than in daughters (F1).

#### 3.2.3. Effects on the *Daphnia* metabolome after F0-exposure

Pairwise comparison by multivariate statistical analyses showed differences from controls in both generation F1 and F2 (FDR-adjusted *p* < 0.05). Opposite to the continuously exposed animals, however, the largest differences were observed in F2. In F1, 21 metabolites were at lower concentrations when compared to controls and none were found up-concentrated (Fig. 2C). In F2, 41 and 16 compounds were at lower and higher concentrations, respectively, when compared to controls (Fig. 2E). This supports the observations made for both ECOD activity and cumulative reproduction, which showed a larger effect in F2 compared to F1. The metabolites that changed concentrations in the F0-exposed treatment group included, for example, tetrahydrocortisol in generation F2 (log2 FC=1.8, FDR-adjusted *p* < 0.05) (Table S5). This metabolite is involved in biosynthesis of steroid hormones, and it was actually equally affected in F1 of the continuously exposed animals (log2 FC=−2.8, FDR-adjusted *p* < 0.05). Juvenile hormone III acid, a metabolite involved in insect hormone biosynthesis and a CYP product, was also affected in F2 of the F0-exposed, but not in F1 (Table S5). In the continuously exposed animals, this metabolite was affected in both generations. This supports the observation of CYP-activity being affected in grand-maternally and potentially germline-exposed (F2), but not in embryonically exposed (F1) animals.

#### 3.2.4. Effects on the transcriptome in generation F2 after F0-exposure

The F2 gene transcription in grandmaternally and potentially germline-exposed animals showed a much larger divergence from the controls than the continuously exposed animals (Fig. 2F+H). In total, 170 genes were less expressed and 127 were more highly expressed (Data S1). Enrichment analysis of gene ontology (GO) terms among the 227 annotated differentially expressed genes using the hypergeometric distribution revealed four GO-terms that were significantly enriched (Bonferroni-adjusted *p*<0.05) in F0-exposed animals relative to controls in the F2 generation (Table 1, Table S7). All enriched GO-terms were related to oxidation-reduction processes and most also to iron-ion containing enzymes. Among the upregulated monooxygenases were four putative CYP-genes coding for CYP4aa1, 4D2O and 6A18; all part of pathways for metabolizing xenobiotics (Lee et al., 2019). Furthermore, the same CYP gene that was upregulated in the continuously exposed F2 animals (GenelD: Dapma7bEVm029432t1) was significantly upregulated (Table 1). Significantly depleted GO-terms were related to the spliceosomal complex, RNA binding and mitochondrial transcription (Table S8). Thus, the functional characteristics of upregulated genes are directly related to the chemical characteristics of prochloraz and the well-documented interaction between the azole moiety and heme-iron (Correia and Monteblanco, 2005). Hence, our data supports that the azole moiety and heme-iron may be a molecular initiating event in an adverse toxic outcome of prochloraz. In addition, our data shows that the same mode of action is affected across generations and in generations beyond the directly exposed generation. Furthermore, the effect on CYPs link the effect in *Daphnia* to the currently accepted AOP for prochloraz in vertebrates. It is, thus, evident that several CYP-dependent biochemical pathways may be affected by the fungicide, similar to the
Table 1
Significantly enriched gene ontology (GO) terms in F0-exposed animals compared to controls using the hypergeometric distribution (Bonferroni-adjusted $p < 0.05$). The enriched GO-term, GeneID, log2 fold change and adjusted $p$-value for the statistical comparison, and finally the differentially expressed genes within this GO-category.

<table>
<thead>
<tr>
<th>Gene ontology</th>
<th>GeneID</th>
<th>Log2FC</th>
<th>p-adj</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation-reduction process</td>
<td>Dapra07EVM0154488</td>
<td>32.5</td>
<td>0.015</td>
<td>Protein fatty acyl-CoA reductase</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0013350</td>
<td>31.0</td>
<td>0.023</td>
<td>Trimethyllysine dioxygenase, mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0187520</td>
<td>18.6</td>
<td>0.005</td>
<td>Glutathione peroxidase-like</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0102010</td>
<td>17.7</td>
<td>0.009</td>
<td>Putative medium chain specific acyl-CoA dehydrogenase, mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0022482</td>
<td>9.1</td>
<td>0.010</td>
<td>D-lactate dehydrogenase, mitochondrial</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0055861</td>
<td>-8.0</td>
<td>0.041</td>
<td>tRNA-dihydroxymethionine(20) synthase (NADP(H))-like protein</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0043958</td>
<td>-8.3</td>
<td>0.036</td>
<td>S-adenosyl-L-methionine-dependent tRNA 4-demethylcytosine synthase</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0048313</td>
<td>-11.5</td>
<td>0.021</td>
<td>Retinol dehydrogenase 13-like</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0122961</td>
<td>-28.5</td>
<td>0.003</td>
<td>3-cwo-5-beta-sterol 4-dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0053171</td>
<td>-37.3</td>
<td>0.011</td>
<td>Ferritin</td>
</tr>
<tr>
<td>Heme binding, iron ion binding</td>
<td>Dapra07EVM0285906</td>
<td>15.1</td>
<td>0.002</td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0064063</td>
<td>11.5</td>
<td>0.027</td>
<td>Membrane-associated progesterone receptor component 2</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0007087</td>
<td>8.4</td>
<td>0.030</td>
<td>Alkylglycerol monoxygenase</td>
</tr>
<tr>
<td>Oxidoreductase activity acting on paired donors with incorporation or reduction of molecular oxygen</td>
<td>Dapra07EVM0038761</td>
<td>17.9</td>
<td>0.036</td>
<td>Egl nine</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0095142</td>
<td>20.1</td>
<td>0.003</td>
<td>Tyrosine 3-monoxygenase</td>
</tr>
<tr>
<td>Monooxygenase activity</td>
<td>Dapra07EVM0149848</td>
<td>18.1</td>
<td>0.012</td>
<td>Putative Cytochrome P450 4a4a1</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0007155</td>
<td>21.1</td>
<td>0.020</td>
<td>Putative Cytochrome P450 4d20</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0151120</td>
<td>33.6</td>
<td>0.022</td>
<td>Putative Cytochrome P450 4d20</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0112265</td>
<td>23.5</td>
<td>0.048</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td></td>
<td>Dapra07EVM0284320</td>
<td>26.2</td>
<td>0.010</td>
<td>Cytochrome P450</td>
</tr>
</tbody>
</table>
metabolome analysis in generation F1 of the continuously exposed animals.

3.3. Are we overlooking the importance of prenatal exposure?

A grandmaternal effect of prochloraz, which we here define as significant differences between controls and F0-exposed animals in F2, but with no difference observed in F1, was found for the cumulative number of offspring (Fig. 1D), cytochrome P450 ECOD activity (Fig. 1G), and up-concentration of metabolites (Fig. 2E). It was further underlined by differential expression of several key genes in F2 (Fig. 2H), which could be directly related to the mode of action of prochloraz (Table 1). This demonstrates, with mechanistic evidence, that effects of prochloraz exposure in D. magna can last beyond the directly exposed generation and that there can be a time gap between the more obvious direct exposure and the emergence of effects in generations that were exposed only at prenatal life stages.

It thereby begs the questions of whether primordial germ cells are an important life stage to consider when evaluating effects of chemical exposure and assessing their risks. During the exposure of the F0-generation, the F2 animals were present as primordial germ cells (PGCs) inside the developing F1s. Effects in F2 could, therefore, arise both because of a direct exposure of the PGCs or indirectly because of an intergenerational transfer of biological information from the developing embryo (F1) to the PGCs (F2). The PGC stage is a sensitive time point, especially for establishment of epigenetic marks that later are essential for the dynamic regulation of gene expression underlying the cellular plastic response to environmental- and developmental cues (Atlasi and Stunnenberg, 2017). Effect of chemicals on the epigenetic landscape of PGCs has for instance been observed in male mice where F0-exposure to the anti-androgenic fungicide vinclozolin changed the expression of ncRNAs in PGCs leading to histopathological effects in testes in F1-F3 (González-Ruíz et al., 2015). In Daphnia, zinc, cadmium and microcystein exposure, gamma-radiation and salinity stress have been observed to change DNA methylation status in association with multigenerational effects (Asselman et al., 2017; Jeremias et al., 2018; Trijau et al., 2009a, 2009b). Epigenetic effects were not evaluated in the present study but several genes coding for epigenetically relevant genes were among the differentially expressed genes in F2 when comparing F0-exposed animals to controls (Table S9).

3.4. Individual plasticity revealed trade-offs

The principal component analysis of gene expression in F2 revealed an interesting pattern in the F0-exposed samples as the individual lineages were gradually more divergent from the controls (Fig. 2F). This pattern in gene-expression variation turned out to correlate with lineage-specific off-spring size within the F2 generation of the F0-exposed lineages (Fig. 1F): The more similar gene expression was to the control animals, the smaller the offspring. This indicates that a gradual plastic adjustment is taking place and that there seems to be a metabolic cost in returning to normal (control) gene expression levels after prochloraz exposure i.e., a trade-off between offspring size and the ability to cope with grandmaternal prochloraz exposure. For further discussion please see Supplementary Information.

3.5. Toxicity is a process

As exemplified, the molecular and physiological effect of chemical exposure reaches to subsequent generations of the directly exposed individual; whether it is a lingering effect in potentially germline-exposed granddaughters or acclimation to the constant exposure. This implies that current risk assessment procedures of for instance pesticides could be neglecting an important aspect of toxicity. It also adds to the concern that toxicity observed in the field might be uncoupled from the measured chemical exposure. Finally, it opens new interesting questions about Daphnia developmental biology. As stated by Tjalling Jager in his paper “Predicting environmental risk: A roadmap for the future”: "Toxicity is not a value, it is a process" (Jager, 2016). An important part of this process is timing and the dynamics here are still not well understood. Future work should focus on multigenerational effects of chemicals, dose-response relationships at environmentally relevant concentrations, and sensitivity of prenatal life stages to elucidate their importance for hazard assessment of chemicals in order to further improve adequate risk assessment of environmental pollutants and protect ecosystems.

4. Author contributions

R.P. and N.C. conceived and designed all the experimental work. R.P produced samples. H.H.D.F.L contributed to the transcriptome methodology and assisted with the data analysis. M.H. contributed to the metabolome methodology and assisted the measurement and characterization. The manuscript was written by R.P. All authors contributed to the discussion of the data, proofread of the manuscript. The overall project was supervised by N.C. and H.H.D.F.L.

5. Funding sources

The authors acknowledge financial support from University of Copenhagen, Department of Plant and Environmental Sciences, PhD scholarship for R. Poulsen and M. Hansen acknowledge the financial starting grant from Aarhus University Research Foundation (AUFF-T-2017-FLS-7-4). The Villum Foundation (Young Investigator Grant, grant number 10,122 to HHDFL), and a The Independent Research Fund Denmark Sapere Aude Research grant no 8049-00086B to HHDFL.

6. Data availability

All data needed to assess the conclusions of the paper are given in the main text, Supplementary Material and data file S1. Metabolomics data is furthermore available in Metabolights under study accession number MTBLS1836 and RNAseq data is available through ArrayExpress under study accession number E-MTAB-9309.

CRediT authorship contribution statement

Rikke Poulsen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. Henrik H. De Fine Licht: Conceptualization, Methodology, Software, Resources, Writing – review & editing, Supervision. Martin Hansen: Methodology, Resources, Validation, Data curtion, Writing – review & editing. Nina Cedergreen: Conceptualization, Methodology, Resources, Data curtion, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgement

We thank research fellows Xiaogang Jiang, Amandine Levastre, Michele Gottardi and Anja Weibel for help with the laboratory work, Andrea Rösch for sharing her knowledge on prochloraz metabolism in invertebrates, Bruno Campos for advice on RNA extractions from Daphnia, Arnar Pálsson and Dagný Asta Rúnarsdóttir for assistance and academic advices in data treatment of RNAseq data.
Supplementary materials


References


