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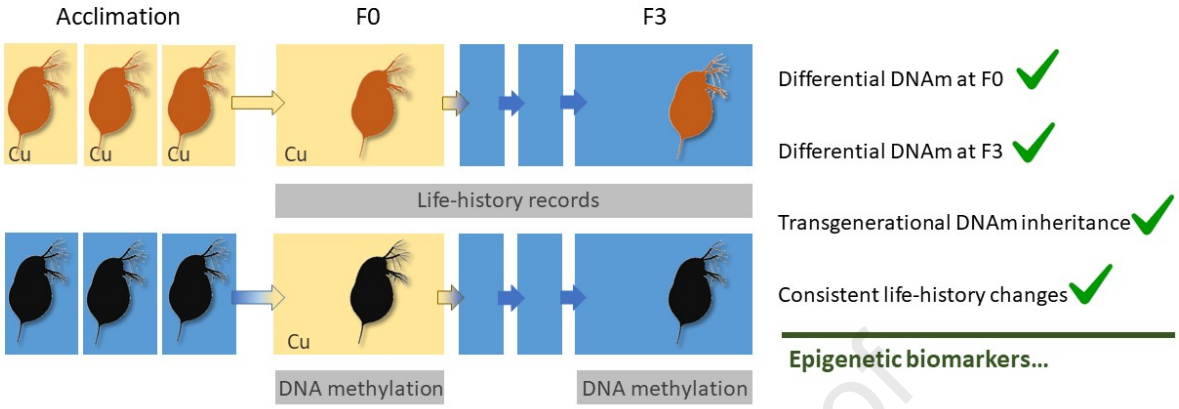

Multigenerational DNA methylation responses to copper exposure in *Daphnia*: potential targets for epigenetic biomarkers?

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Abstract

Epigenetic mechanisms are moving to the forefront of environmental sciences, as environmentally induced epigenetic changes shape biological responses to chemical contamination. This work focused on *Daphnia* as a representative of potentially threatened freshwater biota, aiming to gain an insight into the involvement of epigenetic mechanisms in their response and eventual adaptation to metal contamination. Copper-induced DNA methylation changes, their potential transgenerational inheritance, and life-history traits were assessed. Organisms with different histories of past exposure to copper were exposed to toxic levels of the element for one generation (F0) and then monitored for three subsequent unexposed generations (F1, F2, and F3). Overall, methylation changes targeted important genes for counteracting the effects of metals and oxidative stress, including *dynein light chain*, *ribosomal kinase* and *nuclear fragile X mental retardation-interacting protein*. Also, contrasting overall and gene-specific methylation responses were observed in organisms differing in their history of exposure to copper, with different transgenerational methylation responses being also identified among the two groups, without apparent life-history costs. Taken together, these results demonstrate the capacity of copper to promote epigenetic transgenerational inheritance in a manner related explicitly to history of exposure, thereby supporting the development and incorporation of epigenetic biomarkers in risk assessment frameworks.

Keywords: metal exposure; invertebrates; DNA methylation; epigenetic inheritance; biomarkers

1. Introduction

Freshwaters are amongst the most altered and threatened environments of the planet, and the rapid decline of freshwater biodiversity has been observed worldwide (Albert et al., 2021; Carrizo et al., 2017). Human actions are the major driving force of freshwater transformation, with negative ecological effects being mainly determined by increasing human exploitation of natural resources and industrial activities, as well as by climate change-related effects (Knouft and Ficklin, 2017; Reid et al., 2019). Metals play a very important and ubiquitous role in human industries and societies, with the drainage and discharge of contaminated waters accounting to the increase in the quantity of metals spreading throughout freshwater ecosystems (Carpenter et al., 2011; Chen et al., 2015; Mushtaq et al., 2020). Furthermore, though many metals are essential to life, their environmental persistence, bioaccumulation potential and toxicity towards freshwater organisms has been widely demonstrated (e.g. Ali et al., 2019; Chen et al., 2015; Li et al., 2016). In this context, it has been previously highlighted the importance of evaluating the molecular mechanisms underpinning the toxic effects of metals in freshwater ecosystems and the adaptive capacity of freshwater species facing such stressors (Jeremias et al., 2018a; Reid et al., 2019). Respectively, this effort is vital towards the establishment and reinforcement of feasible protective measures (Jeremias et al., 2018a; Kernan et al., 2011).

Epigenetics comprises both mitotically and meiotically heritable changes in gene expression, excluding those involving alterations in the DNA sequence itself (Bird, 2007; Russo et al., 1996; Skinner, 2011). DNA methylation (DNAm) is perhaps the most widely studied epigenetic mechanism, and this generally refers to the transference of a methyl group to the fifth carbon of a cytosine ring - though other DNA bases can also be methylated (Bird, 2002; Kumar et al., 2018). Moreover, epigenetic mechanisms have been rapidly gaining importance in environmental sciences, with an increasing amount of studies demonstrating their key role in shaping gene-environment interactions, phenotypic plasticity and evolutionary responses (Jeremias et al., 2018a; Skinner, 2015; Vogt, 2017). Interestingly, DNAm is known to respond to a wide range of environmental

stressors, including exposure to metals, and environmentally induced DNAm marks (and their resulting changes in gene expression) can determine specific phenotypic outcomes (Jeremias et al., 2020; Ladd-Acosta, 2015; Skinner, 2011). Furthermore, it should be noticed that the transgenerational inheritance of DNAm marks has now been confirmed in both invertebrate and vertebrate species (Bollati and Baccarelli, 2010; Harney et al., 2022; Jeremias et al., 2018b; Ray et al., 2014). Overall, this evidence supports the use of DNAm marks as epigenetic biomarkers, and they indeed present several advantages over traditional or even transcriptional biomarkers, such as being more accurate, specific, stable and easily measured (Jeremias et al., 2020; Ladd-Acosta, 2015). Besides, epigenetic biomarkers have already been set forward as potential key assets in the Environmental Risk Assessment (ERA) of chemicals (Cotea et al., 2017; Jeremias et al., 2020; Ray et al., 2014; Shaw et al., 2017). In fact, the ERA framework is commonly used to prospectively drive the establishment of regulatory protective benchmarks or to retrospectively guide monitoring and restoration of contaminated sites. DNAm biomarkers potentially serve as molecular tools to define mechanisms of toxicity and establish conservative safety thresholds in prospective assessment, as well as indicators of present and past exposures to stressors in retrospective assessment (Jeremias et al., 2020; Mirbahai and Chipman, 2014; Shaw et al., 2017). In this regard, the study of contaminant-induced methylation responses and their potential transgenerational inheritance are known to be important steps supporting the development of DNAm biomarkers, though these issues remain largely unexplored in ecotoxicological settings (Head, 2014; Jeremias et al., 2020, 2018a; Šrut, 2021).

The freshwater microcrustaceans from the *Daphnia* genus are parthenogenetic organisms that are touted to be ecological and ecotoxicological models, widely considered within regulatory frameworks (Miner et al., 2012; Shaw et al., 2008). More recently, they become recognized as genetic and epigenetic model organisms, in the sense that they are easily clonally propagated in the laboratory, thus allowing the study of molecular modifications without the confounding effects of genetic variation (Harris et al., 2012; Miner et al., 2012; Orsini et al., 2012). Besides, the species is

also increasingly recognized as a valid model for the prospect of mammalian and specifically human health effects resulting from chemicals exposure (Rivetti et al., 2016; Siciliano and Gesuele, 2013). Importantly, DNAm is mainly enriched within the coding regions of genes in *Daphnia*, and previous studies identified the inheritance of DNAm marks resulting from stress exposure (Jeremias et al., 2018b; Kvist et al., 2018; Trijau et al., 2018). In agreement, such epigenetic responses are considered to be key players in the organism's adaptation to stressors (Asselman et al., 2017, 2015; Jeremias et al., 2018b; Lindeman et al., 2019), especially when transgenerational inheritance is established. In *Daphnia* and other invertebrates, transgenerational inheritance is only established in the third non-stressed generation: the environmental exposure of pregnant females (F0 generation) accounts for the direct exposure of progeny (future F1) and germ line of the progeny (future F2) to the same stressor, thus true epigenetic inheritance effects can only be confirmed in the non-exposed F3 (Bell and Stein, 2017; Kovalchuk, 2012; Skinner, 2008). All these features set forward *Daphnia* as a suitable model species for monitoring the link between epigenomic, transcriptomic, and phenotypic responses, allowing researchers to further understand the potential of using epigenetic biomarkers in ERA (Athanasio et al., 2018; Jeremias et al., 2020; Kvist et al., 2018; Thaulow et al., 2020).

In this study, we used *Daphnia magna* to investigate the effects of copper (Cu) exposure on DNAm and potential inheritance of epigenetic marks under this scenario. Copper is amongst the most widespread metal contaminants in freshwater as a result of human activities, and a significant amount of literature is available on the effects of exposure to Cu in different aquatic species, including *Daphnia*, which renders this metal a good model for experimental epigenetics (e.g. Ali et al., 2019; Lopes et al., 2005a). Specifically, we aimed to test the hypothesis that different histories of exposure to copper could determine different epigenetic signatures, which would be carried on into non-exposed generations. Such a confirmation constitutes a valuable add-on towards understanding the potential for epigenetic signatures to serve as biomarkers of exposure and effects in risk assessment, as previously postuled by different theoretical and pratical studies.

2. Materials and Methods

2.1. Daphnia *culturing*

For more than 100 generations, monoclonal cultures of *Daphnia magna* (clone BEAK) were reared in our laboratory under controlled conditions. These comprise culturing in the American Society for Testing and Materials (ASTM) hard water (ASTM, 1980) medium enriched with vitamins and supplemented with an extract of *Ascophyllum nodosum* (an organic additive), under a temperature of 20 ± 2 °C and 16 h/8 h light/dark photoperiod that was provided by cool fluorescent white lights. Culture medium was fully renewed and organisms fed three times a week, with suspensions of *Raphidocelis subcapitata* (ration: 3×10^5 cells mL^{-1}) that were cyclically cultured in Woods Hole MBL (Stein, 1973). This protocol provided naïve organisms (coded hereinafter as Cu^-) for use in the experiments described in section 2.2. Non-naïve organisms (coded hereinafter as Cu^+) for these experiments were reared for three generations - one generation comprised the period from birth to the release of the third brood (the use of the 3rd followed the standards to avoid maternal effects in the offspring responses; e.g. Barata and Baird, 1998), with the neonates therein used to start the subsequent generation - under the same protocol but replacing blank ASTM with ASTM enriched in Cu (same concentration as used also in the following multigenerational experiment; see section 2.2). It is noteworthy, as the support to the definition of the conditioning period, that three to four generations are commonly assumed as sufficient to ensure *Daphnia* acclimation to a given context prior to testing and that such acclimation can occur within conditioning periods as short as one week (Agra et al., 2011; Burton et al., 2020; Müller et al., 2018; Paul et al., 2004).

2.2. *Establishment of exposure level and multigenerational experiment*

The exposure concentration used in the multigenerational experiment was defined following previous immobilisation assessment through a standardized acute toxicity test (OECD, 2004). In short, 3rd brood neonates obtained from bulk cultures, aging less than 24 h, were exposed to a range of Cu concentrations (0.00, 0.010, 0.014, 0.020, 0.027, 0.038, 0.054, 0.075 and 0.105 mg/L) for 48

h, under the previously described conditions of photoperiod and temperature, and without food supply. Treatments were set with 4 replicates that held 5 neonates each. Immobilization was recorded at the end of the test and the EC20, i.e. effective concentration causing 20% of immobilization, was estimated by Probit Analysis to be at 0.021 mg/L (Confidence interval: 0.018-0.026 mg/L). The EC20 level was selected under the standard assumption in ecotoxicological effects-based assessment that the concentration involved corresponds to the lowest capable of inducing a noxious effect in the exposed organism (Lowest Observed Effect Concentration - LOEC surrogate). Moreover, this concentration is within the range of Cu levels found in contaminated freshwaters (Lopes et al., 2005b; Santos et al., 2019; Vidal et al., 2012).

For the multigenerational experiment, monoclonal cultures containing 70 neonates (aging less than 24 h) collected from 3rd brood bulk cultures were established in plastic buckets containing 4 L of test solution (57 mL per organism), under the same temperature, photoperiod, renewal and food schedules as previously described. Three replicates of these cultures were assigned to each of the three experimental treatments (Figure 1): (i) control treatment composed of blank ASTM medium (0 g/L of Cu); (ii) $Cu^{-/+}$, where naïve daphnids were exposed to 0.021 mg/L Cu; (iii) $Cu^{+/+}$, where non-naïve daphnids, i.e. organisms collected from bulk cultures acclimated in Cu-enriched ASTM (0.021 mg/L) for three generations, were exposed to 0.021 mg/L Cu. The exposure was kept as described for one generation (F0), which corresponded to the growth of organisms until releasing of their 3rd brood neonates (see experimental design - Figure 1). These neonates were used to start the corresponding F1 generations ($Cu^{-/+/-}$ and $Cu^{+/+/-}$), the same applying to establish F2 ($Cu^{-/+/-/-}$ and $Cu^{+/+/-/-}$) from F1 and F3 ($Cu^{-/+/-/-/-}$ and $Cu^{+/+/-/-/-}$) from F2, all (F1, F2, F3) reared in blank ASTM, regardless of the exposure treatment held at F0 (Figure 1). During the experiment, F0 and F3 mothers were pooled per replicate bucket shortly after releasing their 3rd brood and stored at −80 °C for DNA extraction (see section 2.3 for details), with a visual inspection being in place to ensure that all collected mothers presented empty brood pouches, thus avoiding the extraction of DNA originating from newly laid parthenogenetic eggs. The decision of sequencing only F0 and F3

treatments (see Figure 1) was made upon the notion that this is the most cost-effective strategy to test the occurrence of true transgenerational inheritance of DNAm marks resulting from Cu exposure in *Daphnia* (Kovalchuk, 2012; Skinner, 2008). Moreover, life-history parameters (mortality, offspring number and broods release) were recorded on a daily basis considering all replicates of all treatments and for all generations involved (F0, F1-F3), thereby allowing the inspection of putative phenotypic effects at the individual and supraindividual level.

2.3. *DNA extraction and reduced representation bisulfite sequencing (RRBS)*

DNA was extracted from 10 pooled organisms collected from each treatment, then stored at -80 ºC, by using the MasterPure Complete DNA and RNA Purification Kit (Epicenter, Madison, WI, USA), according to the instructions of the manufacturer. As previously highlighted, DNA extractions were only performed for F0 and F3 treatments (Figure 1), after which a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used for a preliminary screening on DNA quality. Samples with a 260/230 ratio above 1.7 and a 260/280 ratio between 1.8 and 2.1 were further assessed for DNA concentration and integrity using the Quant-it Picogreen dsDNA assay kit (Thermo Fisher Scientific, MA, USA). Then, genomic DNA was fragmented with the restriction enzyme MspI for following library preparation, bisulfite conversion and sequencing in an Illumina NextSeq 500 platform (Meissner et al., 2005) – see Supplementary protocol 1 for detailed sequencing protocols.

2.4. *Bioinformatic analysis*

Raw sequencing data was deposited in GEO, under the accession number GSE201140. Data quality was assessed with FastQC (version 0.11.9, Babraham Bioinformatics), followed by trimming with Trim Galore! (version 0.6.5, Babraham Bioinformatics), a wrapper tool around Cutadapt and FastQC. In detail, RRBS mode was selected, DNA bases with a Phred scale score below 30 were trimmed and adapter sequences removed. After this, mapping was carried out by using Bismark (version 0.22.3), which allows for bisulfite mapping and methylation calling in a single step

(Krueger and Andrews, 2011). Genome assembly and gene models of *Daphnia magna* were used (Orsini et al., 2016), thus obtaining methylation calls for all cytosines on each strand. The average alignment efficiency was 48%, which is on the lower end of previous RRBS studies with *Daphnia* but in accordance to the low mapping efficiency of bisulfite sequencing data (Asselman et al., 2017; Chatterjee et al., 2012; Jeremias et al., 2018b). At this stage, false positives were identified by determining the methylation rate of unmethylated mitochondrial sequences in all samples and a model based on the binomial distribution - B(n,p) - at each CpG, i.e. DNA methylation of cytosines followed by a guanine-: n referring to the coverage depth of each potentially methylated cytosine and p to the false positive rate. We used the Benjamini-Hochberg procedure to adjust p-values, with values lower than 0.05 being considered true positives. Then, bedtools intersect was used to annotate cytosines within genic regions.

2.5. *Statistical analysis*

Data analysis was run in R version 3.6.1 (R Core Team, 2019). First, the gene models of *D. magna* were used to define genes, comprising intronic and exonic regions (Orsini et al., 2016). Next, the total numbers of methylated cytosines of individual genes were normalized by the total number of cytosines present in those specific genes, thus quantifying methylation levels. Afterwards, the makeBSseqData function from the Bsseq package was utilized to merge the methylation data from the replicated samples and create an object of the BSseq class (Hansen et al., 2012). This served as input for the DMLtest function within the DSS package, using default options to test the existence of differential methylation between treatments at each CpG site (Asselman, 2019; Park and Wu, 2016; Wu et al., 2015). Specifically, this function estimates mean methylation levels and dispersions for each CpG site, and then performs a Wald test under the null hypothesis that the means of the compared treatments are equal (Asselman, 2019; Asselman et al., 2017; Bonasio et al., 2012). Those genes presenting statistically significant CpG methylation were identified by the callDML function from the DSS package, at a significance level of 0.05 - raw p-values and false

discovery rates (FDR) can be found in supplementary Tables S1 and S2. In parallel, the life-history data collected throughout the experiment was used to determine the age at first reproduction (AFR), net reproductive rate (R0) and *per capita* rate of population increase (*r*), calculated on the basis of the Euler-Lotka equation. A one-way ANOVA was then used to assess statistically significant effects of treatments in the life-history parameters, with differences between treatments being tested through a post-hoc Tukey test at a significance level of 0.05.

3. Results

3.1. *Multigenerational life-history effects*

Significant statistical differences (p-value < 0.05) were found when comparing the life-history parameters of the three F0 treatments (Control, $Cu^{+/+}$ and $Cu^{-/+}$), namely regarding population growth and net reproductive rate (Table 1). In particular, for both these parameters, the F0 organisms acclimated in a Cu-enriched medium ($Cu^{+/+}$; non-naïve) showed similar values to those of the Control, while the F0-organisms acclimated in blank ASTM ($Cu^{-/+}$; naïve) showed significantly lower values than the Control (Figure 2). Accordingly, the comparison of F1, F2 and F3 treatments originating from non-naïve and naïve organisms showed that the first group typically presented higher mean values of population growth and net reproductive rates across generations (Figure 2). Nevertheless, in comparison to their respective F0 treatments, both groups presented significantly higher values of population growth and net reproductive rates in the following F1, F2 and F3 generations, which denotes an overall recovery from Cu exposure at the F0 (Table 1, Figure 2). In addition, both groups shared their highest mean values of population growth and net reproductive rate in the F2 and F3 generations, respectively (Figure 2).

On the other hand, no significant effects of any treatment or generation were found regarding age at first reproduction (Table 1). Besides, it is noteworthy that similar life-history records were found when comparing Cu treatments within F3, i.e. naïve and non-naïve organisms recover equally from the Cu challenge at the third non-exposed generation concerning these parameters.

245

246 3.2. *DNA methylation responses*

247 The analysis of mean methylation levels revealed a similar percentage of methylated cytosines in all
248 Cu treatments, both at F0 (0.50, 0.50 and 0.47% for $Cu^{+/+}$, $Cu^{-/+}$ and the control, respectively) and
249 F3 (0.43 and 0.50 for $Cu^{+/+/-/-/-}$, $Cu^{-/+/-/-/-}$, respectively). Still, the F3-$Cu^{+/+/-/-/-}$ treatment was the only
250 one presenting a percent methylated cytosines lower than the Control. In this regard, it is noticeable
251 that while challenged F0 generations ($Cu^{+/+}$ and $Cu^{-/+}$) presented the same mean percent of
252 methylated cytosines (greater than the Control), a decrease in methylation levels was observed at F3
253 only for non-naïve organisms. This suggests that demethylation occurs only in non-naïve organisms
254 and only at the third non-exposed generation. On the other hand, the global methylation remained
255 stable from F0 to F3 generations originating in naïve organisms, i.e. F0-$Cu^{-/+}$ and F3-$Cu^{-/+/-/-/-}$
256 treatments.

257 Gene-specific methylation responses showed that out of the 29 930 gene models available (Orsini et
258 al., 2016), a total of 30 annotated genes were significantly differentially methylated (FDR < 0.05,
259 Table 2) across treatment comparisons (see Table S1 in supportive information for full details on
260 gene-specific methylation differences and statistical summaries).

261 The comparisons made at F0 (i.e. $Cu^{+/+}$ vs. $Cu^{+/-}$, Control vs. $Cu^{+/+}$, Control vs. $Cu^{+/-}$; Figure 3)
262 indicated that all treatments shared two differentially methylated genes: *dynein light chain* and an
263 unannotated gene (Dapma7bEVm645330t). Focusing on annotated genes, it is also interesting to
264 notice that the *Ribosomal protein S6 kinase beta-2* was uniquely differentially methylated when
265 comparing $Cu^{+/+}$ and the Control; the *Nuclear fragile X mental retardation-interacting protein* was
266 differentially methylated when comparing Control with $Cu^{-/+}$ or $Cu^{+/+}$ with $Cu^{-/+}$; the *Lines* gene
267 was differentially methylated exclusively when comparing $Cu^{+/+}$ with $Cu^{-/+}$ (Table 2, Figure 3).
268 Furthermore, when plotting the absolute methylation differences of the genes found differentially
269 methylated, it was clear that the highest median methylation differences occurred when comparing
270 the Control and exposed non-naïve organisms at F0, i.e. Control vs. F0-$Cu^{+/+}$ (Figure 3). On the

other hand, low median methylation differences were generally found for the other comparisons performed (Figure 3), including the comparison between naïve and non-naïve organisms within F0 or F3 (F0-$Cu^{+/+}$ vs. F0-$Cu^{-/+}$ and F3-$Cu^{+/+/-/-/-}$ vs. F3-$Cu^{-/+/-/-/-}$). Regarding F3 in particular, 3 annotated genes were significantly differentially methylated between $Cu^{+/+/-/-/-}$ and $Cu^{-/+/-/-/-}$, namely those coding for the *Dehydrodolichyl diphosphate synthase*, *Gem-associated protein 7* and *Phosphatidylinositol N-acetylglucosaminyltransferase subunit P* (Table 2). Furthermore, naïve and non-naïve F3 organisms shared a single gene (Dapma7bEVm630018t1) differentially methylated relative to the Control (Control vs. F3-$Cu^{+/+/-/-/-}$ and Control vs. F3-$Cu^{-/+/-/-/-}$), while these comparisons presented the highest number of genes significantly differentially methylated: 6 and 7 genes for F3-$Cu^{+/+/-/-/-}$ and F3-$Cu^{-/+/-/-/-}$ vs. Control, respectively (Table 2, Figure 3).

At this stage, the potential existence of DNA methylation inheritance resulting from exposure to elevated Cu levels was investigated. To do so, we focused on the genes differentially methylated from the Control following exposure to Cu at F0 and compared with corresponding patterns found at F3, both for naïve and non-naïve organisms (Table 2 and Figure 4). Regarding non-naïve organisms, it was clear that Cu exposure causes demethylation in four genes at F0, but a transgenerational recovery approaching Control levels is apparent at F3 (Figure 4). Although for the two *dynein light chain* genes Control levels were actually not reached at F3, these levels were even surpassed at F3 for the genes *ribosomal protein S6 kinase beta-2* and Dapma7bEVm029707t1, with this last one presenting significantly different methylation values in F0 and F3 (Table S2, Figure 4). Accordingly, epigenetic transgenerational inheritance can be ruled out for these two genes. On the other hand, the two dynein light chain genes were not differentially methylated when comparing F0 and F3 ($Cu^{+/+}$ vs. $Cu^{+/+/-/-/-}$), thus suggesting transgenerational inheritance of methylation (Table S2, Figure 4).

Interestingly, methylation changes from F0 to F3 generations originating from naïve daphnids did not consistently follow the same patterns previously described for those deriving from non-naïve organisms. In detail, among the five genes differentially methylated from Control in the F0-$Cu^{-/+}$

297 treatment (Table 2), three of them were found to recover to Control methylation levels at F3,
298 namely an unannotated gene (Dapma7bEVm013975t1) and the two *dynein chain* genes previously
299 identified in the analysis of non-naïve daphnids (Figure 4). This recovery to Control levels is
300 confirmed by the absence of these genes from the set found differentially methylated from the
301 Control at F3 (Table 2), complemented by the identification of significant differences in
302 methylation between F3 and F0 ($Cu^{-/+/-/-/-}$ vs. $Cu^{-/+}$; Table S2). Such as for non-naïve organisms (see
303 above), *dynein* genes demethylation was induced by Cu at F0. However, unlike in non-naïve
304 organisms, these genes recovered methylation levels at F3 and differential methylation was
305 observed when comparing F0 and F3 levels, which rules out epigenetic transgenerational
306 inheritance for these genes in the naïve organisms (Figure 4). The same occurred for
307 Dapma7bEV013975t1, denoting hence no transgenerational inheritance for this gene as well. A
308 singular methylation response of naïve organisms was that Cu exposure rather induced
309 hypermethylation at F0 for the genes Dapma7bEV013975t1, Dapma7bEVm630018t1 and *Nuclear*
310 *fragile X mental retardation-interacting* (Figure 4). Remarkably, the gene Dapma7bEVm630018t1
311 and that coding for the *Nuclear fragile X mental retardation-interacting protein* did not recover the
312 methylation levels of the Control by F3. While a demethylation trend was found from F0 to F3 for
313 the gene coding for *Nuclear fragile X mental retardation-interacting protein*, approaching the
314 Control level (Figure 4), its methylation levels were not found to significantly differ when
315 comparing F0 and F3 ($Cu^{-/+}$ vs. $Cu^{-/+/-/-/-}$; Table S2), which suggests epigenetic transgenerational
316 inheritance. This phenomenon is particularly evident however for the gene Dapma7bEVm630018t1.
317 In this case, the hypermethylation caused by the exposure to Cu at F0 was not only kept at F3 but
318 slightly increased (Figure 4), with the absence of significant differences in methylation levels being
319 confirmed when comparing F0 with F3, i.e. $Cu^{-/+}$ vs. $Cu^{-/+/-/-/-}$ (Table S2). Consistently, this gene
320 was indeed identified as differentially methylated with the Control both at F0 and F3 (Table 2).
321
322 **4. Discussion**

The negative impact of Cu towards freshwater organisms and, in particular, *Daphnia* has been relatively well characterized, with studies reporting the toxic effects of Cu exposure on different endpoints, including mortality, reproduction and behaviour (Lahman et al., 2015; Lopes et al., 2005a; Sadeq and Beckerman, 2019; Xie et al., 2006). Despite the molecular mechanisms underpinning toxicity and detoxification pathways remain fairly unexplored, Cu is known to be both cytotoxic and genotoxic by presenting the ability to induce damage to cell membranes, organelles and DNA, which can originate either from direct Cu interaction with biological targets or by the secondary production of reactive oxygen species (ROS), leading to oxidative stress and further negative effects (Anjos et al., 2014; Poynton et al., 2007; Vernon and Jha, 2019). In this regard, increased expression of metallothionein genes and induction of antioxidant enzymes were found in daphnids exposed to Cu, suggesting that these mechanisms are at the forefront of detoxification and defensive pathways (Asselman et al., 2013, 2012; Chain et al., 2019; Poynton et al., 2007).

Interestingly, our results showed that naïve and non-naïve organisms responded differentially to the copper challenge at F0 ($Cu^{+/+}$ and $Cu^{-/+}$) in terms of methylation concerning some genes. In particular, non-naïve F0 organisms showed differential methylation with the Control in one unannotated gene (Dapma7bEVm029707t1) and a gene coding for a ribosomal kinase (*Ribosomal protein S6 kinase beta-2*), while two unannotated genes (Dapma7bEVm630018t1 and Dapma7bEVm013975t1) and a gene coding for *Nuclear fragile X mental retardation-interacting protein* were differentially methylated in naïve F0 organisms. Ribosomal kinase is known to act downstream of the mTOR signalling pathway, which is becoming increasingly recognized as an important mechanism of response to stressors in *Daphnia* by sensing energy and regulating protein synthesis and metabolism (Gomes et al., 2018; Hearn et al., 2020; Liu et al., 2021; Sheng et al., 2012; Song et al., 2016). The *Nuclear fragile X mental retardation-interacting protein* is mainly involved in RNA binding under stress conditions by participating in the formation of stress cell granules, which allows for the rapid modulation of gene expression when cells are subjected to adverse conditions (Laura et al., 2021; Taha et al., 2021). In fact, studies focusing on freshwater and

invertebrate species have widely reported that excess metals are usually accumulated in metal-rich granules, with these mechanisms playing a relevant role in metal detoxification by contributing to prevent the onset of toxicity (Cardon et al., 2019; Eyckmans et al., 2012; Tan and Wang, 2012; Wang et al., 2018).

On the other hand, shared demethylation was found as a response to Cu between naïve and non-naïve organisms at F0 ($Cu^{+/+}$ or $Cu^{-/+}$ vs. Control), namely in two genes coding for dynein light chain proteins. Consistently, increased methylation of both genes was found through the recovery period for both naïve and non-naïve organisms, although never reaching the methylation levels of the Control in F3 non-naïve organisms. Dynein light chain proteins are known to act in microtubule-based movement (i.e. motility of vesicles and organelles along microtubules), while also playing a role in the fate of the spatial distribution of cytoskeletal structures (Kardon and Vale, 2009). Interestingly, the cytoskeletal components of invertebrates are known to be key targets for toxics, with cytoskeletal protection being suggested to be a general mechanism for increased tolerance to environmental stressors (Banni et al., 2016; Gómez-Mendikute and Cajaraville, 2003; Negri et al., 2013). In agreement, previous studies found evidence that metals could bind directly to cytoskeletal proteins causing their denaturation, while ROS also have the ability to damage cytoskeletal components (Gómez-Mendikute and Cajaraville, 2003; Matozzo et al., 2001). Besides, major changes on genes and enzymes responsible for exchanging substance across cell membranes have been reported following exposure to Cu, thus resulting in metabolic alterations; therefore, changes in metabolism may also occur in order to provide the cell energy to mitigate the immediate toxic effects of Cu (Chain et al., 2019; Letelier et al., 2005).

Taking the above findings into account, the differential methylation found when comparing F3 ($Cu^{+/+/-/-}$ or $Cu^{-/+/-/-}$) with the Control should be further discussed. In fact, differential methylation of one annotated gene (Dapma7bEVm630018t1) was shared between naïve and non-naïve organisms at F3, but different methylation responses in such groups occurred in genes mostly involved in the transport of ions, amino acids and phospholipids across the membrane, as well as in

energy metabolism, protein stabilization and microtubule binding and stability (see gene annotation in Table 2). Also interesting, the direct comparison of the F3 groups ($Cu^{+/+/-/-/-}$ vs. $Cu^{-/+/-/-/-}$) showed differential methylation between naïve and non-naïve organisms in a gene coding for a spliceosomal Gem-associated protein, which belongs to a family that is recognized to be involved in alternative mRNA splicing events, namely through exon skipping (Berger et al., 2016; Jong et al., 2008). In fact, differential methylation has been previously associated with alternative splicing events in *Daphnia* after response to *Microcystis aeruginosa* and salinity exposure, being also reported that the transcriptome diversity of different clonal lineages of *Daphnia* exposed to acute Cu concentrations is modulated by alternative splicing events (Asselman et al., 2017; Jeremias et al., 2018b; Suresh et al., 2020). Our results add that the past history of exposure may play the same role as genetic diversity in the onset of alternative splicing events considering that we used the same clone to produce naïve and non-naïve organisms within the experiment.

Overall, the results suggest that DNAm changes resulting from Cu exposure in *Daphnia* are being primarily targeted to genes with important roles in counteracting both the direct effects of Cu and indirect effects resulting from oxidative stress (through a definitive confirmation would require the functional characterization associated to these genes), thus potentially allowing organisms to better cope with this stressor throughout the successive generations (Jeremias et al., 2018b; Trijau et al., 2018), and this clearly implies the role of DNAm as a molecular initiating event for mechanisms of detoxification and adaptation to metal contamination in *Daphnia*. This rationale is further supported by the results of the life-history parameters since a significant increase in population growth and net reproductive rate was observed from F0 to F3 generations of both naïve and non-naïve organisms (note that the F3 performance exceeds, often significantly, the performance of the control regardless of the treatment), showing that the methylation changes are potentially beneficial to withstand the toxic challenge without life-history costs.

There are few studies exploring the effects of Cu exposure on the epigenetic machinery of invertebrates. Most of these focused on soil organisms, with some reporting no epigenetic changes

after Cu exposure while others found changes in overall methylation levels (Bicho et al., 2021, 2020; Noordhoek et al., 2018). We found very small numbers of differentially methylated genes regardless of the comparison in place (see Table 2), especially taking into consideration the higher number of gene-specific methylation changes found in previous multigenerational studies in *Daphnia* that monitored non-exposed generations until the F3 (Jeremias et al., 2018b; Trijau et al., 2018). Still, the present findings are in accordance with previous gene expression studies exploring the effects of metal exposure in *Daphnia*, as a small number of significantly affected genes and very specific toxicant-specific mRNA expression patterns have been consistently reported (Asselman et al., 2013, 2012; De Coninck et al., 2014; Poynton et al., 2008, 2007). The fact that metal exposure induced a small number of gene-specific methylation changes in *Daphnia* highlights a certain degree of specificity in the observed methylation responses that is consistent with previous findings resulting from gene expression studies, thereby suggesting the interplay between the epigenetic and genetic machinery towards mediating the response to metal exposure (Asselman et al., 2013, 2012). Interestigly, in the zebrafish (*Danio rerio*) and Pacif Oyster (*Crassostrea gigas*), two other relevant species for aquatic toxicology research, epigenetic and corresponding genetic changes have been shown to occur due to copper exposure (Dorts et al., 2016; Sussarellu et al., 2018; Tai et al., 2022). Specifically, Tai et al. (2022) showed that Cu exposure in the zebrafish induced alterations in the sperm methylome that were directly associated to changes in gene expression, and that both of these were passed down to the fertilized offspring and ultimately lead to embryonic developmental defects. Furthermore, it is noteworthy that previous studies exploring the use of gene expression profiling to identify novel biomarkers of Cu toxicity towards *D. magna* found several candidates in genes that possess the same biological categories and molecular functions of the ones reported here, namely regarding metal and oxidative stress detoxification and metabolism processes (Table 2; Poynton et al., 2008, 2007). Taken into consideration that gene body methylation, which is by far the main methylation type in invertebrates and *Daphnia* in particular, is known to be associated with higher gene expression levels, there is a likely connection between the observed methylation

changes and phenotypic effects resulting from Cu exposure, thus supporting the development of epigenetic biomarkers (Jeremias et al., 2020; Olson and Roberts, 2014; Rivière, 2014). In this regard, the future analysis of gene expression changes and higher level organismal effects resulting from the observed methylation changes would be of primary importance for further validating the suitability and usefulness of developing DNAm biomarkers in this context (Kvist et al., 2018; Suarez-Ulloa et al., 2015; Torres et al., 2021). Moreover, the analysis of the detected DNAm changes under more realistic scenarios, such as exposure to multiple and different groups of stressors, would constitute an important step towards appraising the adequacy of such potential epigenetic biomarkers (Gao, 2021; Jeremias et al., 2020; Shaw et al., 2017). In this context, methylation-specific PCR could be a valuable technique by allowing the rapid and sensitive assessment of the methylation status of the small number of detected genes under the most different scenarios of exposure (Ku et al., 2011; Ramalho-Carvalho et al., 2018). An increasing amount of studies have been reporting epigenetic transgenerational effects in invertebrates. Yet, many of these epigenetic effects are more likely the result of direct environmental exposures, thus assessing rather the intergenerational epigenetic effects (Heard and Martienssen, 2014; Skinner, 2011). Meeting these demands, previous works showed the inheritance of hypomethylation in a small number of genes following exposure of *Daphnia* to salinity and likely inheritance of hypermethylation after a 25-day γ irradiation (Jeremias et al., 2018b; Trijau et al., 2018). In our study, potential transgenerational hypomethylation inheritance was found for two *dynein light chain* genes in non-naïve organisms. On the other hand, the *Nuclear fragile X mental retardation-interacting protein* and Dapma7bEVm630018t1 showed potential for hypermethylation inheritance in naïve organisms since F0 and F3 methylation levels were both higher than the Control, although significant methylation differences between F3 and the Control were only observed for the last one. Specificially, the transgenerational methylation patterns observed for the gene Dapma7bEVm630018t1 seems to represent the stable germline transmission of differential methylation regions as a result of Cu exposure. This thus adds to an increasing body of literature

reporting the occurrence of differential DNA methylation regions following a variety of toxicant ancestral exposures and their transgenerational inheritance in a toxic specific manner, thereby highlighting the potential of such signatures to provide potential biomarkers for past exposures (e.g. Carvan et al., 2017; Jeremias et al., 2020; Skinner, 2015). Also, similarly to the findings of previous studies, no life-history costs seemed to be associated with this epigenetic transgenerational inheritance phenomenon (Figure 2; Jeremias et al., 2018b; Trijau et al., 2018). Taken together, these results suggest the existence of epigenetic transgenerational inheritance as a result of Cu exposure in *Daphnia*, as well as confirm the occurrence of different transgenerational methylation responses among organisms differing in their life-histories of exposure to Cu (i.e. naïve and non-naïve organisms), reinforcing the potential contribution of epigenetic mechanisms to shape the adaptive capacity of freshwater and invertebrate organisms facing metal contamination, and further supporting the incorporation of epigenetic biomarkers and heritability into risk assessment frameworks (Athanasio et al., 2018; Jeremias et al., 2020, 2018a; Shaw et al., 2017; Šrut, 2021).

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827 **Figure captions**

828 **Figure 1.** Experimental design comprising the acclimation stage (cultures maintained in Cu-
829 enriched or blank ASTM for three generations) to simulate differential contamination history, the
830 exposure stage (F0) and the recovery stage (F1-F3). Arrows represent the flow of 3rd brood neonates
831 from precedent cultures; white boxes represent clean culture medium (blank ASTM) and grey boxes
832 represent the EC20 for Cu (0.021 mg/L) dissolved in ASTM. Plus and minus signs indicate on the
833 history of organisms regarding the present and past culturing in a Cu-enriched medium (+) or in
834 blank ASTM (−). Red box borders indicate the treatments from which DNA was extracted for
835 reduced representation bisulfite sequencing.

836

837 **Figure 2.** Mean population growth (A), net reproductive rate (B) and age at first reproduction (C),
838 with corresponding standard errors, across generations and treatments. Grey and white bars
839 represent, respectively, non-naïve (acclimated in Cu-enriched ASTM) and naïve (acclimated in
840 blank ASTM) generations, while blue bars stand for the Control. Significant differences among
841 treatments within F0 generations (F-test or Tuckey test; $p < 0.05$) are assigned with low-case letters,
842 while significant differences between generations within treatment (Tukey test; $p < 0.05$) are
843 assigned using uppercase letters.

844

845 **Figure 3.** Overview of different methylation patterns found across experimental treatments and
846 generations. Figure A and B: Venn diagrams overlapping the number of genes differentially
847 methylated (FDR < 0.05) when comparing F0 treatments and F3 treatments with the Control,
848 respectively (gene annotations can be found in Table 2)-. Figure C: Absolute methylation
849 differences for genes differentially methylated (FDR < 0.05) considering the comparisons
850 addressed with the Venn diagrams above plus that between naïve and non-naïve organisms at F3.

851

852 **Figure 4.** Changes in methylation levels from F0 to F3 generations (lines added for better visibility)
853 for genes found differentially methylated from the control (FDR < 0.05) in non-naïve and naïve
854 organisms at F0 following Cu exposure (Table 2), with * indicating significant differences in
855 methylation levels (FDR < 0.05) between F0 and F3 generations (Table S2). Error bars represent
856 the standard error of the differences.
857

858 **Tables**

Table 1. ANOVA summaries regarding the effects of treatment within generation or the effects of generation within each treatment in life-history parameters - population growth (r), net reproductive rate (R0) and age at first reproduction (AFR) considering Cu and blank ASTM acclimated organisms. Significant comparisons (p-value < 0.05) are highlighted in bold.

Source of variation	Life-history traits	Degrees of freedom	MS factor	MS error	F ratio	P-values
Cu within F0 (levels: Ctr, $Cu^{+/+}$, $Cu^{-/+}$)	r	2, 6	0.0007008	0.00007037	9.958	**0.0124**
	R0	2, 6	103.9	14.63	7.103	**0.0262**
	AFR	2, 6	1	2	0.5000	0.6297
Cu within F1 (levels: $Cu^{+/+/-}$, $Cu^{-/+/-}$)	r	1, 4	0.0001886	0.0001842	1.024	0.3688
	R0	1, 4	178.5	49.69	3.593	0.1309
	AFR*	--	--	--	--	--
Cu within F2 ($Cu^{+/+/-/-}$, $Cu^{-/+/-/-}$)	r	1, 4	0.0003033	0.0002444	1.241	0.3277
	R0	1, 4	26.04	22.16	1.175	0.3393
	AFR	1, 4	0.6667	0.6667	1	0.3739
Cu within F3 ($Cu^{+/+/-/-/-}$, $Cu^{-/+/-/-/-}$)	r	1, 4	$6.080x10^{-5}$	$6.725x10^{-5}$	0.9042	0.3955
	R0	1, 4	1.793	11.15	0.1608	0.7089
	AFR	1, 4	0.6667	0.6667	1	0.3739
Generation within naïve cultures (levels: F0, F1, F2, F3)	r	3, 8	0.004079	0.0001236	33.01	**<0.001**
	R0	3, 8	518.7	24.24	21.40	**<0.001**
	AFR	3, 8	0.5278	1.417	0.3725	0.7752
Generation within non-naïve cultures (levels: F0, F1, F2, F3)	r	3, 8	0.002588	0.0001483	17.45	**<0.001**
	R0	3, 8	238.3	26.50	8.995	**0.0061**
	AFR*	--	--	--	--	--

859 * Not enough variability within replicates to perform ANOVA for AFR

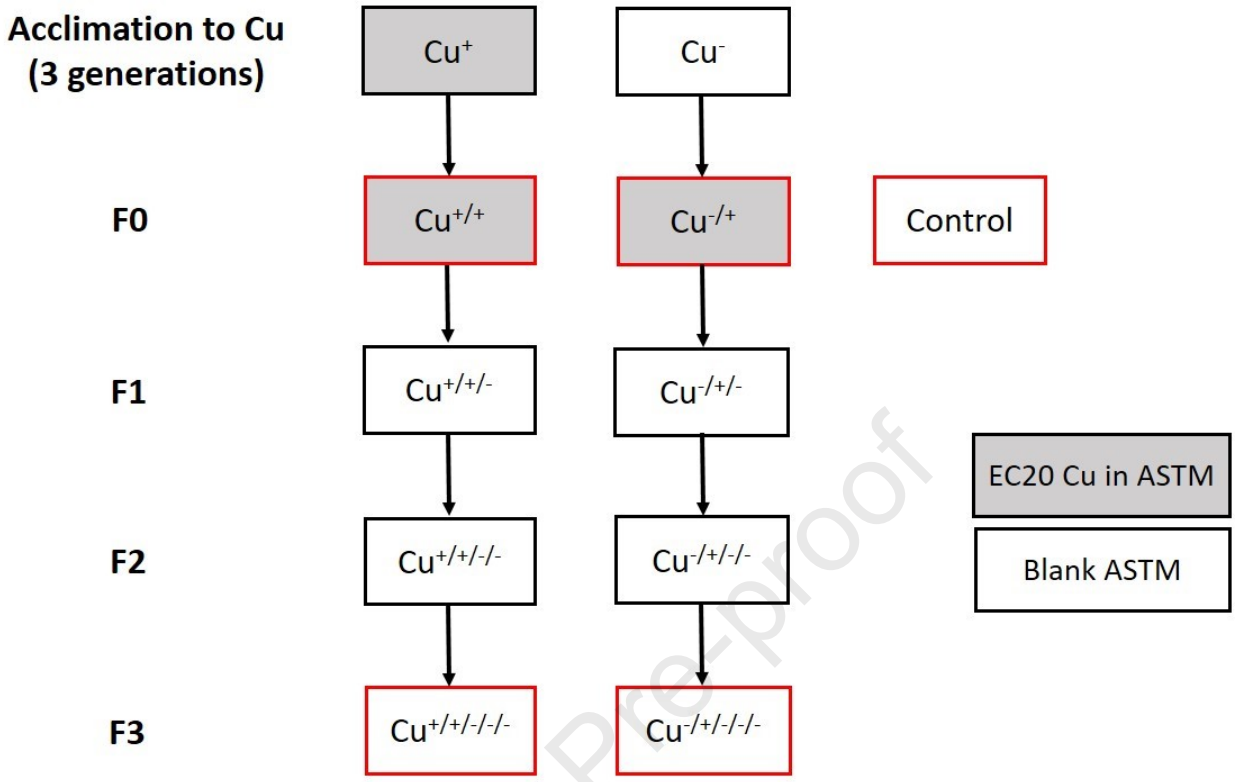
860

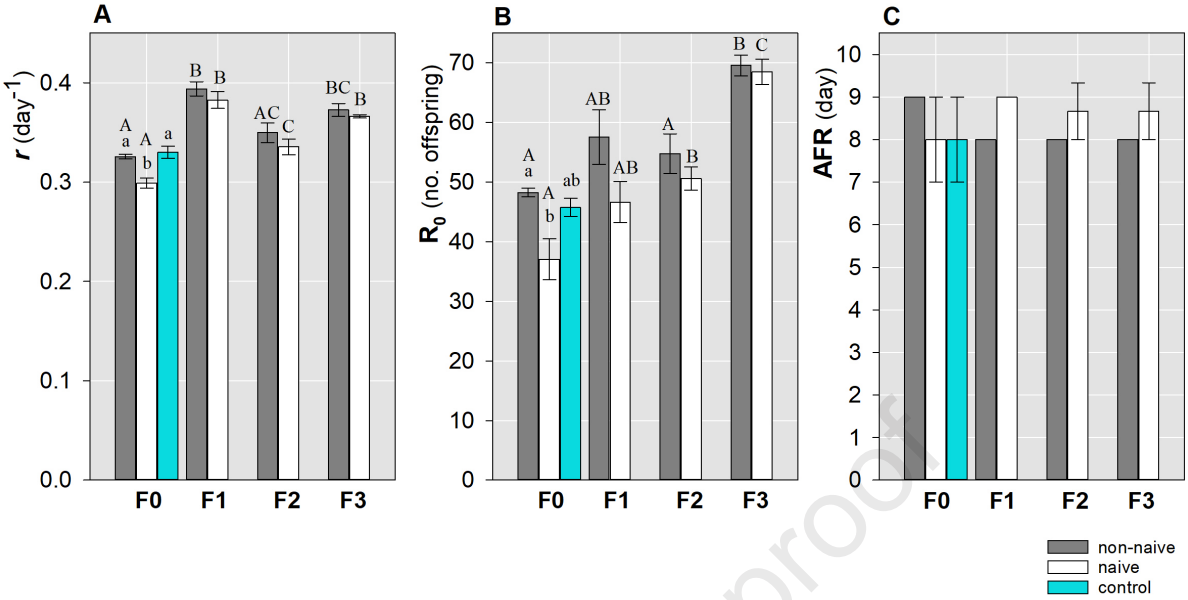
861

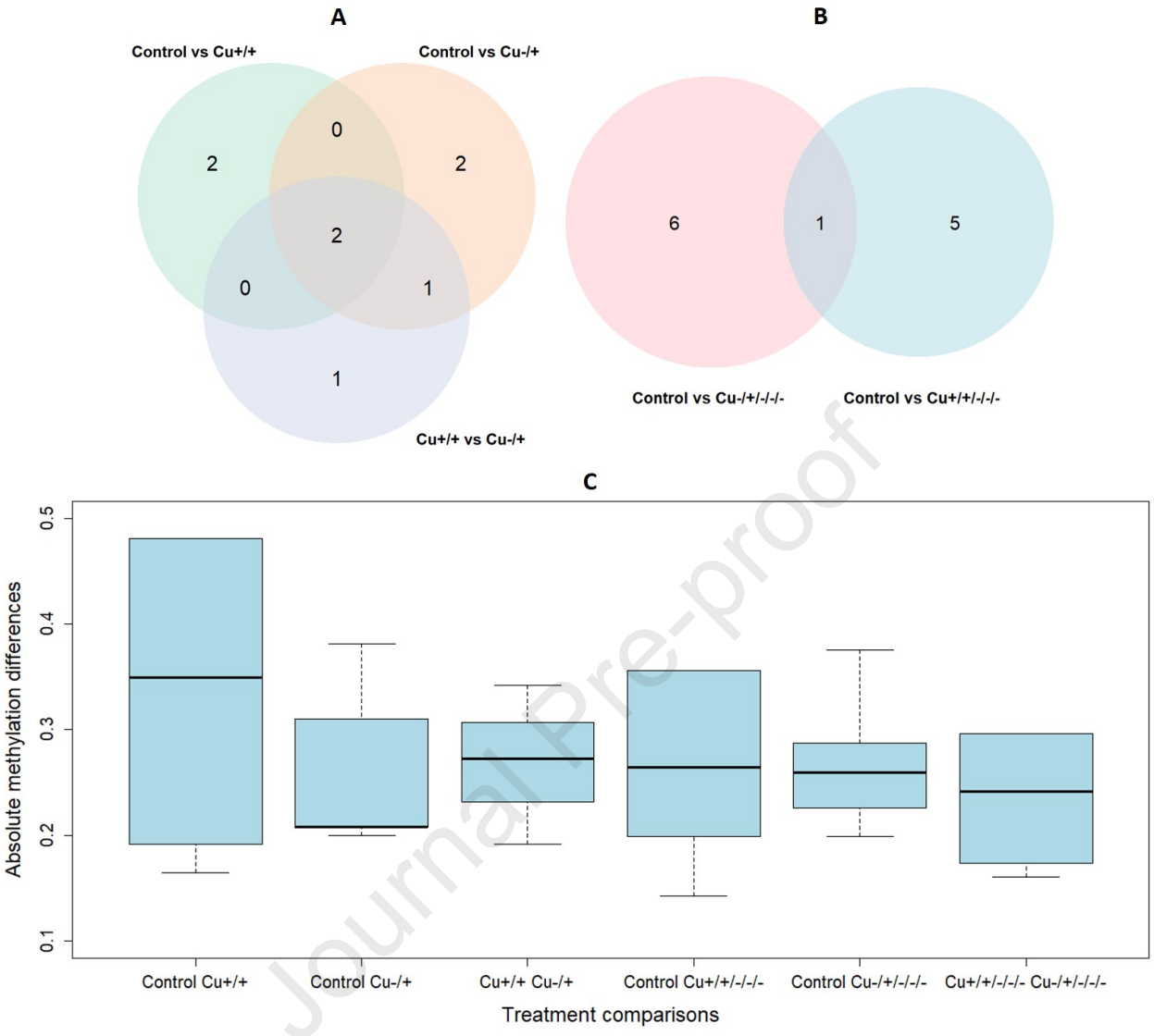
Table 2. Number and annotation/geneID of genes differentially methylated (FDR < 0.05) between the most relevant sequenced treatments, with NA followed by the standard geneID in the reference genome standing for genes without annotation. Details on methylation and statistics can be found in supplementary table S1.

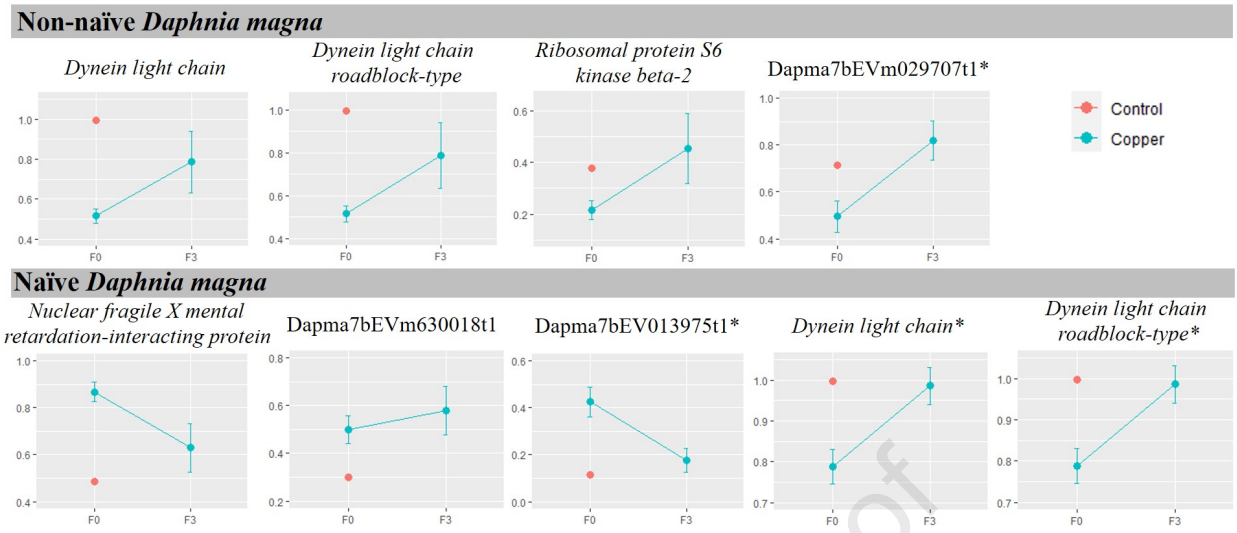
Cu Treatments compared	**Differentially methylated genes**	
	No.	**Annotation**
Control vs. $Cu^{+/+}$ [F0]	4	*Ribosomal protein S6 kinase beta-2*; *dynein light chain roadblock-type*; Uncharacterized protein (Dapma7bEVm029707t1); *dynein light chain*
Control vs. $Cu^{-/+}$ [F0]	5	*Nuclear fragile X mental retardation-interacting protein*; *dynein light chain roadblock-type*; Uncharacterized protein (Dapma7bEVm013975t1); NA (Dapma7bEVm630018t1); *dynein light chain*
$Cu^{+/+}$ vs. $Cu^{-/+}$ [F0]	4	*Lines*; *Nuclear fragile X mental retardation-interacting protein*; *dynein light chain roadblock-type*; *dynein light chain*
Control vs. $Cu^{+/+/-/-}$ [F3]	6	*V-type proton ATPase subunit D*; *Phosphatidylinositol N-acetylglucosaminyltransferase subunit P*; *Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase*; *DNL-type zinc finger protein*; NA (Dapma7bEVm630018t1); NA (Dapma7bEVm645458t1)
Control vs. $Cu^{-/+/-/-}$ [F3]	7	*Sideroflexin-4*; *Phospholipid scramblase*; *Dynactin subunit*; Uncharacterized protein; *Phospholipid scramblase*; NA (Dapma7bEVm630018t1); *Dynactin 5*
$Cu^{+/+/-/-}$ vs. $Cu^{-/+/-/-}$ [F3]	4	*Dehydrodolichyl diphosphate synthase*; *Gem-associated protein 7*; *Phosphatidylinositol N-acetylglucosaminyltransferase subunit P*; NA (Dapma7bEVm645458t1)

862









Highlights

- Direct and inherited effects of Cu in DNA methylation of *Daphnia* were explored
- Methylation changes targeted genes that offset metal toxicity and oxidative stress
- Distinct methylation effects noticed in daphnids differing in Cu exposure history
- Exposure history promoted transgenerational inheritance in a specific manner

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: