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Exposure to copper during larval development has intra- and trans-generational influence on fitness in later life

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ABSTRACT

Anthropogenic pollution has a disadvantageous influence on various life-history traits. Although direct effects are well known, potential fitness-related trans-generational costs are less studied. Previously, empirical findings have demonstrated that environmental conditions faced by the parental generation have an effect on the traits expressed by their offspring. Here, to study this conjecture larvae of the common fruit fly (*Drosophila melanogaster*) were either exposed to a sub-lethal concentration of copper or reared on uncontaminated larval medium. Adult flies were kept under uncontaminated conditions. For the next generation, individuals were mated with their own group and their offspring were either exposed to copper or fed with uncontaminated larval medium. We found that in the parental generation copper exposure reduced fecundity compared with uncontaminated controls. In the progeny, females suffered impaired fecundity only if their larval condition differed from the conditions experienced by their parents. If the progeny was raised under similar conditions than the parental generation, no effect on fecundity was discovered, suggesting acclimatization to the prevailing conditions after short-time copper exposure (two generations). Our results demonstrate that exposure to an environmental stressor like heavy metals causes intra-and trans-generational fitness costs. Further, individuals may be able to acclimatize in prevailing contaminated conditions, but this might in turn debase fitness under uncontaminated conditions. Our findings are consistent with the prediction of the adaptive parental effects hypothesis which states that parents may produce offspring that are more successful under conditions faced by their parents.

1. Introduction

Heavy metal pollution caused by anthropogenic action is an increasing problem all over the world. Especially in the vicinity of metal industry concentrations found in nature can reach notably high levels (Fonseca et al., 2013; Spurgeon et al., 1994). Amounts discovered in organisms are found to correlate positively with those measured in ambient environment (Laskowski, 1991), which indicates that heavy metals can easily transfer from environment to organisms. Especially non-essential heavy metals are thought to accumulate in body tissues, whereas vital trace elements such as surplus copper are suggested to be more easily processed (Hunter and Johnson, 1982; Jinhui et al., 2019; Timmermans and Walker, 1989). Even though copper is essentially needed (Harrison et al., 2000; Walker et al., 2006), high concentrations are harmful and can have disadvantageous impact on expressed traits. Several previous studies have demonstrated the immediate effects of heavy metal exposure on various life-history traits such as prolonged development time, smaller individual size and alteration of immune system activity (Al-Momani and Massadeh, 2005; Donker, 1992; Moe et al., 2001; Plachetka-Bozek et al., 2019; Simkiss et al., 1993; Sorvari et al., 2007; Wu and Yi, 2015; Zidar et al., 2004). Furthermore, previous studies have found evidence for the direct fitness costs of experienced heavy metal exposure. Individuals exposed to metal suffer debased fertility and reproductive success (Moe et al., 2001; Spurgeon et al., 1994). It has been addressed that detoxification enzymes, which are essentially needed for processing disadvantageous compounds (Dubovskiy et al., 2011; Wu and Yi, 2015), restrain valuable resources (Calow, 1991; Posthuma and van Straalen, 1993) which is why less can be allocated to other functions. As a result, this can lead to a reduction in fitness or other traits.

As explained above, environmental conditions faced by individuals have an effect on their performance. Under stressful conditions, individuals are thought to allocate resources between costly features (Sheldon and Verhulst, 1996). This kind of trade-off between different

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functions has formerly been documented in a variety of species (Lazzaro et al., 2009). Previous studies have demonstrated the direct costs of different life-history traits under conditions in which costs could not be compensated. For example, in the study by Moret and Schmid-Hempel (2000) those bumblebee (*Bombus terrestris*) workers whose immunity was activated expressed weaker survival under starved conditions, suggesting an increase in energy demand due to immune activation. Similarly, Freitak et al. (2003) discovered that immune activation of the white cabbage butterfly (*Pieris brassicae*) during the pupal stage had an influence on standard metabolic rate. This finding also supports the notion that increased expenses of a costly trait can be covered from other functions. Even though there is quite well documented evidence for the direct influence of costly environmental stress on different life-history traits, the potential trans-generational effects have not gained attention until recently.

In addition to the fact that experienced conditions have a direct impact on a variety of life-history traits, the influence of conditions faced by the parents may reach to the next generations. Indeed, an increasing number of studies report that environmental conditions faced by the parental generation have an impact on distinct life-history traits expressed by their offspring (e.g. Crill et al., 1996; Kristensen et al., 2008; Pölkki et al., 2012; Sadd et al., 2005; Valtonen et al., 2012; Vijendravarma et al., 2010). According to the 'adaptive parental effects hypothesis' parents reared under an environmental stressor produce offspring that are better fit to those conditions experienced by their parents, whereas the 'parental stress hypothesis' predicts that stress may result in reduced offspring quality (Mousseau and Fox, 1998; Vijendravarma et al., 2009). Nevertheless, in the light of growing evidence, it seems that experienced environmental conditions can produce epigenetic adjustment (Bonduriansky and Head, 2007). It has been suggested that such trans-generationally transmitted changes in phenotype are transmitted to the next generation through non-genetic inheritance that regulates genome activity. Such mechanisms are, for instance, DNA methylation, histone modifications and many others which are considered to be epigenetic factors (Skinner and Guerrero-Bosagna, 2009). As mentioned earlier, an upregulated immune system requires energy (Freitak et al., 2003; Moret and Schmid-Hempel, 2000; Rolff and Siva-Jothy, 2003) as well as detoxification processes that also demand resources (Widdows and Donkin, 1991). This could lead to the assumption that under stressful conditions exposure to heavy metals might result in fitness-related trade-offs in reproductive success through resource allocation between costly traits, which could potentially be trans-generationally transmitted to the next generation.

In this study, we have experimentally tested potential fitness-related costs of larval heavy metal exposure on adult reproductive success by testing fecundity of individuals exposed to a sub-lethal concentration of copper. We have chosen larval exposure, since they are less mobile and their diet is likely to consist of single source which is why they are more likely to suffer heavy metal exposure than adults (Shorrocks, 1975). In addition, based on the findings of previous studies, surplus copper can be depleted from body tissues (Timmermans and Walker, 1989). Therefore, we expect that larval exposure is no longer apparent in adults but that the potential effects observed here are likely based on trade-offs between costs suffered during early development. We have examined possible intra- and trans-generational effects by rearing the parental generation of the common fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae; Meigen, 1830) either on food supplemented with copper or on uncontaminated larval medium. The next generations of the two treatments were either reared on contaminated or uncontaminated medium. Fecundity was determined by counting the produced eggs of females within each treatment (Simmons and Bradley, 1997).

2. Materials and methods

2.1. The stock population

This work was done by using wild originated laboratory stock flies which were captured from Rauma, Finland, in 2006. The stock comprises thousands of individuals maintained under controlled light (12 L: 12 D) and temperature (23 ± 1 °C) conditions. Stock jars (2 L) contained 200 ml of larval medium (1 L distilled water, 80 g semolina, 10 g agar, 20 g brewer' yeast, 150 g syrup and 7.5 ml nipagin) with yeast culture on top for adults.

2.2. The parental generation and creating the next generation

This study was conducted during autumn 2012 when altogether 600 virgin flies (300 males and 300 females) were randomly collected from the stock. Three-day-old adults were allowed to mate and lay eggs for 24 h in a cage containing vials of larval medium with yeast topping. Eggs (parental generation) were collected into 30 ml vials containing 10 ml of larval medium supplemented with either distilled water (uncontaminated condition) or 50 μg of copper sulphate solution per 1 g of larval medium (contaminated condition; Sigma Aldrich®, copper $CuSO_4$ 10.00 g for 1000 ml standard solution, diluted in deionized water). Altogether, 22 vials were prepared for each treatment (uncontaminated and contaminated). Each vial contained 20 eggs (altogether 440 individuals per treatment). At the time of eclosion, adult flies were collected every 6 h (three times per day) during the light period into vials containing 5 ml of larval medium and yeast culture. Males and females were collected separately to ensure virginity. To ease handling, flies were anesthetized with CO_2 and ice. At the age of three days, flies from both treatments were either used for testing fecundity (one pair from each vial, see the next chapter) or for generating the next generation (F1, see the next paragraph).

The rest of the flies (not used in fecundity test) from both treatment groups were used to produce the next progeny. Flies from both treatment groups were moved into separate cages and were allowed to mate. Cages contained larval medium vials with yeast for egg laying. After 24 h eggs were collected and moved into 30 ml vials containing 10 ml of either uncontaminated food or contaminated food supplemented with a sub-lethal concentration of copper (see above). So that, eggs from uncontaminated parents were moved either onto contaminated food (treatment control; henceforth referred to as: 0→50) or uncontaminated food (hence: control, 0→0) and similarly eggs from copper contaminated parents were placed either onto copper containing food (50→50) or uncontaminated larval food (50→0). When the flies started to hatch they were checked every 6 h during the light period and adults were placed into 30 ml vials containing 5 ml of larval medium with yeast culture (see above).

2.3. Measuring development time and fecundity

For both generations development time was calculated by subtracting the time elapsed between oviposition and adult eclosion. Hatching adults were checked every 6 h during the light period until eclosion ceased. In both generations, development time was determined from randomly selected vials for each treatment (parental generation: uncontaminated 15 vials and contaminated 15 vials; the next generation: uncontaminated 10 vials and contaminated 10 vials). Individuals' size was determined by measuring thorax length of adult flies under a light microscope with an ocular micrometer.

At the age of three days flies were subject to fecundity tests. Randomly selected female and male flies from (one pair from each of the original vial) the same treatment were paired and put into vials containing larval medium with yeast (in the parental generation, yielding 22 pairs per treatment; in the next generation 30–34 pairs per treatment). After copulation, males were removed and females were allowed

to lay eggs for 24 h (Okada et al., 2011; Simmons and Bradley, 1997). Removal of the male fly was made without anesthetization. The number of eggs produced by each female was determined under a light microscope by counting the eggs twice. Fecundity was tested similarly for both generations. Some of the pairs in both generations did not copulate and hence, pairs were excluded from the experiment.

2.4. Statistical analysis

Normality of variables was estimated with the Shapiro-Wilks test and homogeneity was examined with Levene's test. Because fecundity has been found to correlate with female size (Honěk, 1993), we corrected fecundity for size by regressing the number of produced eggs (fecundity) against female size; the residuals were tested for normality and used as a dependent variable in further analyses. In the parental generation, differences between treatment groups were analyzed with Independent Samples *t*-test. In the next generation, one-way ANOVA was used to compare fecundity between the treatment groups. Because equality of variances was not fulfilled (the next generation), statistics were reported using the Brown-Forsythe correction. In further pairwise comparisons statistical tests in which equality is not assumed were shown (Independent Samples *t*-test).

Development time was analyzed by using Cox regression (Cox proportional hazards regression) and further pairwise comparisons were conducted with Kaplan-Meier analysis. Parental exposure, exposure of the offspring and sex were used as categorical covariates. Non-significant interactions were removed from the final model. Results of all pairwise comparisons were Bonferroni corrected by multiplying received *P*-values with the number of groups for controlling the type I error. All tests were made with IBM SPSS Statistics 21 for WINDOWS.

3. Results

3.1. Fecundity and development time of the parental generation

Continuous exposure to copper during larval development had an effect on the fecundity of adult flies (*t*-test: t = 3.362, df = 42, P = 0.002). Individuals exposed to copper during their larval development produced fewer eggs as adults than the uncontaminated control group (Fig. 1, Table 1).

When analyzing development time, no significant interaction between copper exposure and sex (OR = 0.992, Wald = 0.002, df = 1, $P < 0.965$) was found. Individuals exposed to copper developed slower compared to the uncontaminated control group (OR = 6.515, Wald = 129.168, df = 1, $P < 0.001$, Fig. 2). The influence of sex on development time was marginally significant (OR = 1.292, Wald = 3.646, df = 1, $P < 0.056$). There was a trend of females developing faster than males (Fig. 2, Table 1).

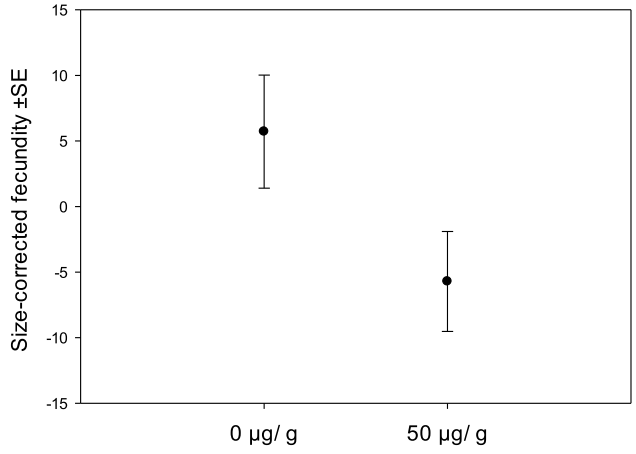

Fig. 1. Size-corrected fecundity (mean number of eggs ±SE) of the parental generation. Females reared either on contaminated (50 µg/g) or uncontaminated (0 µg/g) food.

Table 1
Absolute values of fecundity and development time for the two generations. Fecundity measured for females.

Copper (µg/g)	sex	mean	SE	SD	*n*
Parental generation					
Fecundity (mean number of eggs)					
0		102.66	3.799	20.458	19
50		88.41	2.629	13.659	19
Development time (in days)					
0	♀	9.80	0.033	0.357	13
	♂	9.91	0.033	0.396	13
50	♀	10.84	0.053	0.561	15
	♂	11.07	0.053	0.558	15
The offspring					
Fecundity (mean number of eggs)					
0→0		98.57	2.843	15.573	29
0→50		84.32	6.015	30.073	24
50→0		80.33	5.999	29.390	24
50→50		94.19	2.493	16.160	34
Development time (in days)					
0→0	♀	9.79	0.036	0.346	10
	♂	10.13	0.047	0.451	10
0→50	♀	10.90	0.081	0.722	10
	♂	11.17	0.073	0.661	10
50→0	♀	9.93	0.036	0.370	10
	♂	10.26	0.039	0.355	10
50→50	♀	11.27	0.054	0.570	10
	♂	11.53	0.061	0.054	10

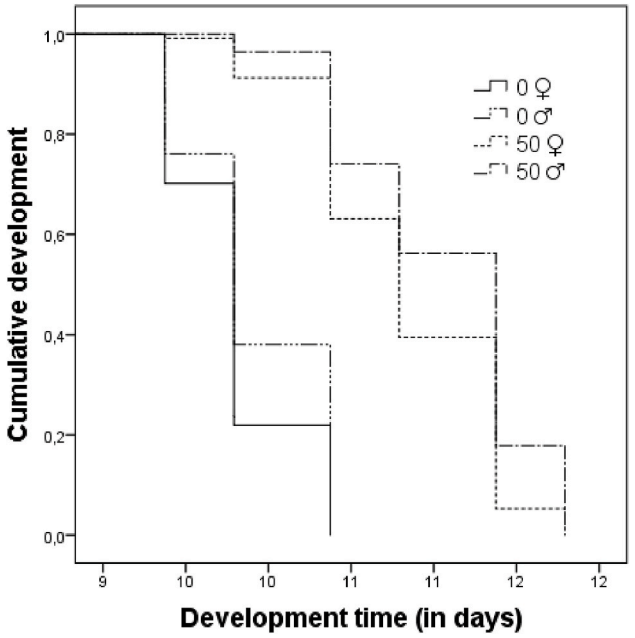

Fig. 2. Cumulative development time of males and females of the parental generation reared either on copper contaminated (50 µg/g) or uncontaminated (0 µg/g) larval medium.

3.2. Fecundity and development time of the next generation

Treatment groups differed from each other in their fecundity ($F_{3,70} = 3.677$, P = 0.016). Parental exposure to copper had a trans-generational effect on the fecundity of their offspring (0→ 0 vs. 50→0: t = 2.912, df = 51, P = 0.005). Individuals whose parents were exposed to copper produced significantly less eggs even if they were reared on uncontaminated larval food compared to the uncontaminated control group

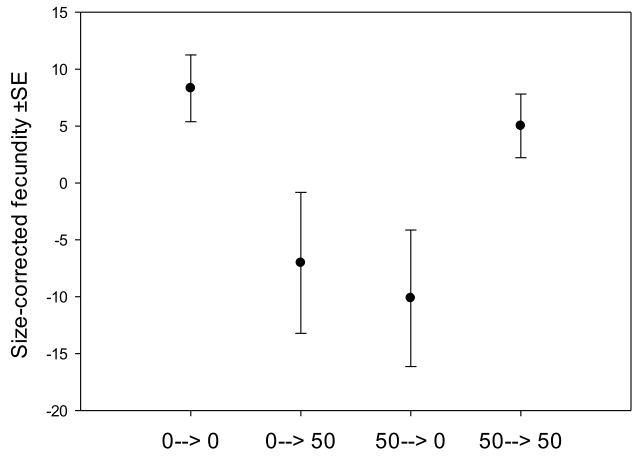

Fig. 3. Fecundity (mean number of eggs ± SE) of the next generation reared either on contaminated (50 μg/g) or uncontaminated (0 μg/g) larval food, and whose parents were reared either on uncontaminated (0 μg/g) or contaminated (50 μg/g) larval food.

(Fig. 3). However, the controls did not differ from the group that was reared two generations under copper contaminated food (0→0 vs. 50→50: t = 0.813, df = 61, P = 0.419). Individuals reared two generations under contaminated food produced as many eggs as those reared on uncontaminated larval food (Fig. 3, Table 1). As expected, treatment controls differed from the controls in their fecundity (0→0 vs. 0→50: t = 2.363, df = 51, P = 0.022).

For the final model of development time, all non-significant factors were excluded. A significant interaction between exposure of the progeny and sex was discovered (OR = 1.552; Wald = 8.324; df = 1, *P* = 0.004). Parental exposure (OR = 1.338; Wald = 14.746; df = 1, *P* = <0.001) as well as direct exposure (OR = 6.181; Wald = 190.740; df = 1, *P* = <0.001) had an effect on the progeny's development time (Table 1). Also sexes differed significantly from each other in their development time (OR = 1.319; Wald = 6.608; df = 1, *P* = 0.010), females developed faster than males (Table 1) except for the treatment control group (0→50), in which the difference in development time between males and females was not significant (Table 2). Because of the sex difference, further multiple comparisons were conducted separately for each of the sexes. Among both females and males, all treatment groups (0→50, 50→50) differed in their development time when compared with the control group (0→0), except for the treatment group 50→0 (Fig. 4a and b). In addition, a difference in development time between groups 0→50 and 50→50 was detected (Table 3).

4. Discussion

4.1. Fecundity and development time of the parental generation

We discovered that exposure to copper had an adverse effect on fecundity of the parental generation. Individuals exposed to copper during their larval development produced fewer eggs as adults compared with the uncontaminated control group. Our findings are in line with earlier studies in which exposure to heavy metals was found to cause fitness-related costs (Moe et al., 2001; Spurgeon et al., 1994). However, the mechanism at work has remained unclear. Nevertheless, heavy metal exposure during larval development comes with direct costs, since the processing of harmful compounds consumes valuable resources (Widdows and Donkin, 1991) which is why there might be fever resources remaining, for example, for the development of ovarioles. In addition, in our previous study we discovered that larval exposure to a sub-lethal copper concentration upregulates immune activation as adults (Pölkki et al., 2012). Since maintenance of the immune system consumes energy (Valtonen et al., 2010), one could expect that the upregulation of immunity due to heavy metal exposure might also be costly, and expenses of this costly trait would be covered by allocating resources from other functions. Based on these arguments, one would presume that some resources are no longer available for reproduction.

As expected, individuals of the parental generation who were reared on copper contaminated larval medium developed more slowly compared with the ones kept on uncontaminated control medium. The most likely explanation for the longer development time is the direct toxic effects of surplus copper. Detoxification processes as well as handling the disadvantageous compounds requires energy (Widdows and Donkin, 1991). This kind of energy demand must be allocated from other traits especially under conditions where resources are somehow limited, which is the most likely case here. Since, copper was received via larval diet, which is why concentrations in body tissues are likely to increase with food consumption. Therefore, under contaminated conditions this may result in longer development time and/or resource allocation between costly life-history traits.

Table 2
Sex differences in development time in the next generation within treatment groups. Bonferroni corrected results of the Log Rank (Mantel-Cox) are presented[a].

Copper (μg/g)	χ^2	*df*	*P*
0→0	**26.395**	**1**	**<0.004**
0→50	5.665	1	0.068
50→0	**32.989**	**1**	**<0.004**
50→50	**7.010**	**1**	**0.032**

[a]Significant results in bold (P ≤ 0.05.

4.2. Fecundity and development time of the next generation

Our study revealed that the effects of copper exposure on fecundity can be trans-generationally transmitted to the next generation. Individuals whose parents were exposed to copper produced fewer eggs than the uncontaminated control group, if they were reared under uncontaminated conditions (0→0 vs. 50→0). These findings are consistent with our recent study in which exposure to copper was found to have a trans-generational effect on the encapsulation response of the blow fly, *Protophormia terraenovae*. Individuals whose parents were exposed to a sub-lethal concentration of copper expressed a similar effect in encapsulation response, even if they were reared under uncontaminated conditions (Pölkki et al., 2012). Interestingly, previous studies suggest that immune activation might correlate positively with the rate of detoxification enzyme levels (Dubovskiy et al., 2011; Sun et al., 2008; Zvereva et al., 2003). Together with our previous study in which upregulated immune activation caused by copper exposure was found to be trans-generationally transmitted to the next generation (Pölkki et al., 2012), this could lead to the assumption that the next generation might express enhanced levels of detoxification enzymes. If we would consider this from an evolutionary point of view, it would be beneficial if parents exposed to a certain environmental condition, such as anomalous pathogen load or heavy metal concentrations, could prepare their offspring by producing a progeny that has better ability to cope in similar conditions. However, this is an advantage only if the present conditions predict the future conditions and the offspring face similar environmental burden than their parents (Sadd et al., 2005). In the present study, the observed fitness costs might be more related to the increasing demands of detoxification enzymes and handling the surplus copper. If this was to be true, the offspring would gain when reared under similar contaminated conditions as their parents. This hypothesis receives support below but remains still highly speculative.

We found evidence for acclimatization after short-term (two generations) exposure to copper. Interestingly, individuals reared two generations on copper contaminated larval medium did not differ in their fecundity when compared with the control group that was reared two

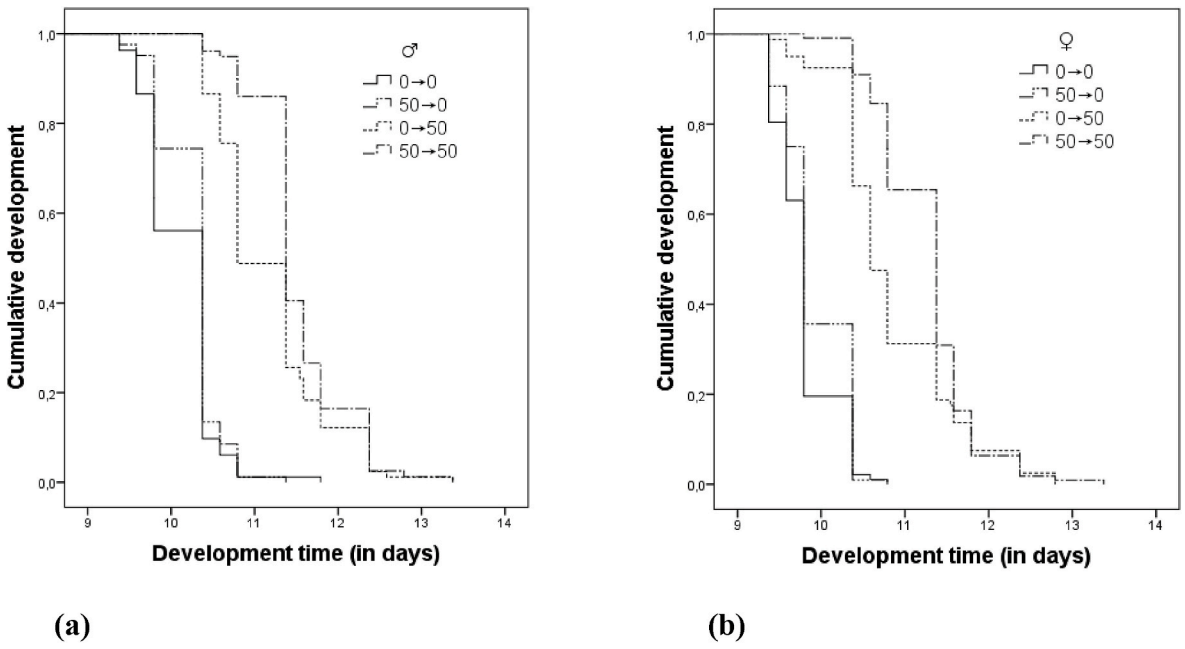

Fig. 4. Cumulative development time of the next generation for females (a) and males (b) reared either on uncontaminated control food (0 μg/g) or exposed to copper (50 μg/g) and whose parents were either reared on uncontaminated (0 μg/g) larval medium or exposed to copper (50 μg/g).

Table 3
Effects of parental and direct larval exposure to a sub-lethal concentration of copper on development time. Results of the Log Rank (Mantel-Cox) are presented[a].

Copper (μg/g)	χ^2	*df*	*P*
Females			
0→0 vs. 0→50	113.307	1	**<0.008**
0→0 vs. 50→0	4.921	1	0.216
0→0 vs. 50→50	209.493	1	**<0.008**
0→50 vs. 50→50	10.796	1	**0.008**
Males			
0→0 vs. 0→50	104.534	1	**<0.008**
0→0 vs. 50→0	3.825	1	0.400
0→0 vs. 50→50	139.472	1	**<0.008**
0→50 vs. 50→50	8.416	1	**0.032**

[a]Significant results in bold ($P \leq 0.05$).

generations under uncontaminated conditions. One explanation for this, as already discussed above, could be that the next generation might express upregulated detoxification enzyme levels or otherwise be more tolerant towards copper. However, the 'parental stress hypothesis' predicts that parents reared under unfavorable nutrition conditions should produce lower quality offspring (Vijendravarma et al., 2010). Since females did not differ from the control group in their fecundity, 'the adaptive parental effects hypothesis' seems more plausible. One thing to keep in mind is that production of the detoxification enzymes requires energy (Calow, 1991) but here the toxins are received via food, and therefore, higher the food consumption is, the greater is the copper intake received through the larval diet. If offspring conditions correspond to those faced by their parents offspring might be more prepared of handling the toxins and be more successful under contaminated conditions, but otherwise this trait becomes costly (Sadd et al., 2005). Even though individuals seemed to be able to compensate the energy deficit, more time might be needed for processing the toxic compound. In copper contaminated conditions higher copper tolerance is beneficial. However, as reported in the study by Kristensen et al. (2008) these kinds of traits might become costly if conditions differ from the conditions leading to acclimatization. Further, acclimatization to one condition is advantageous only if the present conditions corresponds future conditions.

It remains unclear whether effects were maternally or paternally inherited, since both parents were reared in the same environment. However, maternal effects in particular are considered to be prominent factors affecting offspring phenotype (Badyaev and Uller, 2009). Potential transmission mechanisms are epigenetic factors, i.e. offspring phenotype is altered through non-genetic inheritance (Bonduriansky and Head, 2007; Skinner and Guerrero-Bosagna, 2009) which were not tested here. However, the exact mechanism remains elusive and needs further experiments. Nevertheless, the concentration used in this experiment was preliminarily tested to have no effect on overall survival. However, selected concentration corresponds to those found in nature nearby metal industry (e.g. Spurgeon et al., 1994). Usage of a sub-lethal dosage excludes the possibility that the observed results are altered by mortality-related selection towards improved copper tolerance through increased mortality levels in copper exposure groups (see Pölkki et al., 2012, 2014).

In the next generation direct exposure to copper had a negative effect on development time when the groups (0→50 and 50→50) were compared with the uncontaminated control (0→0). This result is similar to the parental generation and can also be explained by the direct harmful effects of copper exposure (see above). However, when comparing the group 0→50 with the treatment group 50→50 they differed in their development time, the latter one being slower. Although individuals exposed to copper for two generations (50→50) did not differ in their fecundity from the controls, whereas the treatment control (0→50) differed. In theory, this could mean that after two generations of exposure individuals might indeed be able to handle the toxicants better, but that they might need more time to compensate the direct costs and therefore develop more slowly. Support for this hypothesis comes from the uncontaminated group (50→0) whose fecundity was lower compared with the controls (0→0). However, these groups did not differ in their development time, which could indicate an increase in other expenses that stem from intensified detoxification mechanisms. Furthermore, a sex difference in development time was detected. In this species, females usually eclose earlier than males.

To summarize, larval exposure to copper had a deleterious effect on adult fecundity. Also, parental exposure to copper had a trans-

generational influence on the fecundity of their offspring, if the offspring were reared under uncontaminated conditions. In case the progeny was exposed to a similar concentration of copper than their parents, no difference in fecundity was discovered. Our results suggest a short-term acclimatization that could be a result of physiological adaptation to heavy metal exposure through phenotypic plasticity. However, acclimatization might weaken fecundity under uncontaminated conditions. Our results may follow the adaptive parental effects hypothesis: by parents being able to prepare their offspring to cope with conditions similar to their own (Sadd et al., 2005; Vijendravarma et al., 2010). However, the proximate and ultimate mechanisms behind the observed results remain unclear, which should definitely be tested in future studies. Our results show that exposure to heavy metals influences fecundity and therefore can have an impact on reproductive success and overall fitness. One thing to keep in mind is that these kinds of trans-generational effects leading to acclimatization are beneficial only if the present or parental environment predicts future (Sadd et al., 2005). Otherwise, under different environmental conditions they might become unnecessary or even costly and the observed or measured traits, such as fecundity here, might result as opposite (Kristensen et al., 2008).

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.

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