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A multigenerational study can detect the evolutionary response to BaP exposure in the non-biting freshwater midge Chironomus riparius

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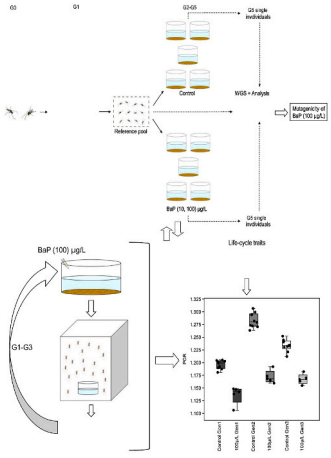
HIGHLIGHTS

- BaP exposure increases the mutation rate of *C. riparius*.
- BaP exposure is detrimental for the fitness and the population dynamics of *C. riparius*.
- Multi-generational studies are essential to assess evolutionary implications of anthropogenic substances on biodiversity.

GRAPHICAL ABSTRACT



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ABSTRACT

The release of polycyclic aromatic hydrocarbons (PAHs) into the environment is posing a threat to ecosystems and human health. Benzo(*a*)pyrene (BaP) is considered a biomarker of PAH exposure and is classified as a Group 1 carcinogen. However, it was not known whether BaP is mutagenic, i.e. induces inherited germline mutations. In this study, we used a recently established method, which combines short-term mutation accumulation lines (MAL) with whole genome sequencing (WGS) to assess mutagenicity in the non-biting midge *Chironomus riparius*. The mutagenicity analysis was supplemented by an evaluation of the development of population fitness in three successive generations in the case of chronic exposure to BaP at a high concentration (100 µg/L). In addition, the level of ROS-induced oxidative stress was examined *in vivo*. Exposure to the higher BaP concentration led to an

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increase in germline mutations relative to the control, while the lower concentration showed no mentionable effect. Against expectations, BaP exposure decreased ROS-level compared to the control and is thus probably not responsible for the increased mutation rate. Likewise, the higher BaP concentration decreased fitness measured as population growth rate per day (PGR) significantly over all generations, without signs of rapid evolutionary adaptations. Our results thus highlighted that high BaP exposure may influence the evolutionary trajectory of organisms.

1. Introduction

The release of anthropogenic substances into the environment usually causes negative effects at all levels of biological organization, from the molecule up to the community and ecosystem (Zhang et al., 2018). The unifying factor is their effect on population fitness, which on the one hand integrates all processes within individuals and on the other hand is decisive for the organism's role in the ecological community and thus for the ecosystem (Sæther and Engen, 2015; Shaw et al., 2008). However, as species are not static but evolve, anthropogenic stressors with a negative impact on population fitness also represent selection factors that can trigger evolutionary adaptation (Charlesworth, 1971; Murray, 1990). The same stressors can also induce mutations that, although essential for evolution, in the vast majority of cases have a negative impact on fitness (Baer et al., 2005; Eyre-Walker et al., 2006). These long-term consequences, which alter not only current demography but also the future evolutionary trajectory of species, are the focus of the emerging research field of evolutionary ecotoxicology.

Polycyclic aromatic hydrocarbons (PAH) represent one of the most important polluting compounds, ubiquitously present in all environmental compartments (Adeniji et al., 2019). Their heterocyclic aromatic ring structure, hydrophobicity, and thermostability make them highly persistent in the environment (Patel et al., 2020). Moreover, PAH represents a legacy of contemporary society as their increasing presence in the environment was associated with car use and urban sprawl (Van Metre et al., 2000). BaP is one of the most prevalent PAH pollutants in the urban environment. It is formed during the incomplete combustion of organic matter like wood and certain types of fossil fuels such as petrol and diesel and reaches the environment mainly in exhaust gasses, as well as in cigarette smoke (Bukowska et al., 2022; Phillips, 1983). It accumulates in the sediment carried by runoff water, particularly if the runoff encounters vehicles, road surfaces, or deposits of combustion byproducts. Moreover, it has been described as genotoxic and carcinogenic in mammalian cells and has already been proven to inhibit DNA repair, thereby likely promoting mutagenicity. It is the only PAH classified as carcinogenic in Group 1 (Baan et al., 2008). Benzo[*a*]pyrene has been shown to increase ROS production and induce oxidative stress (Baan et al., 2008). Under conditions of oxidative stress, ROS levels can become excessive and cause damage to biomolecules such as DNA, proteins, and lipids (Dröge, 2002). Its accumulation in tissue caused adverse metabolic, neuronal, and genotoxic effects in Chironomid and Aedes larvae (Kagan and Kagan, 1986; Vicentini et al., 2017).

However, it is not known whether BaP has a mutagenic effect on germ cells upon environmental exposure. Instead of mutations in somatic cells, mutations in germ cells are passed onto offspring for generations even if exposure to the substance has ceased. Despite being the "fuel of evolution", mutations have usually a detrimental effect on the fitness of organisms (Lynch and Gabriel, 1990). Thus, measuring the mutagenic effect of a toxic substance in germ cells is crucial because it can irreversibly change the evolutionary trajectory of the population under investigation. Indeed, mutagens behave differently from other toxicants, which may exert a genotoxic action, but without necessarily inducing an increased rate of mutagenicity. Ecotoxicological tests represent a key tool for determining the toxicity of a substance and its potential impact on the environment. However, these tests usually do not focus on the direct measurement of mutagenicity. In this regard, considering the evolutionary effects of anthropogenic emissions increases the scope of environmental assessments and allows for an integrated assessment of emerging pollution problems.

Although it is reliable to test mutation rates in microorganisms or in cell cultures, these do not consider the real complexity of a multicellular organism, let alone an organism as a living system (Kirkland et al., 2007; Knaap et al., 1988). However, the use of next-generation sequencing (NGS) technology for the direct assessment of the mutagenicity of chemicals is currently not yet widespread (Beal et al., 2019; Du et al., 2017; Wamucho et al., 2019). There is a lack of established and standardized protocols, which means that thousands of chemicals currently in use in Europe are not comprehensively evaluated for their potential health and environmental risks (Doria and Pfenninger, 2021). In the present study, we describe the application of a recently introduced mutation rate test (Oppold and Pfenninger, 2017) as an effective ecotoxicological mutagenicity test tool for metazoan organisms. The test is a combination of short-term mutation accumulation (MA) lines, whole genome sequencing (WGS), and dedicated data analysis. For this, we used *Chironomus riparius,* a non-biting freshwater midge that is widely distributed in Europe, Asia, and North America. It is used as a test species in ecotoxicological assessment of water and sediments and four validated tests are included in the OECD Guidelines for the Testing of Chemicals (OECD, 2010). *C. riparius* has already been used as a model organism for mutagenicity tests, evaluating the toxicity of Cadmium (Cd) in a multigenerational scenario (Doria and Pfenninger, 2021). In addition, the genomic analysis was complemented by a multigenerational study over three successive generations, assessing changes in population fitness over time, in particular, whether constant exposure leads to rapid adaptation, as has been shown for other stressors in *C. riparius* (Foucault et al., 2019; Nowak et al., 2009; Pfenninger and Foucault, 2020). Because oxidative stress is known as a major agent to induce mutations (Aitken and Krausz, 2001), we measured oxidative stress through the quantification of reactive oxygen species (ROS).

2. Materials and methods

2.1. Test compound

Benzo[*a*]pyrene (BaP) (Merck, Germany) was prepared by diluting it with dimethyl sulfoxide (DMSO) and kept as a 1% stock solution. The BaP exposure was continued for 5 generations at concentrations of 10 μg/L and 100 μg/L. The concentrations were chosen, because the lower value represents high environmentally relevant concentrations (Bukowska et al., 2022), and the higher was a just not yet lethal concentration in an exploratory 24 h acute test with freshly hatched larvae (data not shown). BaP was added to the medium in corresponding amounts at the beginning of every generation.

2.2. Chemical analysis

To determine the final concentration of BaP, sediment, and water samples were collected and sent to Fresenius SGS Institut GmbH laboratory for chemical analysis. Water samples were analyzed using gas chromatography combined with mass spectrometric detection (GC-MS) (DIN 38407-39:2011–09). Sediment samples were analyzed according to DIN EN 14346:2007–03 by gas chromatography and subsequent determination by mass spectrometry (GC-MS) (DIN IDO, 18287:2006–05).

2.3. Mutation accumulation lines experimental design

For the experiment, one egg rope was raised under optimal conditions to avoid premature selection pressure. After the successful reproduction of the G1 generation, egg ropes from the G2 generation were collected to establish the next generation. The hatched clutches were placed in glass bowls (20 cm diameter, 14 cm height) containing 1.5 cm of sand and 1.150 L of medium with a conductivity between 550 and 650, and a pH value of around 8. The four groups were Control, 10 μg/L, 100 μg/L, and Solvent, with 15 replicates each. For conducting the next generation, only one egg clutch was chosen. The mutation accumulation lines were kept at 22 ± 0.5 °C, 60% humidity, and 16:8 light and dark periods. The bowls were aerated 24 h a day, and evaporation of the medium was avoided by adding distilled water and adjusting the conductivity when necessary. Because of the swarm fertilization of *C. riparius* and the impossibility of determining the parents of specific egg clutches, adults of the first generation were collected and used as a pooled reference. At the end of the fifth generation, one randomly chosen female was collected for DNA extraction.

2.4. Life cycle test

A chronic toxicity test was conducted according to the OECD Guideline 233 to determine the toxicity of benzo[*a*]pyrene (BaP) to *C. riparius* over a test period of 28 days (OECD, 2010). The same experimental design was used with minor modifications to mutation accumulation lines. Five egg clutches were selected to initiate the experiment, and 30 first-instar larvae were placed in glass bowls. After placing 30 individuals, BaP was added to the medium at a final concentration of 100 μg/L. In every generation, the glass bowls were replaced with new sand, medium, and 100 μ/L BaP was freshly added, keeping the exposure concentration constant. Both the control and the treatment were subjected to a life cycle test every generation. The number of emerged adults was counted to determine mortality, which corresponds to the subtraction of the emerged from the exposed larvae. Adult sexes were recorded daily to determine the mean emergence time (EmT50), i.e. when 50% of the females emerged. The number of adults that emerged and their sexes were recorded daily to determine the mean emergence time (EmT50). For fertility determination, all emerged individuals were placed in breeding cages according to their experimental groups. The number of fertile laid eggs were counted, and the egg numbers were determined according to. Non-fertile eggs rarely occur in experiments, with the exception of certain experimental conditions that, for instance, strongly influence sex ratios. Finally, all measured parameters were summarized in the population growth rate (PGR) (Nemec et al., 2013).

2.5. Whole genome sequencing and bioinformatic analysis

DNA extraction was done with the Blood and Tissue QUIAGEN Kit by following the manufacturer's instructions. The whole genome sequencing of both the reference pool and individuals was established using the study (Doria and Pfenninger, 2021). Clean reads of individual females of each mutation accumulation line and ancestors were analyzed using the best practices of the GATK pipeline (McKenna et al., 2010). First, the reads were paired with Pear (Zhang et al., 2014). The reference genome v.4 (unpublished data) was used for mapping with bwa-mem. Picard v.1.123 (https://broadinstitute.github.io/picard/) was used for marking and removing duplicates, and low-quality reads were removed using samtools with default parameters. The target lists for realignments, vcf file creation, variant filtration, and base recalibration were created using GATK. The bam files were merged with samtools merge (Danecek et al., 2021), and accuMUlate (Winter et al., 2018) was used with the same reference genome and merged bam files. The output was filtered with a custom bash script using the following parameters: probability of a mutation (≥ 0.90), probability of one mutation (≥ 0.90), probability of correct descendant genotype (≥ 0.90), N mutant in wt (=0), mapping quality difference (≤ 2.95), and stand bias (≥ 0.05). The filtered mutation positions were then validated using IGV. The mutation rate was estimated by multiplying the number of mutations per generational passage by the callable sites. Finally, we used a Bayesian implementation of the Poisson test to compare the mutational rates between treatments with the R package BayesianFirstAid (Bååth, 2014).

2.6. ROS detection and image analysis

For each condition (control, 10μ/L, and 100μ/L), 10 L3 larvae were collected and placed in 24 well plates. Plates were filled with 2.5 ml medium as described in Foucault. CellROX Orange (Thermo Fisher cat. no. C10443) reagents were used to identify ROS products. CellROX is an oxidative stress reagent that is cell-permeable and suitable for live cell ROS measurements. Within the reduced state, they are non-fluorescent but after oxidation by ROS, they exhibit fluorogenic signals at 545/565 nm for CellROX Orange. The reagent is localized within the cytoplasm and can detect 5 different ROS types (hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite anion, and superoxide anion). After placing larvae on the well plates, 0.75 μl of CellROX Orange was used per larva.

Well-plates were placed in a climate chamber with a 16:8 light/dark cycle with 550 lux light intensity without aeration under 20 °C. After 24 h of treatment, well plates were placed in a styrofoam box to avoid the temperature change effect. The ROS was measured in a live larva with ZEISS Axio Imager 2 under 10× magnification. The images were taken with AxioVision Rel. v.4.8. For fluorescence images, an HXP 120C fluorescence lamp was used with maximum light intensity (Item Number: 423,013-9010-000). Fluorescence images were obtained from the larva under filter set "43 HE" (BP 550/25 HE, FT 570 HE, BP 605/70 HE, Item Number 489043-9901-000) with 1 s exposure. This specific filter excites blue light around 550 nm, transmitting emitted red fluorescence above 570 nm filtering out the remaining blue excitation light and allowing only red fluorescence around 605 nm.

The fluorescence field images were analyzed by ImageJ Fiji (v. 2.15.0). Images were uploaded to ImageJ as an image sequence and converted to 8-bit grayscale from RGB Color images to avoid color difference and only calculate light intensity. The same threshold was applied to all images (Threshold: 23). After setting the threshold measure function was used. The mean values of each image were taken as fluorescence intensity. The fluorescence intensity we measured is not the actual ROS amount within the cell but the current amount of reagent entered in the cell and oxidized by binding to ROS which is the remaining amount after the cell's antioxidant system scavenges ROS. The data were analyzed with the R package BayesianFirtsAid (Bååth, 2014).

2.7. Data analysis

The statistics program PAST® software (version 4.15) was used to create the graphs. The R package BayesianFirstAid (Bååth, 2014) was used for the *t*-test to compare life-cycle parameters between treatments.

3. Results

3.1. Final BaP concentration

Final BaP concentrations of group 100 μg/L resulted in a BaP sediment content of 0.63 μg/kg after the first generation. After the second generation, 0.19 μg/kg BaP, and after the third generation, 0.20 μg/kg was detected. The final concentration of BaP was undetectable in all generations of the 10 μg/L treatment.

3.2. Mutation rate and spectrum

The mean read coverage per MAL ranged from 41.61× to 87.83× and the number of callable sites ranged from 33,153,776 to 169,912,242 (Table 2). In total, 2104 candidate mutations were identified, which were subsequently filtered down to 104 credible mutations. In 100 μg/L BaP, 5 transversion, and 27 transition mutations were identified with TV/TS ratio of 0.19. In 10 μg/L BaP, 4 transversion, and 19 transition mutations were identified, and TV/TS ratio was 0.21. For the control and solvent groups, 6 transversion and 19 transition mutations, and 8 transversion and 16 transition mutations, respectively, were identified (Table 1). For control, the TV/TS ratio was 0.31, and for SOL 0.5. The ratio between the treatments and the control groups was not significantly different in Fisher's exact test ($p = 0.1292$).

The mutation rate estimate for the Control was $\mu = 3.16 \times 10^{-9}$ (95% HDI 2.1 × 10–9 and 4.6 × 10^{-9}), and for 100 μg/L BaP was $\mu = 3.51 \times 10^{-9}$ (95% HDI 2.6 × 10–9 and 5.3 × 10^{-9}). The control/100 μg/L BaP rate ratio was 1.2 as shown in Fig. 1, with 75.7% certainty this ratio being larger than 1. The rate estimate for 10 μg/L BaP was $\mu = 2.68 \times 10^{-9}$ (95% HDI 1.8 × 10–8 and 4.2 × 10–8). The control/10 μg/L BaP rate ratio (Fig. 1) was 1.1 (36.3% certainty). For the solvent group, the rate estimate was $\mu = 2.6 \times 10^{-9}$ (95% HDI 1.8 × 10–8 and 4.1 × 10–8) as well with a rate ratio of 1.1 (33.8% certainty).

3.3. Life cycle test

To increase statistical power, we combined the control and SOL groups, as there were no mentionable differences between the groups in any fitness parameters (mortality, EMT50, and fertility). In the first generation (G1), mortality differences were generally very low, with no evidence of significant differences between control and treatment (100 μg/L BaP) (median = −13, 95% HDI between −59 and 29). In the second generation (G2), a large difference of means (−11, 95% HDI -19 and −2.2) was found between control and treatment, with a posterior probability of 98.7% that control was smaller than treatment. This evidence had a large effect size of significantly increased mortality in treatment (Cohen's d = −2). The third generation (G3) also showed a large difference of means (−16, 95% HDI -24 and −8.1) between control and treatment, with a posterior probability of 99.7% that the control was had a lower mortality than the treatment (Fig. 2a). This evidence had a very large effect size (d = −2.8) (Table 3).

In the first generation no mentionable EmT50 difference was found between control and treatment (Fig. 2b) (−0.24, 95% HDI -1.4 and 0.91), as well as in the second and third generation (0.001, 95% HDI -1.9 and 1.7; −0.45, 95% HDI -1.7 and 0.86) (Table 3).

Regarding fertility, in all three generations small differences of means (G1 = 0.39, 95% HDI -0.13 and 0.87; G2 = 0.6, 95% HDI 0.15 and 1.1; G3 = 0.36, 95% HDI 0.19 and 0.54) were observed between control and treatment, with a posterior probability of 94.7%; 99.2% and 99.9% that control was bigger than treatment, respectively. This evidence had a large effect size of significantly reduced fertility in all generations in the treatment (G1 d = 1.2; G2 d = 1.7; G3 d = 2.3). (Fig. 2c) (Table 3).

In the first generation, differences in daily PGR were generally low, with no evidence of relevant differences between control and treatment (0.02, 95% HDI -0.2 and 0.05). In the second generation, a small difference of means (0.08, 95% HDI 0.03 and 0.12) was found between control and treatment, with a posterior probability of 99.7% that control was bigger than treatment (Fig. 2d). This evidence had a very large effect size (d = 2.7). In the third generation, again a small difference of means (0.07, 95% HDI 0.05 and 0.09) was found between control and treatment, with a posterior probability of 100% that control was bigger than treatment. This evidence had a huge effect size (d = 5.1) (Table 3).

Table 1
Mutation spectrum of *C. riparius* after 5 generations of 100 μg/L BaP exposure.

Treatments	Transitions		Transversion			
	G<>A	C<>T	A<>C	C<>G	A<>T	G<>T
100 μg/L	11	16	–	–	3	2
10 μg/L	6	13	1	1	1	1
CT	6	13	1	–	5	–
SOL	8	8	1	1	4	2

Table 2
Mean coverage, number of callable sites, number of single nucleotide mutations (SNM), and mutation rate (μ) per treatment of *C. riparius*.

Treatment	Mean Coverage	No. of Callable Sites	No. of SNM	Mean μ Rate
100 μg/L	49.9	1.7E+08	32	3.51E-09
10 μg/L	52.4	1.6E+08	23	2.68E-09
Control	51.6	1.6E+08	25	3.16E-09
Solvent	47.8	1.7E+08	24	2.60E-09

3.4. Reactive oxygen species measurements

The fluorescence images were analyzed with ImageJ to determine the red color density and intensity. The control group showed significantly higher fluorescence intensity than the treatment group 10 μg/L (30, 95% HDI 23 and 37, with posterior a probability of 100%, d = 4.3) and 100 μg/L (29, 95% HDI 23 and 37, with a posterior probability of 100%, d = 4.2). There was no significant difference between the BaP treatments (−0.53, 95% HDI -1.1 and 0.74) (Fig. 3).

4. Discussion

Our study investigated the microevolutionary responses of the midge *C. riparius* to BaP exposure, assessed by directly measuring mutagenicity and population fitness in a multigenerational setup. The results of our study showed that multigenerational exposure to BaP resulted in a non-significant increase in mutations at the lower exposure concentration (10 μg/L) and a 1.2-fold increase compared to the control at the higher concentration (100 μg/L). This concentration, however, exceeded the levels typically found in natural habitats (Bukowska et al., 2022) and was intended as the positive control. Nevertheless, the observed increase in mutation rate was 1.2-fold rather modest. Temperatures with which *C. riparius* is regularly confronted in their habitat (e.g. 12 °C and 26 °C), showed much more pronounced effects, leading to 2.79- and 4.54-fold increases in mutation rate, respectively (Waldvogel and Pfenninger, 2021). Analysis of the mutational spectrum in both control and treatment groups revealed a shift towards transitions at the expense of transversions in Fisher's exact test ($p = 0.1292$), while no significant difference in the mutation spectrum ratio was observed between the groups. Therefore, although BaP in environmentally unrealistically high concentrations induces an increased mutation rate, it is likely no important driver of the mutation rate in natural habitats. However, organisms like *C. riparius* are subjected to multiple environmental stressors in the wild (Pfenninger and Foucault, 2020). Such complex scenarios in natural populations may exert additive, interactive, or even inhibitory effects on mutation rates, thus warranting further investigation. Our results showed that exposure to BaP at higher concentrations (100 μg/L) exerted a mutagenic effect and that this rather modest effect can be reliably traced already after a few generations. As only the number of generational passages is relevant, the time necessary to perform the test could be shortened, if more MAL were used. Likewise, the test could also be adapted for different test species e.g. from other realms. We could thereby show that it is possible to develop a practical and easy-to-implement pipeline for rapid detection of germ cell mutagens in a metazoan test organism. Further multigenerational studies on the effect of BaP and other potentially mutagenic substances on mutational rates are essential to better assess evolutionary effects and influence on the living environment.

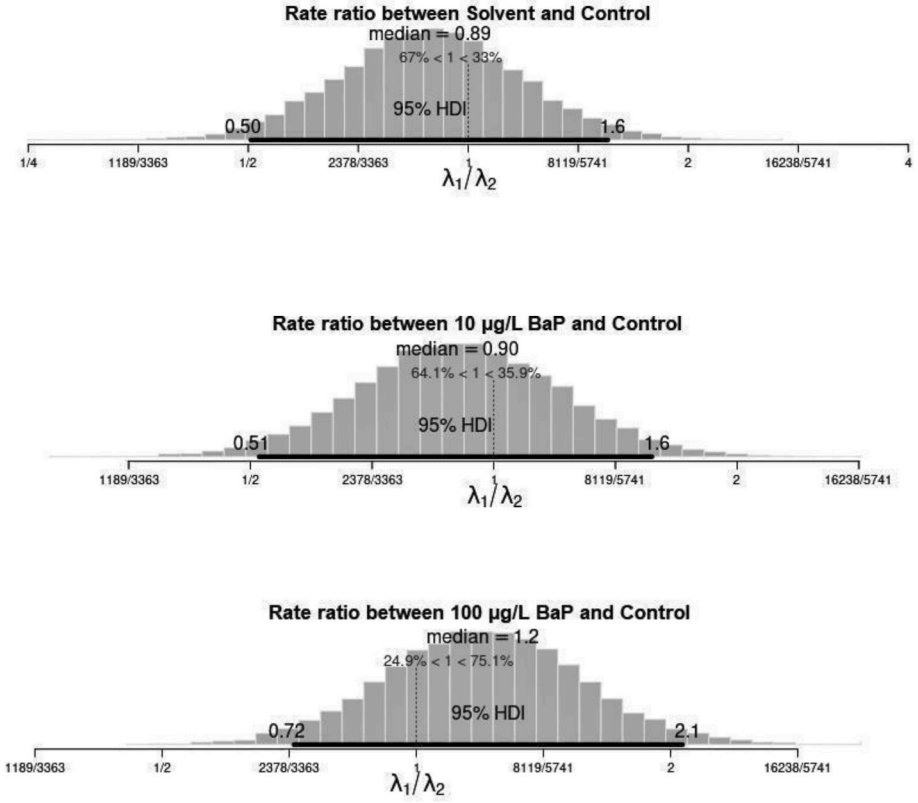

Fig. 1. Posterior distributions for rate ratios between solvent, 10 µg/L, and 100 µg/L BaP relative to the control group.

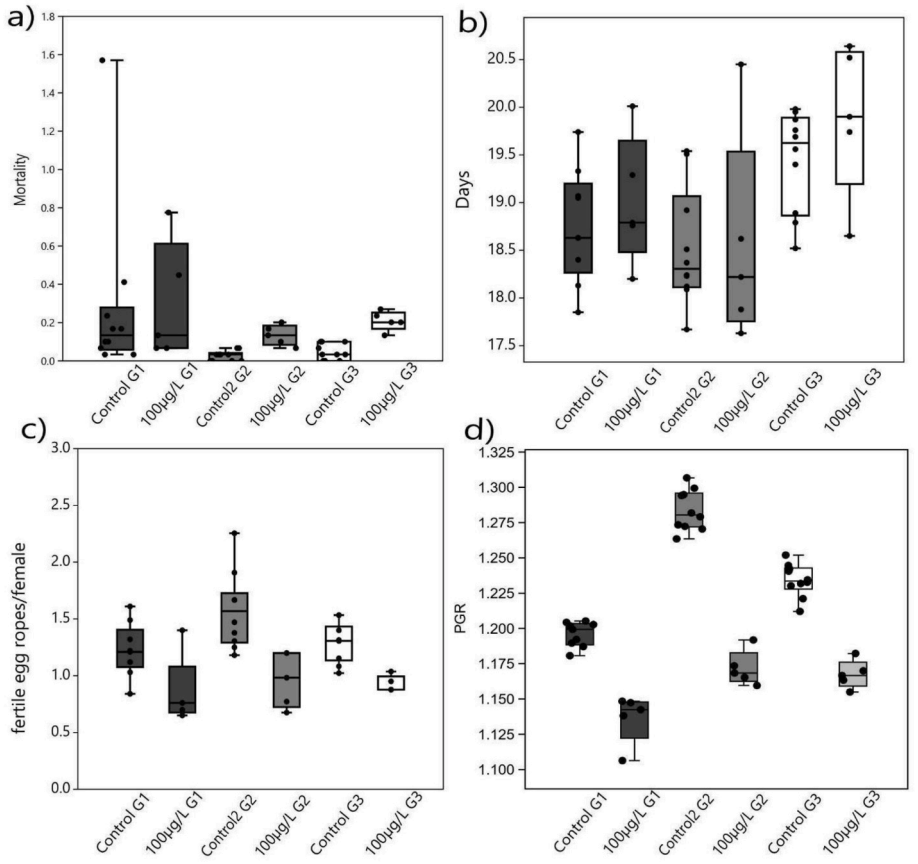

Fig. 2. *C. riparius* effects on a) mortality, b) Emt50, c) fertility, and d) PGR after exposure to control and 100µ/L of BaP for three consecutive generations.

Table 3
Summary of Bayesian statistics for Emerged Adults, EmT50, Fertility, and PGR within generations between the control group and 100 μg/L group.

Mortality					
Generation	Mean of Control (95% HDI)	Mean of 100 μg/L (95% HDI)	Mean Difference	PP (100 μg/L > Control)	Effect Size (95% HDI)
G1	14 (4.2–23)	27 (-14–71)	−13 (-59–29)	22.2%	−0.50 (−1.8–0.79
G2	2.6 (0.60–4.7)	13 (5.1–22)	−11 (−19–−2.2)	1.3%	−2 (−4–−0.17)
G3	4.6 (1.4–7.8)	21 (13–28)	−16 (−24–−8.1)	0.3%	−2.8 (−4.9–−0.76)
EmT50					
G1	19 (18–19)	19 (18–20)	−0.24 (−1.4–0.91)	30.4%	−0.31 (−1.5–0.89
G2	18 (18–19)	18 (17–20)	0.00077 (−1.9–1.7)	50.1%	0.00066 (−1.2–1.2)
G3	19 (19–20)	20 (19–21)	−0.45 (−1.7–0.86)	19.4%	−0.54 (−1.8–0.68)
Fertility					
G1	1.2 (1.0–1.3)	0.83 (0.39–1.3)	0.39 (−0.13–0.87)	94.7%	1.2 (−0.33–2.8)
G2	1.6 (1.3–1.8)	0.97 (0.57–1.3)	0.60 (0.15–1.1)	99.2%	1.7 (0.30–3.2)
G3	1.3 (1.1–1.4)	0.92 (0.81–1.0)	0.36 (0.19–0.54)	99.9%	2.3 (0.81–3.9)
PGR					
G1	1.2 (1.2–1.2)	1.2 (1.1–1.2)	0.016 (−0.023–0.054)	85.8%	0.73 (−0.64–2.1)
G2	1.2 (1.2–1.2)	1.1 (1.1–1.2)	0.08 (0.031–0.12)	99.7%	2.7 (0.46–4.8)
G3	1.2 (1.2–1.2)	1.2 (1.2–1.2)	0.067 (0.048–0.085)	100%	5.1 (1.9–8.3)

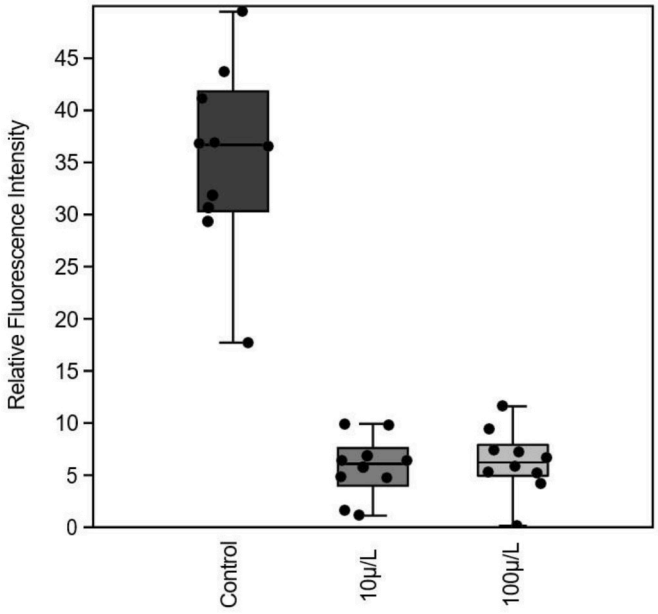

Fig. 3. Comparison of relative fluorescence intensity among control, 10μ/L, and 100μ/L.

While we could not show a mutagenic impact of environmentally relevant concentrations of BaP, the picture was different when the direct fitness effects of BaP were considered. Life cycle traits were assessed in a multi-generation test conducted with *C. riparius* exposed to nominal BaP concentrations of 0 (control) and 100 μg/L. Mortality, EmT50, fertility, and PGR of control and exposed groups fluctuated between generations. Despite the lack of significance, there was a tendency towards increased mortality in the first generation. Furthermore, there was a significant increase in mortality in the second and third generations. This effect could be due to the lipophilic properties of BaP, which is absorbed by the sediment (Leversee et al., 1982). Due to their sediment-bound lifestyle, chironomid larvae are exposed throughout the larval stage to pollutants in the sediment, thus absorbing the pollutant and accumulating it (Markert et al., 2003). During larval development, lipids are mobilized in the pupal stage and BaP previously stored in adipose tissue is released. Therefore, the release of BaP could be the cause of the observed increased adult mortality (Du et al., 2014).

The results of EmT50 showed no significant effects for the experimental groups compared to the control, for all generations analyzed. Therefore, no effect of BaP exposure on the developmental time of *C. riparius* was detected. However, fertility decreased significantly in all generations and was further significantly reduced in the third. Reduced fertility is a common consequence of exposure to various contaminants (Nowak et al., 2007). Regarding BaP, multigenerational studies with other organisms have yielded similar results, showing reduced fertility and reproduction in successive generations exposed to BaP (Mohamed et al., 2010; White et al., 1999). Mohamed et al. (2010) reported a decrease in sperm production in male mice exposed to BaP in the adult generation. In this case, the effects were even intergenerational, as the effect was felt up to the F2 generation without the G2 or G3 generation being exposed to BaP. In another study, larvae of the freshwater fish *Pimephales promelas* were exposed to BaP, and toxic effects on reproduction were observed in subsequent generations (White et al., 1999).

The PGR clearly decreased with BaP exposure, mainly due to the observed loss of fertility. In all generations, the BaP-exposed group had a significantly lower PGR value than the control. Although the effect of BaP as a stressor of *C. riparius* was confirmed, the PGR value was above the critical value of 1 d^{-1} despite the high stress. However, as we assessed the PGR per day, even a small decrease may have dramatic influences in the long run. For example, a population growing 8% less per day would have after only 14 days half the size of an undisturbed growing population.

While many organisms are unable to adapt to changing environmental conditions, *C. riparius* is known for its adaptability to environmental stressors (Doria et al., 2022; Khosrovyan et al., 2022). However, in the present study, we could not confirm an adaptation of *C. riparius* to BaP exposure. One reason might be that the effect of BaP exposure might not have been detrimental enough to trigger a rapid adaptation response, so detecting potential adaptation would have required more generations. Other, mutually non-exclusive reasons might have been i) lacking genetic variation for the particular trait in the laboratory population, ii) adaptation happened but the accumulation of mutations may have had an accumulating negative effect on fitness, thus veiling the adaptation. Further studies with a modified experimental design would be required to test this hypothesis.

The direct assessment of ROS activity by fluorescence intensity was performed with living *C. riparius* larvae exposed to nominal BaP concentrations of 0 μg/L (control), 10 μg/L, and 100 μg/L. Some studies on BaP exposure suggested that DNA damage is caused by ROS production (Saunders et al., 2006; Vicentini et al., 2017). In a study conducted on *Chironomus sancticaroli Strixino & Strixino* larvae, it was found that BaP

exposure caused genotoxic effects and biochemical changes usually associated with oxidative stress (Vicentini et al., 2017). Contrary to these results, our study, however, showed lower ROS levels in both treatments than in the control group. This discrepancy might have two reasons: First, the study by Vincentini et al. did not measure ROS activity directly, but antioxidative enzyme activity. However, such activity was shown to be time-dependent rather than concentration-related, as exposure to 10 μg/L and 100 μg/L BaP initially increased antioxidant enzyme activity in the first 24 h, but subsequently reduced to control level after 48 h (Guo et al., 2021). Second, the direct measurement as employed here presents the equilibrium between metabolic ROS production and antioxidant activity, it is possible that BaP either had a negative impact on metabolism or stimulated antioxidant activities to an extent that pushed the level under that of the control. In any case, our results suggested that the observed increase of mutation rate was not related to an increased ROS production, but rather involved other, unknown processes.

5. Conclusion

The results of our multigenerational study show that high BaP exposure led to an increased mutation rate and negatively impacted the demographic dynamics of *C. riparius*, thus altering the eco-evolutionary trajectory of the population. A rapid adaptive effect could not be observed but cannot be excluded over longer time frames. The results of this study thus demonstrated the importance of multigenerational studies for ecological risk assessment, as the evolutionary effects of most ecotoxicologically relevant chemical inputs such as BaP are still unknown.

CRediT authorship contribution statement

Burak Bulut: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Lorenzo Rigano:** Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Halina Binde Doria:** Conceptualization, Data curation, Project administration, Writing – review & editing. **Gajana Gemüth:** Conceptualization, Data curation. **Markus Pfenninger:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142242.

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