Advancing evolutionary ecotoxicology: single to complex contaminant exposure in C*hironomus riparius*

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ZUSAMMENFASSUNG

Im Anthropozän haben menschliche Aktivitäten wie weitverbreitete Verschmutzung, Habitatdegradation und Klimawandel Ökosysteme tiefgreifend verändert. Dabei handelt es sich nicht um bloß vorübergehende Störungen, sondern um starke Selektionskräfte, die die evolutionären Bahnen exponierter Organismen verändern können.

Die traditionelle Ökotoxikologie hat sich in erster Linie darauf konzentriert, kurzfristige toxische Effekte zu bewerten und sichere Expositionsgrenzwerte festzulegen, um Überleben und Wachstum zu gewährleisten. Auch wenn dieser Fokus für regulatorische Rahmenwerke wesentlich ist, weist er erhebliche Einschränkungen bei der Berücksichtigung der langfristigen evolutionären Auswirkungen chronischer Exposition gegenüber anthropogenen Stressoren auf. Infolgedessen gelingt es traditionellen Ansätzen häufig nicht zu berücksichtigen, wie persistente Kontamination evolutionäre Veränderungen über Generationen hinweg vorantreiben kann. Populationen, die kontinuierlich Umweltverschmutzung ausgesetzt sind, können adaptive Veränderungen durchlaufen, die nicht nur ihre unmittelbare Lebensfähigkeit, sondern auch ihre genetische Diversität und ihr langfristiges evolutionäres Potenzial beeinflussen. Im Laufe der Zeit können solche Veränderungen Kaskadeneffekte auslösen, die das ökologische Gleichgewicht stören, wodurch Ökosysteme anfälliger für zusätzliche Stressoren und weniger widerstandsfähig gegenüber ihrer Regeneration werden.

Diese Konzepte bilden das Fundament der evolutionären Ökotoxikologie, eines aufstrebenden Forschungsfeldes, das Evolutionsbiologie, Populationsgenetik und Ökotoxikologie in einem einheitlichen Rahmen integriert. Ihr Hauptziel ist es zu verstehen, wie chronische Schadstoffexposition genomweite und populationsbezogene Veränderungen antreibt. Obwohl die konzeptuellen Grundlagen bereits vor fast zwei Jahrzehnten entwickelt wurden, blieb die evolutionäre Ökotoxikologie aufgrund technologischer Einschränkungen weitgehend theoretisch, was ihre Integration in die Umwelt-Risikoabschätzung (ERA) verzögerte und die Fähigkeit einschränkte, langfristige ökologische Auswirkungen vorherzusagen. Darüber hinaus ist Umweltkontamination in realen Szenarien von Natur aus komplex. Schadstoffe wie Schwermetalle, Mikroplastik und chemische Gemische wirken selten

isoliert, sondern üben vielschichtige Selektionskräfte aus, die über eine einfache additive Toxizität hinausgehen. Diese kombinierten Stressoren verändern sowohl phänotypische Merkmale als auch genetische Strukturen über aufeinanderfolgende Generationen hinweg und treiben Populationen dazu, Resistenzmechanismen zu entwickeln oder lokal auszusterben.

Meine Doktorarbeit zielte darauf ab, diese Lücke zu schließen und die evolutionäre Ökotoxikologie von einem theoretischen Konzept zu einer praktisch anwendbaren Wissenschaft mit direkten Implikationen für ERA und den Erhalt von Ökosystemleistungen weiterzuentwickeln. Um dieses Ziel zu erreichen, entwarf ich eine Reihe von Experimenten, die sowohl Einzelkontaminanten als auch Sedimente aus städtischem Oberflächenabfluss untersuchten, welche realistische und chemisch komplexe Umweltgemische darstellen. Die Zuckmückenart *Chironomus riparius*, eine etablierte Modellorganismus in der Süßwasser-Ökotoxikologie, wurde aufgrund ihres kurzen Lebenszyklus, ihrer hohen Reproduktionsrate und ihrer Empfindlichkeit gegenüber Schadstoffen ausgewählt, was sie besonders geeignet für evolutionäre Studien macht.

Während Schwermetalle (wie Cadmium) und Mikroplastik weitgehend hinsichtlich ihres mutagenen Potenzials untersucht wurden, blieben polyzyklische aromatische Kohlenwasserstoffe (PAKs), obwohl sie Hauptbestandteile von Sedimenten aus städtischem Oberflächenabfluss sind, weitgehend unerforscht. Um diese Lücke zu schließen, konzentrierte sich Kapitel 1 auf die Exposition von *C. riparius*-Populationen gegenüber Benzo[*a*]pyren (BaP), einem repräsentativen PAK, in zwei Konzentrationen (10 µg/L und 100 µg/L) über drei Generationen hinweg. Sein mutagenes Potenzial wurde mithilfe von kurzzeitigen Mutationsakkumulationslinien (MAL) und Ganzgenomsequenzierung (WGS) bewertet, was eine signifikante Zunahme von Keimbahnmutationen bei 100 µg/L zeigte, während bei 10 µg/L keine nachweisbaren Effekte beobachtet wurden. Um Fitnessveränderungen zu bewerten, maß ich wichtige Lebenszyklusmerkmale, darunter Mortalität, mittlere Emergenzzeit (EmT50), Fruchtbarkeit, Larvengröße und Populationswachstumsrate (PGR) über mehrere Generationen hinweg. Die chronische Exposition gegenüber hohen BaP-Konzentrationen führte durchweg zu einer Reduktion der PGR, ohne Anzeichen einer Anpassung. Überraschenderweise führte die BaP-Exposition auch zu einem Rückgang der Produktion reaktiver Sauerstoffspezies (ROS), was darauf hindeutet, dass oxidativer Stress nicht der Hauptmechanismus für die beobachtete Mutagenität war.

Zur Erweiterung dieser Ergebnisse untersuchte ich in Kapitel 2 das mutagene Potenzial von Sedimenten aus städtischem Oberflächenabfluss, einer chemisch vielfältigen und heterogenen Matrix, die außerdem reich an organischer Substanz ist. Diese komplexe Mischung entsteht durch die Sedimentation von Partikeln, die durch städtischen Regenwasserabfluss transportiert und in Sedimentationsbecken abgelagert werden – künstliche Teiche, die strategisch in der Nähe stark befahrener Gebiete angelegt sind. Mithilfe derselben MAL-WGS-Methodik setzte ich *C. riparius* über fünf Generationen hinweg einer niedrigen (0,5 %) und einer hohen (10 %) Konzentration des Sediments aus. Beide Expositionsstufen führten zu einem Anstieg der Mutationsrate um 50 % im Vergleich zur Kontrollgruppe, unabhängig von der Konzentration. Die Analyse der Mutationsspektren zeigte eine starke Ähnlichkeit mit den durch BaP ausgelösten Profilen, was darauf hindeutet, dass PAK-ähnliche Verbindungen im Sediment Hauptverursacher waren. Die chemische Charakterisierung bestätigte das Vorhandensein verkehrsbedingter Schadstoffe, darunter verschiedene PAKs, Reifen- und Straßenabriebspartikel (TRWPs), Schwermetalle und Mikroplastik in Konzentrationen, die mit ähnlichen Matrices übereinstimmen, was die Repräsentativität und ökologische Relevanz des Sediments unterstreicht.

Um die Auswirkungen von Sedimenten aus städtischem Oberflächenabfluss weiter zu untersuchen, bewertete ich in Kapitel 3 deren akute Toxizität in *C. riparius* über eine Reihe von Konzentrationen (0,5 %, 1 %, 2 %, 5 %, 10 %, 20 %) hinweg, wobei wichtige Lebenszyklusmerkmale innerhalb einer Generation gemessen wurden. Die Exposition gegenüber dem Sediment führte bereits bei niedrigen Konzentrationen zu einem Anstieg der Mortalität um fast 30 %, wobei der Effekt jedoch nicht streng konzentrationsabhängig war. Darüber hinaus wurden deutliche Rückgänge der Fruchtbarkeit, verzögerte EmT50-Werte und veränderte Larvengrößen festgestellt, was auf signifikante subletale Effekte hinweist. Die Berechnung der PGR zeigte zudem eine konzentrationsabhängige Abnahme, was die ökologischen Risiken städtischer Sedimentkontamination unterstreicht.

In Kapitel 4 führte ich einen evolutionären Lebenszyklustest in Kombination mit dem Evolve-and-Resequence-(E&R)-Ansatz an *C. riparius* durch. Ziel war es zu ermitteln, ob eine chronische Exposition gegenüber Sedimenten aus städtischem Oberflächenabfluss adaptive Evolution über mehrere Generationen hinweg antreiben kann. Zwei Konzentrationen – niedrig (0,5 %) und hoch (10 %) – wurden getestet, um mögliche unterschiedliche evolutionäre Reaktionen zu erfassen. Lebenszyklusmerkmale

wurden über sieben Generationen hinweg verfolgt, wobei nichtlineare Fitnessverläufe, transgenerationale Effekte, Leistungswiederherstellungen und Hinweise auf Trade-offs in der Lebensgeschichte festgestellt wurden – alles Anzeichen adaptiver Veränderungen. Zur Aufdeckung der genetischen Grundlagen dieser Reaktionen verfolgte ich Veränderungen in der Allelfrequenz über die Zeit. Die genomische Analyse zeigte signifikante Allelfrequenzverschiebungen (AFCs) an mehreren Loci und identifizierte Kandidatenhaplotypen, die für breite Stressantwortpfade angereichert waren und unter Selektionsdruck standen. Diese Muster deuten auf eine schnelle polygenetische Anpassung an städtische Sedimentkontamination hin und zeigen, wie chronische Exposition genomweite evolutionäre Veränderungen antreiben kann. Bemerkenswerterweise traten ähnliche Selektionseffekte auch in den Kontrollpopulationen auf, was darauf hindeutet, dass bereits die Laborbedingungen Selektionsdruck ausübten.

Meine Gesamtergebnisse stellen konventionelle Annahmen der ERA infrage, die Empfindlichkeit gegenüber Schadstoffen oft als generationenübergreifend konstant betrachten. Durch den Nachweis einer multigenerationellen Anpassung an komplexe Schadstoffgemische unterstreichen meine Ergebnisse die Notwendigkeit, evolutionäre Prinzipien in Umweltbewertungsrahmen zu integrieren, um langfristige ökologische Risiken besser vorhersagen zu können.

Meine Ergebnisse festigen die Rolle der evolutionären Ökotoxikologie als praktische und wirkungsvolle Wissenschaft, die theoretische Spekulationen überwindet und reale Anwendungen unterstützt. Die präsentierten Belege untermauern einen grundlegenden Paradigmenwechsel: Umweltkontaminanten sind nicht nur toxische Substanzen, sondern starke Treiber evolutionärer Veränderungen, die genetische Vielfalt und Populationsdynamiken umgestalten. Diese evolutionären Auswirkungen zu erkennen ist entscheidend – nicht nur für den Erhalt der Biodiversität, sondern auch für den Schutz jener Ökosystemleistungen, die das menschliche Wohlergehen sichern.

SUMMARY

In the Anthropocene, human activities such as widespread pollution, habitat degradation, and climate change have profoundly transformed ecosystems. These are not mere transient disturbances but represent powerful selective pressures that can alter the evolutionary trajectories of exposed organisms.

Traditional ecotoxicology has primarily focused on short-term effects of single contaminants, defining safe exposure levels from endpoints such as survival and growth. While this focus is essential for regulatory frameworks, it has significant limitations in addressing the long-term evolutionary impacts of chronic exposure to anthropogenic stressors. As a result, traditional approaches often fail to account for how persistent contamination can drive evolutionary changes across generations.

Populations continuously exposed to environmental pollutants may undergo adaptive changes that affect not only their immediate viability but also their genetic diversity and long-term evolutionary potential. Over time, such shifts can trigger cascading effects that disrupt ecological balance, leaving ecosystems more vulnerable to additional stressors and less resilient in their capacity for recovery.

These concepts form the cornerstone of evolutionary ecotoxicology, an emerging field that integrates evolutionary biology, population genetics, and ecotoxicology into a unified framework. Its primary objective is to understand how chronic pollutant exposure drives genome-wide and population-level changes. Although its conceptual basis was established nearly two decades ago, evolutionary ecotoxicology remained largely theoretical due to technological constraints, delaying its integration into environmental risk assessment (ERA) and limiting the ability to predict long-term ecological impacts.

Moreover, contamination in real-world scenarios is inherently complex. Pollutants such as heavy metals, microplastics, and chemical mixtures rarely act in isolation, instead imposing multifaceted selective pressures that transcend simple additive toxicity. These combined stressors reshape both phenotypic traits and genetic architectures across successive generations, driving populations to develop resistance mechanisms or face local extinction.

My doctoral research aimed to bridge this gap, advancing evolutionary ecotoxicology from theoretical concepts to a practically applicable science with direct implications for ERA and the preservation of ecosystem services. To achieve this goal, I designed a series of experiments targeting both single-contaminant exposure and urban runoff sediments,

representing realistic and chemically complex environmental mixtures. The non-biting midge *Chironomus riparius*, a well-established sentinel species in freshwater ecotoxicology, was used as the model organism due to its short life cycle, high reproductive rate, and sensitivity to pollutants, all of which make it particularly suitable for evolutionary studies.

While heavy metals, such as cadmium, and microplastics have been widely investigated for their mutagenic potential, polycyclic aromatic hydrocarbons (PAHs) remain understudied, despite being major constituents of urban runoff sediment. To address this gap, Chapter 1 focused on the exposure of *C. riparius* populations to benzo[*a*]pyrene (BaP), a representative PAH, at two concentrations (10 µg/L and 100 µg/L) over three generations. Its mutagenic potential was assessed using short-term mutation accumulation lines (MAL) and whole-genome sequencing (WGS), which revealed a significant increase in germline mutations at 100 µg/L, but no detectable effects were observed at 10 µg/L. To evaluate fitness alterations, key life-cycle traits were measured across three generations, including mortality, mean emergence time (EmT50), fertility, larval size, and population growth rate (PGR). Chronic exposure to high BaP concentrations consistently led to reductions in PGR, with no signs of adaptation. Surprisingly, BaP exposure also resulted in decreased levels of reactive oxygen species (ROS) production, indicating that oxidative damage was not the primary mechanism driving the observed mutagenicity.

To expand on these results, in Chapter 2 I explored the mutagenic potential of urban runoff sediment, a chemically diverse and heterogeneous matrix also rich in organic matter. This complex mixture originates from the sedimentation of particles transported by urban runoff, accumulating in sedimentation basins, which are artificial ponds strategically located near high-traffic areas. Using the same MAL-WGS methodology, I exposed *C. riparius* to a low concentration (0.5%) and a high concentration (10%) of urban runoff sediment, over five generations. Both exposure levels induced a 50% increase in mutation rates compared to controls, regardless of concentration. Comparative mutational spectra analysis indicated a strong resemblance to the profiles triggered by BaP, suggesting that PAH-like compounds in the sediment were major contributors. Chemical characterization confirmed the presence of traffic-derived contaminants, including various PAHs, tyre and road wear particles (TRWPs), heavy metals, and microplastics, at levels consistent with similar matrices, highlighting the sediment's representativeness and ecological relevance.

To further investigate the impact of urban runoff sediment, in Chapter 3 I evaluated its acute toxicity over a range of concentration (0.5%, 1%, 2%, 5%, 10%, 20%) in *C. riparius*, measuring key life-cycle traits in a single generation. Exposure to urban runoff sediment

increased mortality by nearly 30%, even at low concentrations, though the effect was not strictly concentration dependent. Moreover, substantial declines in fertility, delayed EmT50, and altered larval size were observed, suggesting significant sub-lethal effects. PGR calculations further indicated a concentration-dependent decrease, highlighting the ecological risks of urban runoff contamination.

In Chapter 4, I performed an evolutionary life-cycle test combined with the Evolve and Resequence (E&R) approach on *C. riparius*. The aim was to determine whether chronic exposure to urban runoff sediment could drive adaptive evolution across multiple generations. Two sediment concentrations, low (0.5%) and high (10%), were tested to capture potential differential evolutionary responses. Life-cycle traits were monitored over seven generations, revealing non-linear fitness trajectories, including transgenerational effects, performance recovery, and evidence of life-history trade-offs, indicative of adaptive changes. To uncover the genetic basis of these responses, I tracked allele frequency changes over time. Genomic analysis revealed significant allele frequency changes (AFCs) across multiple loci, identifying candidate haplotypes enriched for broad stress-response pathways under selective pressure. These patterns indicated rapid polygenic adaptation to urban runoff contamination, showing how chronic exposure can drive genome-wide evolutionary changes. Notably, similar selection signals also emerged in control populations, suggesting that laboratory conditions themselves imposed selective pressures.

My overall results challenge conventional assumptions of ERA, which often view sensitivity to pollutants as static and unchanging across generations. By demonstrating multigenerational adaptation to complex contaminant mixtures, my results highlight the importance of integrating evolutionary principles into environmental assessment frameworks to improve long-term ecological risk predictions. My findings solidify the role of evolutionary ecotoxicology as a practical and impactful science, transcending theoretical speculation to guide real-world applications.

The evidence presented underscores a critical paradigm shift, showing that environmental contaminants are not simply toxic agents but powerful drivers of evolutionary change that reshape genetic diversity and population dynamics. Recognizing these evolutionary impacts is essential not only for biodiversity conservation, but also for preserving the ecosystem services that sustain human well-being.

GENERAL INTRODUCTION

1. Human-Driven Environmental Change and the Rise of the Anthropocene

The history of life on Earth is characterized by a continuous transformation, a dynamic narrative of adaptation, diversification, and extinction shaped by the interaction between abiotic and biotic forces.

Over millions of years, climatic fluctuations, tectonic activity, and geological processes have remodelled landscapes and influenced the distribution of ecosystems, while evolutionary pressures have driven species adaptation and structured ecological interactions (Carroll et al., 2007; Erwin, 2009; Sharp, 1982). Within this broader evolutionary trajectory, the relatively recent emergence of *Homo sapiens* introduced an ecological agent of unprecedented transformative magnitude (Boivin et al., 2016).

As early as the Pleistocene, archaeological evidence shows that human groups actively modified their environments through controlled fire use, cooperative hunting, and targeted landscape alterations, leaving the earliest discernible imprints on ecosystems (Boivin et al., 2016; Ellis et al., 2021; Thompson et al., 2021). These activities reflected not only adaptation to environmental conditions but also the emergence of deliberate human ecosystem management, shaped by cultural values and practical needs (Ellis et al., 2021).

The transition into the Holocene, characterized by greater climatic stability, facilitated the independent development of agriculture and animal domestication across several regions (Adams and Faure, 1997; Feynman and Ruzmaikin, 2007). This led to significant transformations in land use, including vegetation clearance, habitat fragmentation, and water management, progressively reshaping natural landscapes (Ellis and Ramankutty, 2008; Kaplan et al., 2011; Montgomery, 2007).

As human societies grew in complexity, so did their capacity to manage and exploit natural systems. Early state societies in Mesopotamia, the Indus Valley, and the Mediterranean basin developed extensive infrastructure for irrigation, transportation, and urban planning, reflecting an emerging vision of nature as a domain to be systematically organized (Adams, 1981; Possehl, 2002; Scott, 2017). This utilitarian perception reached a sophisticated expression in Roman civilization, where natural resources were systematically integrated into administrative and economic frameworks through monumental projects such as aqueducts, road networks, and drainage systems (Alcock, 1995; Squatriti, 1998). In medieval and early

modern Western Europe, environmental control became increasingly systematic, driven by expanding bureaucracy and global trade, leading to formal resource management (Braudel et al., 1992; Hoffmann, 2014).

By the eighteenth century, significant transformations in human–environment interactions had occurred, reflecting broader social and technological changes that would characterize the Industrial Revolution (Crutzen and Stoermer, 2021; McNeill, 2001). Originating in Britain and spreading across Europe and beyond, this era marked a profound shift away from localized economies toward industrial production concentrated in factories and powered by fossil energy sources such as coal, oil, and natural gas (Allen, 2009; Broadberry and O'Rourke, 2010; Malm, 2016; Sieferle, 2001). This new energy regime sustained industrial growth, while rapid population expansion intensified pressure on natural resources, leading to widespread deforestation and land conversion to support urban and industrial infrastructure (McNeill, 2001; Wohl, 2020).

The widespread combustion of fossil fuels released massive amounts of greenhouse gases into the atmosphere, fundamentally altering atmospheric composition and driving global climate change and ecosystem instability (Hansen et al., 2006; IPCC, 2023; Mann et al., 1999; Ruddiman, 2008). Simultaneously, the intensification of agriculture accelerated habitat loss, chemical pollution, and nutrient runoff, placing additional stress on terrestrial and aquatic ecosystems (Tilman et al., 2001; Vitousek et al., 1997). The cumulative effect and increasing intensity of these anthropogenic pressures contributed to the conceptualization of the Anthropocene (Crutzen and Stoermer, 2021), defined by the unprecedented scale at which human activity dominates and alters Earth system processes (Steffen et al., 2007; Zalasiewicz et al., 2008).

A defining feature of this epoch is the widespread and long-term release of synthetic and industrial chemical contaminants into the environment. These include polycyclic aromatic hydrocarbons (PAHs), heavy metals, microplastics, pesticides, and pharmaceuticals, primarily originating from industrial, agricultural, and urban sources (Crutzen, 2002; Hale et al., 2020; Steffen et al., 2007; Wilkinson et al., 2022). Their global accumulation currently represents one of the most persistent and far-reaching consequences of human activity, with profound implications for ecological stability and resilience (van Straalen and Feder, 2012).

2. Integrating Toxicology and Ecology: The Emergence of Ecotoxicology

By the mid-twentieth century, the environmental impacts of synthetic chemical contamination became undeniably evident. Industrial disasters such as the mercury poisoning

in Minamata, Japan (Harada, 1995), and the catastrophic fires on the Cuyahoga River in the United States (Stradling and Stradling, 2015) demonstrated that pollution was no longer a localized concern, but a widespread and systemic threat to both ecosystems and human health. These incidents, along with the growing global awareness of environmental degradation in the post-World War II era, underscored the urgent need to better understand how pollutants interact with living systems, exposing a critical gap in the scientific knowledge of the time.

In 1969, René Truhaut, a French toxicologist and public health expert, introduced the term ecotoxicology to define a new scientific field focused on assessing the effects of toxic substances within ecological frameworks (Truhaut, 1975). This marked a conceptual shift, extending toxicology beyond its traditional anthropocentric and individual-level focus and laying the foundation for a more integrated approach to chemical risk assessment.

Until that time, toxicology and ecology had largely evolved as separate scientific domains. Toxicology was primarily rooted in biomedical sciences, focusing on identifying dose–response relationships and establishing thresholds of adverse effects in humans or in laboratory organisms used as proxies for human health (Klaassen et al., 2010). Ecology, on the other hand, explored the structure and dynamics of populations, communities, and ecosystems but lacked the mechanistic tools necessary to examine the biological impacts of chemical contaminants at higher levels of biological organisations (Ecological Understanding, 2007). As a result, neither discipline was fully equipped to evaluate the broader ecological consequences of pollution (Cairns, 2014). Ecotoxicology emerged to bridge this gap, integrating mechanistic toxicological insights with ecological perspectives to investigate how chemical stressors affect organisms at multiple levels of biological organisation, from molecular pathways to ecosystem processes (Newman, 2019).

Since its early development, ecotoxicology has become central to Environmental Risk Assessment (ERA), the systematic evaluation of the probability and severity of adverse effects resulting from environmental exposure to chemicals (De Luca Peña et al., 2022). To quantify these effects, ecotoxicologists rely on standardised bioassays that generate measurable endpoints crucial for regulatory decision-making. Commonly used metrics include the LC50 (Lethal Concentration causing mortality in 50% of test organisms), the EC50 (Effective Concentration inducing a specific sublethal effect in 50% of organisms), and the NOEC (No Observed Effect Concentration), which define thresholds below which chemicals are generally considered unlikely to pose significant ecological risk (Forbes and Calow, 2002; Rand and Petrocelli, 1985; Stephan, 1977).

These assays are typically performed on a relatively limited set of model species, selected primarily for practical reasons such as ease of laboratory culture and short life cycles. These characteristics facilitate consistent testing protocols and ensure reliable data comparisons across studies. In aquatic ecotoxicology, *Daphnia magna*, a small freshwater crustacean known for its high reproductive rate and extensive toxicological data, has become a cornerstone test species (OECD, 2012, 2004). Alongside this invertebrate model, vertebrate species like the zebrafish (*Danio rerio*) and the fathead minnow (*Pimephales promelas*) are widely used in both acute and chronic toxicity tests, offering crucial insights into the effects of pollutants on vertebrate development and physiology (Nagel, 2002; OECD, 2025; Wang et al., 2021).

Nevertheless, relying on a limited group of standard species presents inherent limitations, as these organisms reflect only a small portion of the ecological diversity found in natural environments. To address this constraint and improve ecological relevance, ecotoxicologists have progressively incorporated additional species into testing protocols. For example, *Chironomus* larvae, which inhabit sediments and play significant roles in freshwater food webs, have become valuable indicators for assessing sediment and benthic contamination, thus linking laboratory results more closely to ecological impacts observed at the community level (OECD, 2023, 2010).

To ensure that toxicity data remain scientifically robust and comparable across jurisdictions, international regulatory bodies have established comprehensive guidelines for ecotoxicological testing. Organisations such as the Organisation for Economic Co-operation and Development (OECD), the United States Environmental Protection Agency (USEPA), and the Society of Environmental Toxicology and Chemistry (SETAC) have developed detailed protocols covering experimental design, species selection, measurement endpoints, statistical analyses, and criteria for data validity (Rosenbaum et al., 2008; USEPA, 1998). While these regulatory frameworks are crucial for maintaining scientific credibility and supporting regulatory decisions, their stringent requirements can also impose constraints, potentially limiting the use of non-standard species or innovative methodologies that might better capture the complexity and variability of natural ecosystems (Newman, 2019).

Although faced with significant challenges, ecotoxicology has established itself as a cornerstone of contemporary ERA, providing researchers and regulatory bodies with reliable tools to assess the ecological impacts of chemical pollution and to guide strategies for protecting ecosystem integrity (Forbes and Calow, 2002; Malaj et al., 2014; Newman, 2019).

3. Evolutionary Perspectives and Exposure Complexity in Ecotoxicology

One of the key limitations of conventional ecotoxicological approaches lies in their limited capacity to account for evolutionary dynamics unfolding over extended timescales (Newman, 2019; Sibly and Calow, 1989). Most testing protocols focus on single-species exposures conducted over short periods, typically lasting from a few hours to several days, or at most a single generation (Newman, 2019; OECD, 2012, 2004). These tests implicitly assume that organismal sensitivity to toxicants remains static, overlooking the potential for both physiological acclimation and heritable evolutionary responses under prolonged contaminant pressure (Brady et al., 2017; Dallinger and Höckner, 2013; Rodríguez-Romero et al., 2021).

However, empirical evidence shows that sustained chemical exposure can drive heritable genetic change over short timescales, challenging the assumption of static sensitivity (Klerks and Levinton, 1989; Oziolor and Matson, 2015).

One of the first documented cases dates to the early 20th century, when pesticide resistance was observed in the scale insect *Quadraspidiotus perniciosus* (Melander, A. L., 1914). In the following decades, additional cases of resistance were documented, including DDT-tolerant vertebrates such as laboratory mice (Ozburn and Morrison, 1962), as well as amphibians and freshwater fish inhabiting intensively treated agricultural landscapes (Boyd et al., 1963; Boyd and Ferguson, 1964).

More recent studies have provided increasingly ecologically realistic examples of evolved tolerance in wild aquatic organisms. For instance, Klerks and Levinton (1989) showed that *Limnodrilus hoffmeisteri,* a sediment-dwelling oligochaete worm, has developed tolerance to cadmium in contaminated sites along the Hudson River, exhibiting both physiological and genetically determined resistance. Similarly, Nacci et al., (2010) discovered that *Fundulus heteroclitus*, a small estuarine fish species, has evolved resistance to high levels of polychlorinated biphenyls (PCBs) in heavily polluted coastal habitats, with evidence pointing to adaptive alterations in the aryl hydrocarbon receptor (AHR) pathway and associated detoxification mechanisms.

Together, these observations have contributed to a growing body of evidence that challenges the traditional view of evolution as a slow and gradual process. Instead, they support the understanding that intense anthropogenic selective pressures can induce rapid evolutionary change over ecologically relevant timescales (Carroll et al., 2007; Hendry and Kinnison, 1999). This conceptual shift has been reinforced by advances in evolutionary theory and, more recently, by the development of molecular, population, and ecological genomics, which have

significantly improved our understanding of how selection operates under environmental stress (Ellegren, 2014; Stapley et al., 2010).

Furthermore, while adaptation to a single stressor appears feasible in several cases, emerging evidence suggests that simultaneous adaptation to multiple stressors, such as chemical pollutants combined with rising temperatures or other ecological changes, may be considerably more challenging (Todgham and Stillman, 2013; Shahid et al., 2024). This limitation implies that, although evolvability can provide some species with the capacity to withstand individual stressors, populations exposed to complex and interacting environmental pressures may still experience declines or face the risk of local extinction (Briski et al., 2025a; Holmstrup et al., 2010; Todgham and Stillman, 2013).

3.1 Evolutionary Ecotoxicology in the Genomic Era

Although significant progress has been made in integrating evolutionary perspectives into ecotoxicology, their application remained limited and largely underdeveloped during the early 2000s. Technical and methodological limitations, such as the absence of tools to monitor genome-wide variation across generations, constrained the ability to detect and quantify contaminant-driven evolutionary responses in natural populations (Forbes and Calow, 2002). The advent of high-throughput sequencing and advanced analytical approaches has progressively overcome these barriers, enabling the integration of molecular and population-level data into ecotoxicological research (Barrick et al., 2017).

Building on these developments, evolutionary ecotoxicology has emerged as an interdisciplinary field combining evolutionary biology, population genetics, and ecotoxicology to study heritable responses to chemical stress across generations (Brady et al., 2017; Coutellec and Barata, 2011; Straub et al., 2020). By combining evolutionary theory with genomic and computational tools, it is now possible to investigate adaptation with unprecedented precision.

Experimental evolution approaches such as mutation accumulation (MA) lines and Evolve and Resequence (E&R) provide robust frameworks to explore contaminant-induced evolutionary change.

In MA experiments, replicate lineages are propagated from a single ancestor under minimal selection, promoting the accumulation of spontaneous mutations. Whole-genome sequencing at the end of the experiment allows for the estimation of mutation rates and spectra under different exposure regimes (Bulut et al., 2024; Oppold and Pfenninger, 2017; Rigano et al., 2025b).

In E&R experiments, replicate populations are exposed to defined selection pressures over multiple generations. Sampling and sequencing (e.g., Pool-Seq) at successive time points enable the detection of allele frequency shifts and the identification of loci under selection (Kofler et al., 2011a, 2011b; Rigano et al., 2025a; Schlötterer et al., 2015).

When combined with phenotypic data, including survival, development, fertility, and population growth, these approaches provide a comprehensive picture of evolutionary responses to pollution (Bulut et al., 2024; Rigano et al., 2025a; Sibly and Calow, 1989). This strengthens the ability to establish causal links between exposure and heritable change.

Together, these methodological advances offer a solid foundation for investigating evolution in polluted environments (Spurgeon et al., 2020; van Straalen and Feder, 2012), and supporting the long-overdue integration of evolutionary principles into ecological risk assessment and long-term monitoring (Straub et al., 2020).

4. Contaminants as Agents of Evolutionary and Ecological Change

The biological effects of contaminants are multifaceted, acting across multiple levels of biological organization, from molecular disruptions to alterations in individual fitness, population structure, and ultimately, ecosystem functioning (Chapman, 2002; Newman, 2019).

At the molecular level, contaminants may compromise genomic integrity and stability through several mechanisms, including the formation of DNA adducts, induction of strand breaks, and interference with replication and repair pathways essential for maintaining genome integrity (Belfiore, 2001; Newman, 2019). These genotoxic effects can generate mutations, some of which, if occurring in germline cells, may become heritable and introduce novel genetic variation into populations (Loewe and Hill, 2010). While most mutations are neutral or deleterious, under strong contaminant-driven selection certain variants may confer fitness advantages, providing the substrate for rapid adaptation (Eyre-Walker and Keightley, 2007; Rigano et al., 2025b; Scott and Sloman, 2004).

Many contaminants also increase intracellular levels of reactive oxygen species (ROS), leading to oxidative damage to lipids, proteins, and nucleic acids, and disrupting cellular redox homeostasis (Dröge, 2002; Lushchak, 2011; Rigano et al., 2024). In addition, they can interfere with transcriptional and translational processes, altering gene expression and compromising essential metabolic and cellular functions (Ankley et al., 2010; Newman, 2019). These molecular effects can translate into selective pressures at the population level. Sustained exposure may select for tolerant genotypes, promoting progressive shifts in allele frequencies. Over relatively short evolutionary timescales, this process can lead to adaptive divergence

between exposed and unexposed populations (Oziolor and Matson, 2015; Rigano et al., 2025a). While these adaptations are beneficial in some ways, they frequently come with fitness trade-offs, including reduced fertility, delayed development, or increased sensitivity to other stressors, which may ultimately constrain long-term evolutionary success (Berger et al., 2017; Bulut et al., 2024; Rigano et al., 2025b).

Beyond direct genetic alterations, epigenetic mechanisms such as DNA methylation, histone modification, and small RNA regulation may contribute to transgenerational phenotypic variation without altering DNA sequence (Heard and Martienssen, 2014). These epigenetic modifications can modulate gene expression in response to contaminant exposure and may thus influence adaptive responses and heritability across multiple generations, presenting an additional layer of complexity in contaminant-induced evolution (Manikkam et al., 2012; Vandegehuchte and Janssen, 2014).

At the ecosystem level, contaminant-driven alterations in population structure and function can propagate through food webs, destabilizing trophic interactions and impairing essential ecological processes such as nutrient cycling, primary production, water purification, and carbon sequestration (Cardinale et al., 2012; Fleeger et al., 2003; Malaj et al., 2014). As population health integrates processes across molecular, physiological, and demographic scales, contaminant-induced changes often initiate cascading effects that ultimately alter community dynamics, biodiversity patterns, and ecosystem resilience (Chapman, 2002; Newman, 2019; Sibly and Calow, 1989).

These evolutionary and ecological processes unfold in inherently dynamic environments, where organisms are rarely exposed to a single contaminant in isolation. Instead, they experience complex mixtures of chemicals alongside fluctuating abiotic conditions such as temperature, pH, or oxygen availability. Such interactions often generate non-linear outcomes that cannot be predicted from single-compound studies, complicating both ecological forecasting and evolutionary inference (Crain et al., 2008; Reuben et al., 2022; Schäfer et al., 2023).

Collectively, the dynamic interactions among these multi-scale biological responses underscore the dual role of contaminants as both immediate ecological stressors and potent evolutionary agents (Sibly and Calow, 1989). Their capacity to simultaneously disrupt organismal performance and shape adaptive trajectories highlights the importance of explicitly incorporating evolutionary perspectives into assessments of contaminant-driven biological change.

5. Urban Runoff Sediments: From Representative Contaminants to Complex Environmental Mixtures

Urban areas, characterized by dense populations and intense vehicular traffic, present significant environmental challenges due to the diverse array of contaminants mobilized via urban runoff. Among these pollutants, tyre and road wear particles (TRWPs), polycyclic aromatic hydrocarbons (PAHs), heavy metals, and microplastics, ultimately accumulate in urban runoff sediments. These sediments represent a heterogeneous matrix of organic material and anthropogenic pollutants with important ecological consequences (Chiaia-Hernández et al., 2022; Kayhanian et al., 2012; Paul and Meyer, 2008).

TRWPs primarily originate from the mechanical abrasion of vehicle tyres against road surfaces, releasing a mixture of microscopic to macroscopic particles (Baensch-Baltruschat et al., 2021; Kole et al., 2017). Chemically, they consist of synthetic polymers (e.g., styrene-butadiene rubber), inorganic fillers (e.g., carbon black, silica, zinc oxide), and a wide array of chemical additives used in tyre manufacturing (Baensch-Baltruschat et al., 2021; Kole et al., 2017). Moreover, TRWPs can adsorb additional pollutants from the urban atmosphere and road surfaces, including PAHs, heavy metals, and persistent organic pollutants, thereby amplifying their environmental hazard (Baensch-Baltruschat et al., 2021).

The accumulation of these contaminants is strongly influenced by hydrological processes. During precipitation events, stormwater runoff mobilizes particles and dissolved substances from road surfaces and urban landscapes, transporting TRWPs, chemical pollutants, mineral particles, and organic matter through engineered drainage networks (Eriksson et al., 2007; Marsalek, 2014). Many of these materials are captured in urban runoff sedimentation basins, engineered ponds designed to retain suspended solids and associated pollutants before their release into natural water bodies (Liu et al., 2017). If not intercepted, these contaminants may be directly discharged into rivers, lakes, and coastal waters, where they accumulate in aquatic sediments (Baensch-Baltruschat et al., 2021).

Among the various pollutants typically found in urban runoff, polycyclic aromatic hydrocarbons (PAHs) are of particular concern due to their persistence, bioaccumulation potential, and known toxic effects. These compounds mainly originate from the incomplete combustion of organic matter, such as vehicle exhaust and industrial emissions, as well as from tyre wear (Grung et al., 2016; Tobiszewski and Namieśnik, 2012).

The environmental implications of urban runoff sediments are substantial and long-lasting. TRWPs contribute to microplastic pollution while PAHs, heavy metals, and associated

chemicals introduce toxic compounds into aquatic systems (Baensch-Baltruschat et al., 2021; Kole et al., 2017). The leaching of these substances poses multiple toxicological risks to aquatic organisms, including genotoxicity, oxidative stress, endocrine disruption, and physical damage (Bouredji et al., 2023). Additionally, their persistence and bioavailability raise concerns for long-term ecological impacts. These contaminants can be ingested by sediment-dwelling organisms, resulting in bioaccumulation and trophic transfer throughout food webs, potentially reaching higher trophic levels and humans (Baensch-Baltruschat et al., 2021; Bouredji et al., 2023; Meland et al., 2024).

More broadly, urban runoff sediments, enriched with TRWPs, PAHs, heavy metals, and organic material, represent a pervasive and persistent form of anthropogenic pollution arising from the interaction between vehicular activity and urban hydrology (Baensch-Baltruschat et al., 2021; Kole et al., 2017). Their complex and evolving chemical composition, combined with their long-term persistence and ecological consequences, highlights the need for comprehensive management strategies.

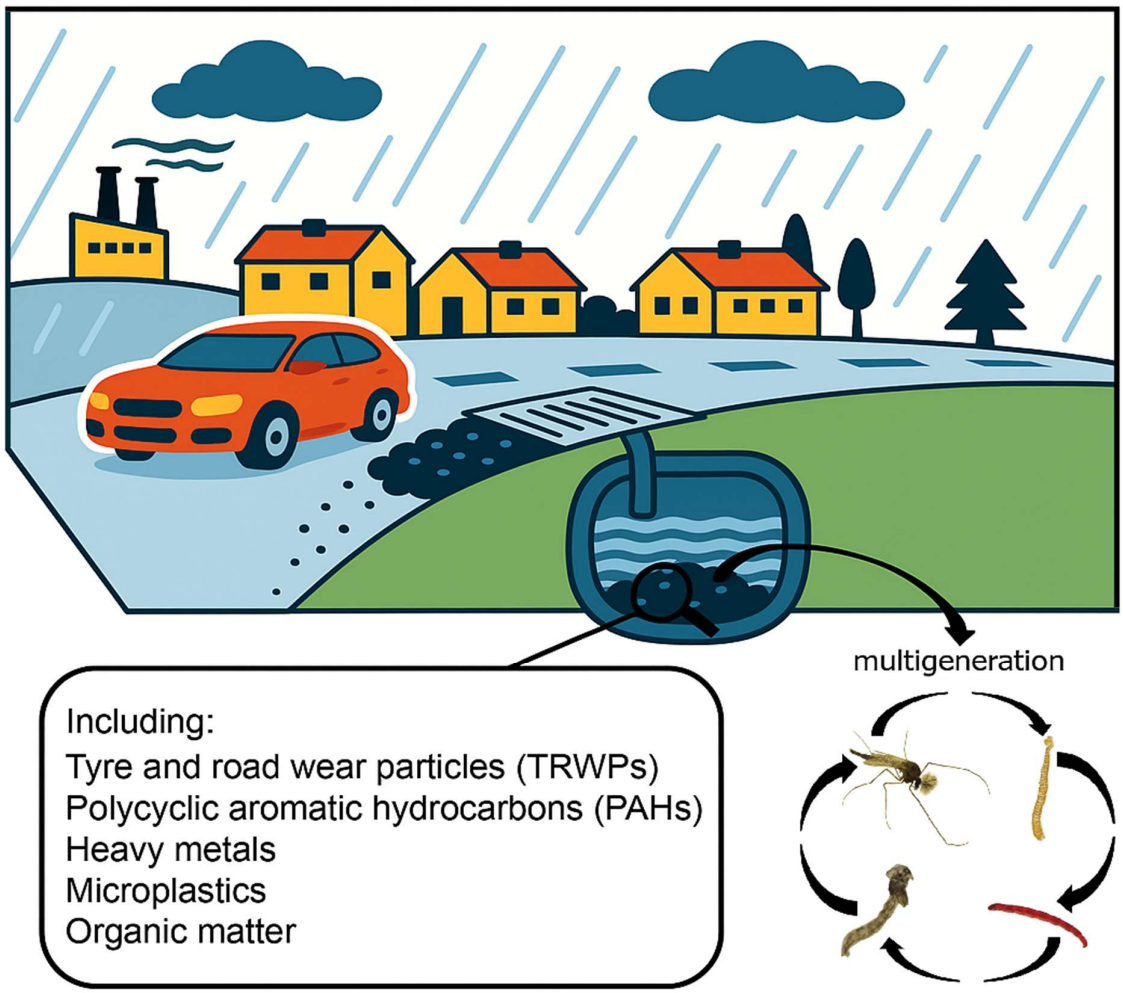

Figure 1. Schematic representation of urban runoff sediment generation and its relevance for evolutionary ecotoxicology using *Chironomus riparius* as a model species.
Urban runoff sediments accumulate in retention basins and consist of complex mixtures of contaminants and organic matter, primarily transported during precipitation events. *C. riparius* populations were exposed to these mixtures across multiple generations to investigate the evolutionary consequences of chronic chemical stress.
Adapted by the author, with original image elements courtesy of Quentin Foucault and Markus Pfenninger.

6. The Model Organism: *Chironomus riparius*

For my doctoral research, I employed the non-biting midge *Chironomus riparius* (Order: Diptera; Family: Chironomidae) as a model species due to its established relevance in

ecotoxicology and its suitability for investigating eco-evolutionary dynamics (Bickham, 2011; Foucault et al., 2019).

Chironomid midges, including *C. riparius*, are ecologically important components of freshwater ecosystems, where they act as primary consumers of organic detritus and contribute significantly to nutrient cycling (Armitage et al., 1995). Their ability to tolerate moderate pollution levels has made them valuable bioindicators in environmental monitoring (Ha and Choi, 2008; Heiri, 2013)

C. riparius is widely used in ecotoxicological testing, supported by internationally recognized protocols developed by agencies such as the OECD and the US EPA. These tests typically rely on the larval stage to assess sediment toxicity, with endpoints including survival, growth, emergence timing, and reproductive output (OECD, 2023, 2010). Its benthic lifestyle and sensitivity to a range of environmental contaminants, including heavy metals, pesticides, and polycyclic aromatic hydrocarbons, support its continued use in water and sediment quality assessment (Ristola et al., 1999).

The species exhibits a holometabolous life cycle with distinct egg, larval, pupal, and adult stages, all of which can be maintained under controlled laboratory conditions (Armitage et al., 1995; Foucault et al., 2019). Its short generation time, high fecundity, and ease of cultivation facilitate the maintenance of large and genetically diverse populations in experimental settings. This is essential for studying evolutionary processes, as it permits the retention of standing genetic variation, reduces the influence of random genetic drift, and enables reproducible multigenerational experiments (Bulut et al., 2024; Doria et al., 2022a; Pfenninger and Foucault, 2020).

The capacity of *C. riparius* to develop tolerance to various contaminants has been documented in multiple studies. These adaptive responses are often associated with enhanced detoxification capacity, such as the upregulation of glutathione S-transferases, or with modifications in physiological regulation (Doria et al., 2021; Marziali et al., 2019; Rigano et al., 2025a). These traits reinforce its utility in investigating contaminant-driven evolutionary responses.

Recent developments in genomic resources have further expanded the value of *C. riparius* as a model for evolutionary ecotoxicology. The availability of a high-quality reference genome (Schmidt et al., 2020), along with extensive single nucleotide polymorphism datasets and transcriptomic profiles, now enables genome-wide analyses of contaminant-induced changes (Bulut et al., 2024; Doria et al., 2022b; Rigano et al., 2025a, 2025b). Furthermore, increasing evidence of transgenerational and epigenetic responses to chemical stress

underscores the species' potential for elucidating long-term molecular mechanisms of adaptation (Klerks and Levinton, 1989; Vandegehuchte and Janssen, 2014)

Altogether, *C. riparius* offers an effective combination of ecological relevance, experimental flexibility, and genomic accessibility. This makes it a robust and versatile model system for exploring the evolutionary impacts of chemical stress in freshwater environments.

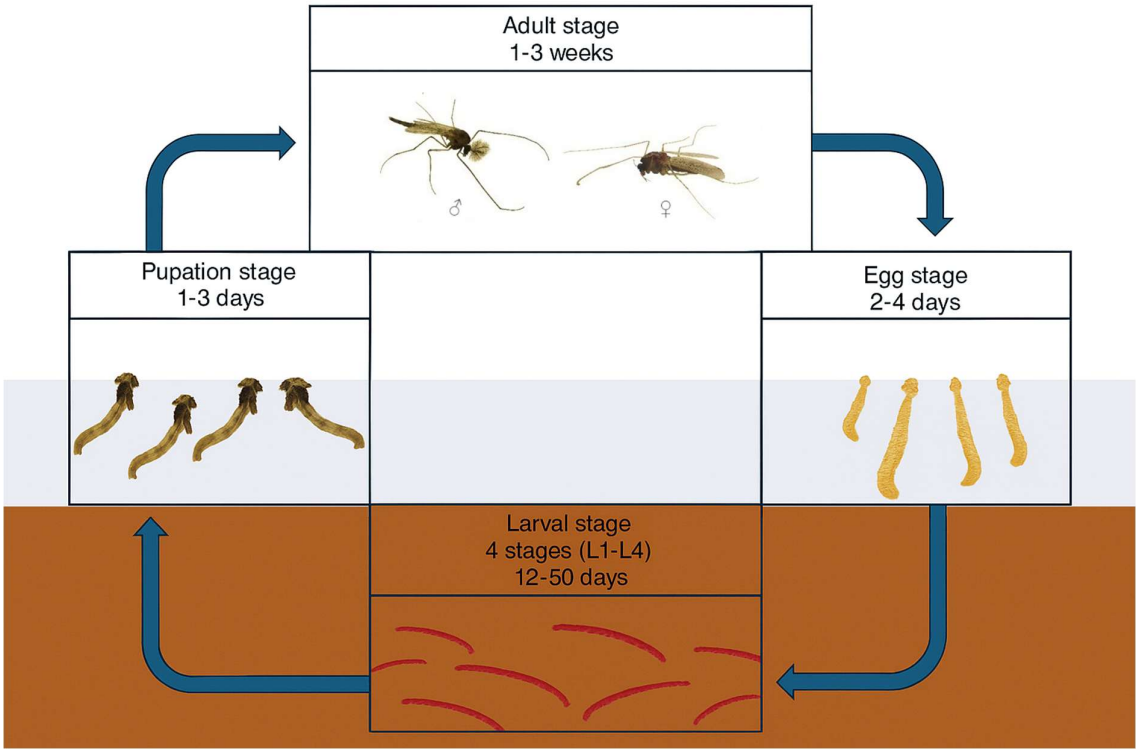

Figure 2. Life cycle of the freshwater midge *Chironomus riparius*.

It comprises four stages: egg, larva, pupa, and adult. The adult is the only aerial stage, while the others are strictly aquatic.

Adapted by the author, with original image elements courtesy of Quentin Foucault and Markus Pfenninger.

7. Aim of this thesis

This doctoral research aimed to advance evolutionary ecotoxicology by integrating multigenerational experimental designs with high-resolution genomic analyses to investigate how chronic contaminant exposure can influence evolutionary trajectories in exposed populations.

The broader objective was to strengthen the empirical and methodological foundations of the field, supporting its development as a quantitative and mechanistically informed

discipline. Although not directly focused on regulatory applications, my research was conceived with the perspective that evolutionary ecotoxicology could contribute to more realistic and predictive ecological risk assessment frameworks.

To this end, populations of *C. riparius* were exposed to two distinct contamination scenarios. The first involved benzo[a]pyrene (BaP), a single well-characterized compound frequently detected in urban runoff, tested under chronic multigenerational exposure. The second consisted of urban runoff sediments, a chemically complex field-derived mixture investigated both under acute and multigenerational exposure. This comparative design made it possible to directly assess how single-substance and mixture exposures influence evolutionary responses.

Evolutionary responses were investigated through a combination of multigenerational phenotypic assays and whole-genome sequencing with integrative bioinformatics approaches, addressing the following research questions:

i) Fitness and life-history effects

Does chronic exposure to BaP and to urban runoff sediments alter fitness-related and life-history traits across generations? Does acute exposure to urban runoff sediments affect these traits and oxidative stress, thereby providing a baseline for interpreting evolutionary responses?

ii) Mutation rate and mutational spectrum

Does long-term exposure to chemical stressors affect germline mutation rates or alter the spectrum of mutational events? Could such effects be mediated by oxidative DNA damage caused by reactive oxygen species (ROS)?

iii) Genomic signatures of selection

Are prolonged selective pressures associated with consistent shifts in allele frequencies consistent with selection and potentially indicative of adaptive evolution?

iv) Coupled evolutionary dynamics

How do phenotypic and genomic changes co-evolve over generations, and what do they reveal about adaptive evolution under contaminant pressure?

By addressing these questions within an integrated experimental and analytical framework, this thesis establishes a foundation for understanding how contaminants can

influence evolutionary processes in exposed populations. Thereby, it strengthens the empirical and methodological basis of evolutionary ecotoxicology and defines key directions for its future development as a quantitatively robust and mechanistically grounded discipline.

CHAPTER 1

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

Burak Bulut, Lorenzo Rigano, Halina Binde Doria, Gajana Gemüth, Markus Pfenninger

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Appendix to Chapter 1

Declaration of contributions

Publication title: *A multigenerational study can detect the evolutionary response to BaP exposure in the non-biting freshwater midge Chironomus riparius*
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Authors and contributions:

- **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.
  *Joint first authors.

Introductory note

While the mutagenic potential of heavy metals (e.g. cadmium) and microplastics has been widely investigated, polycyclic aromatic hydrocarbons (PAHs) remain comparatively understudied, despite their abundance in urban runoff sediments. Among them, benzo[a]pyrene (BaP) is considered a model compound due to its known carcinogenic properties and environmental relevance.

The aim of this first chapter was to assess whether multigenerational exposure of *C. riparius* populations to BaP could trigger detectable evolutionary and fitness-related responses. Populations were exposed to two concentrations (10 µg/L and 100 µg/L) across multiple generations, and mutation rate, key life-history traits, and oxidative stress markers (ROS) were measured.

This study addressed the central question of how chronic exposure to a major PAH can shape population-level responses across generations. The results established a reference point for

evaluating the evolutionary and ecotoxicological impacts of other classes of pollutants examined in later chapters. In this way, the work presented here links BaP exposure to the broader goal of understanding how multiple stressors influence population resilience in aquatic insects.

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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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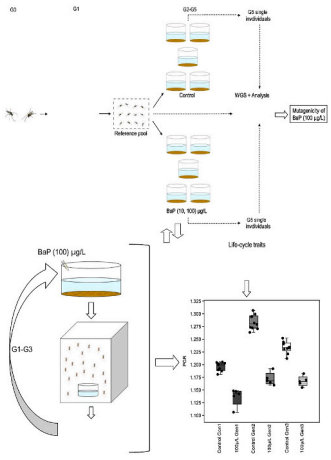
HIGHLIGHTS

- 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.

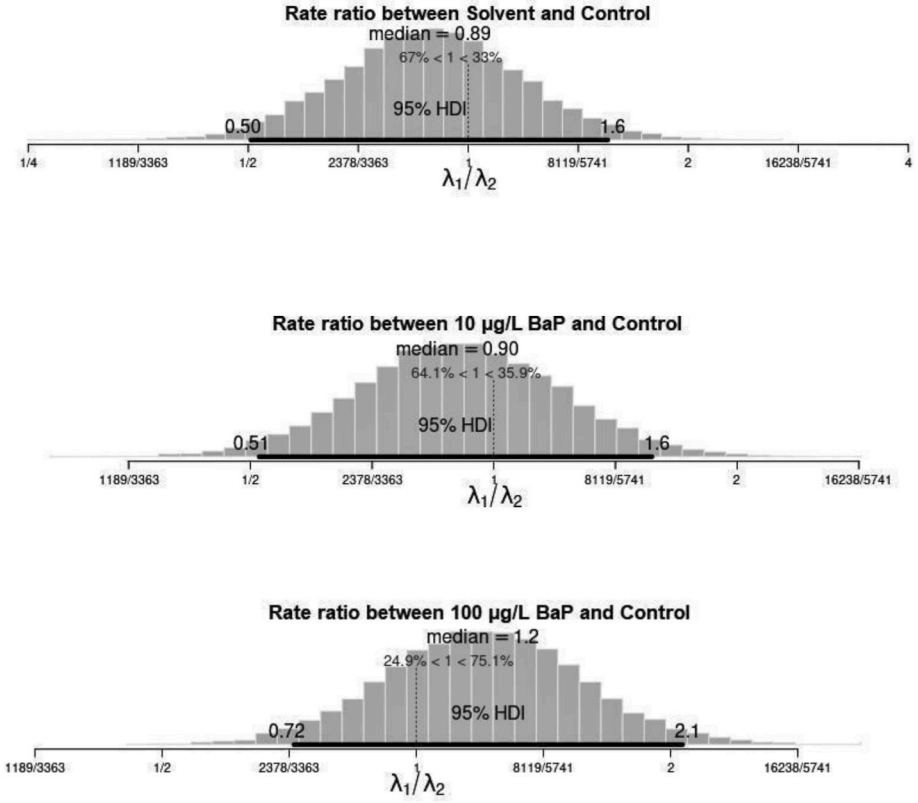

Fig. 1. Posterior distributions for rate ratios between solvent, 10 µg/L, and 100 µg/L BaP relative to the control group.

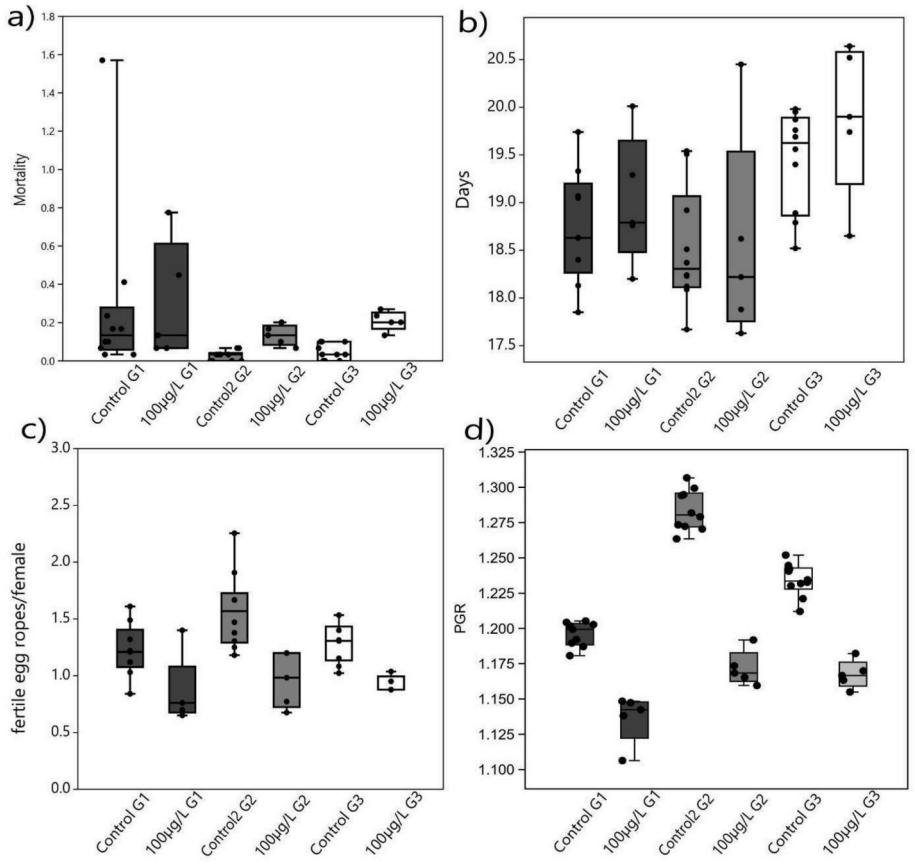

Fig. 2. *C. riparius* effects on a) mortality, b) Emt50, c) fertility, and d) PGR after exposure to control and 100µ/L of BaP for three consecutive generations.

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)

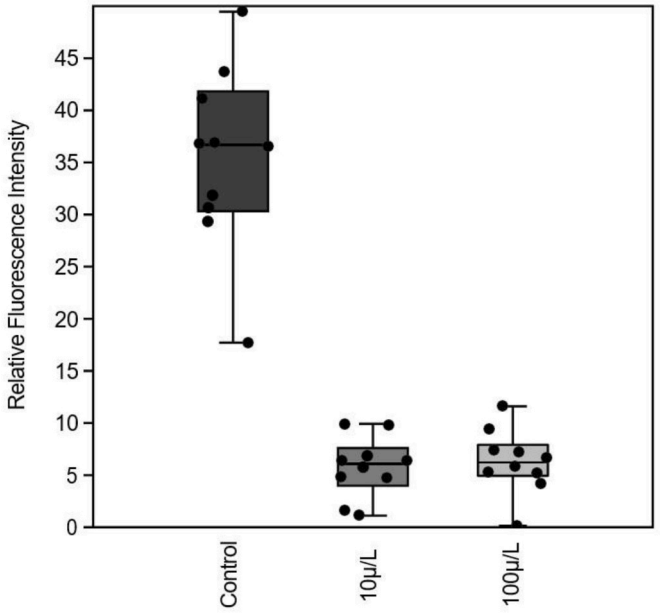

Fig. 3. Comparison of relative fluorescence intensity among control, 10μ/L, and 100μ/L.

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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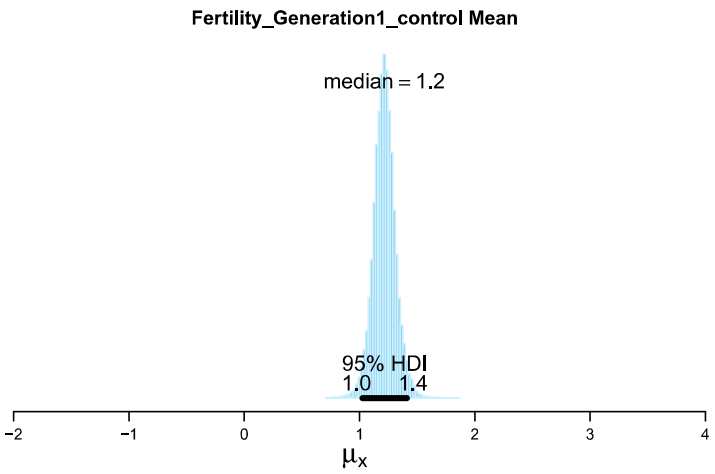
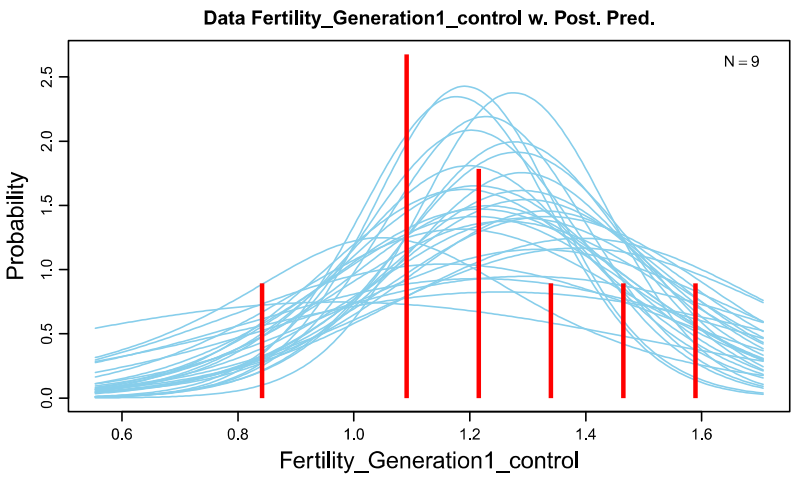
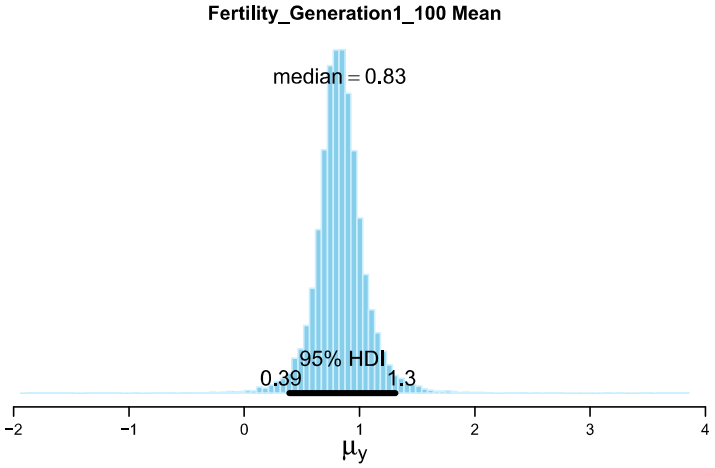
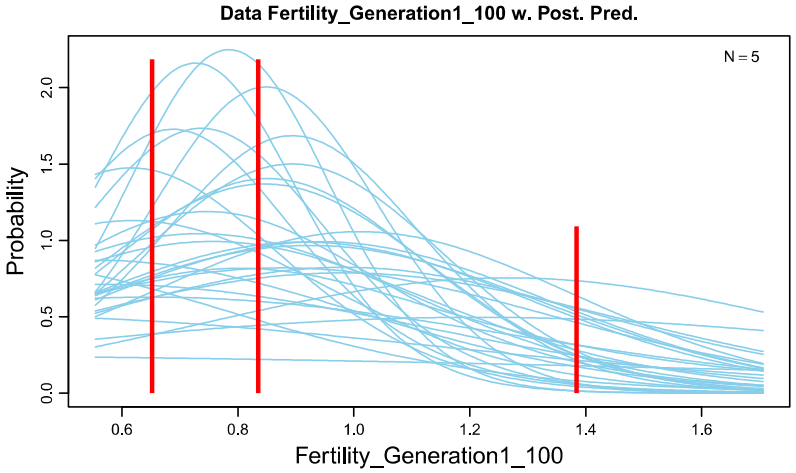
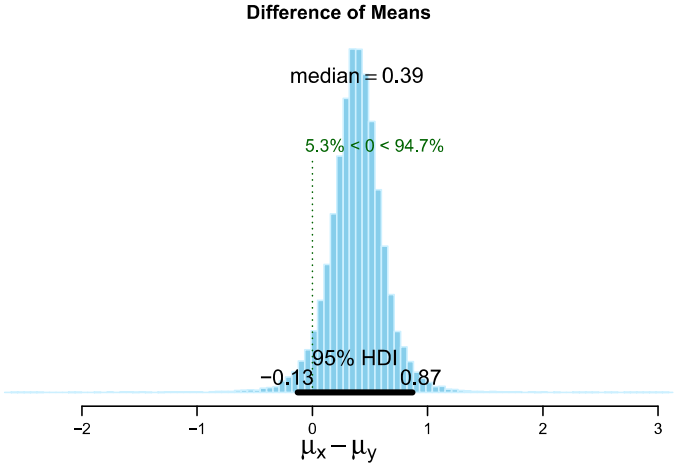
Supplementary data

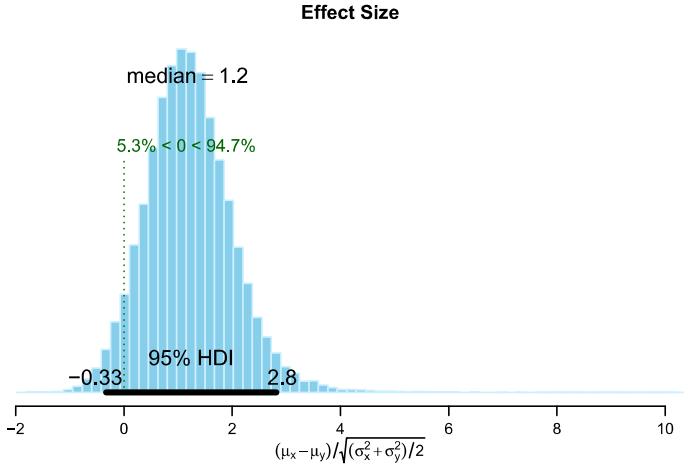

Fertility_Generation1_control Mean
median = 1.2
95% HDI
1.0
1.4
μx

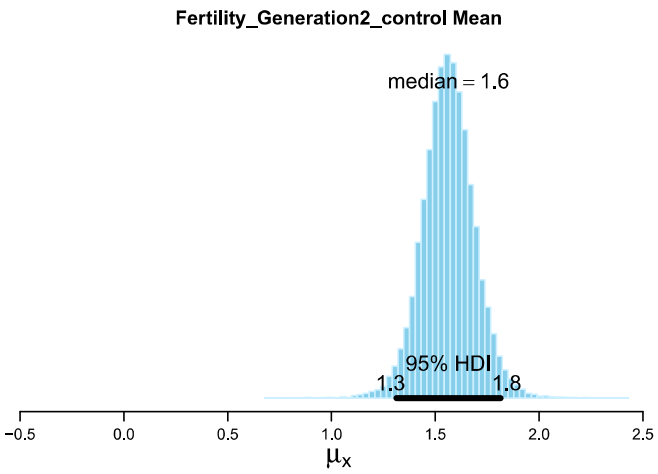

Data Fertility_Generation1_control w. Post. Pred.
N = 9
Probability
Fertility_Generation1_control

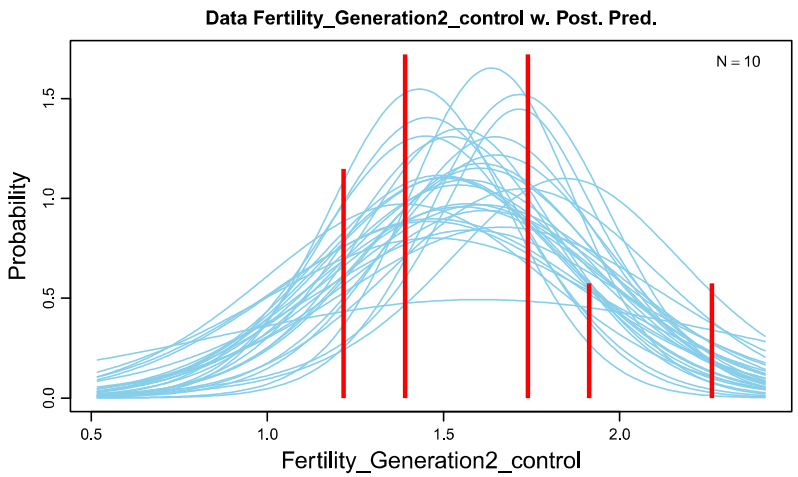

Fertility_Generation1_100 Mean
median = 0.83
95% HDI
0.39
1.3
μy

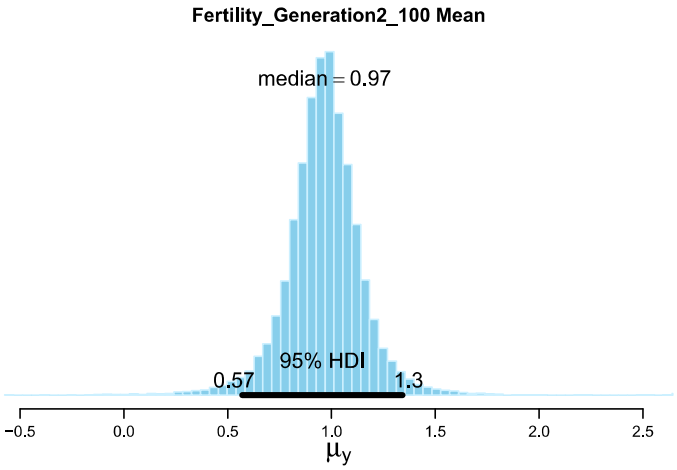

Data Fertility_Generation1_100 w. Post. Pred.
N = 5
Probability
Fertility_Generation1_100

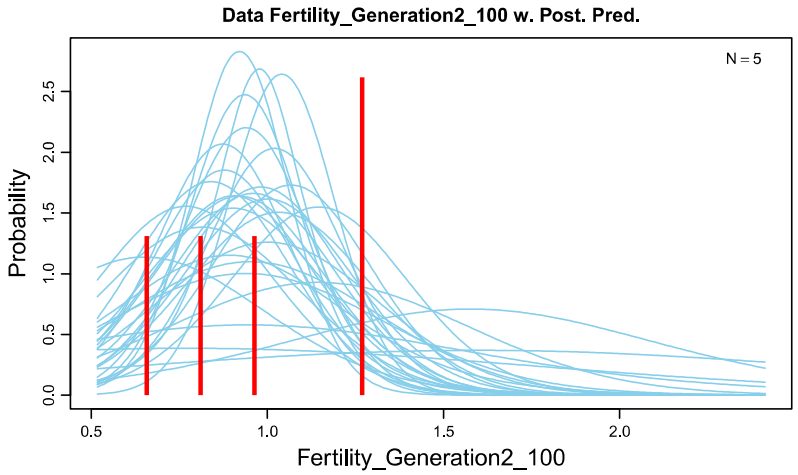

Difference of Means
median = 0.39
5.3% < 0 < 94.7%
95% HDI
−0.13
0.87
μx − μy

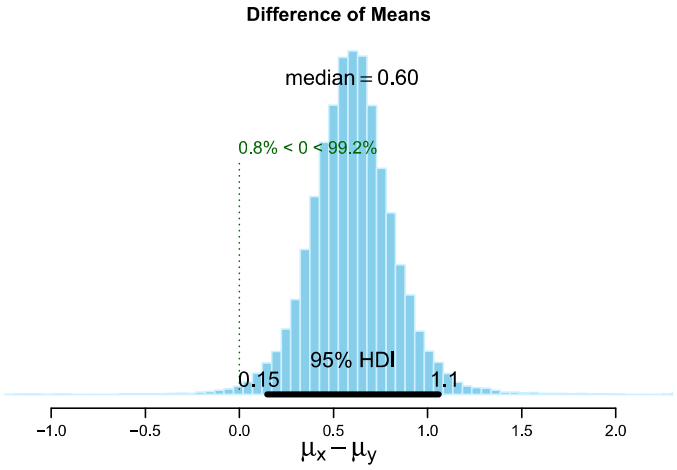

Effect Size
median = 1.2
5.3% < 0 < 94.7%
95% HDI
−0.33
2.8
(μx − μy)/√((σx² + σy²)/2)

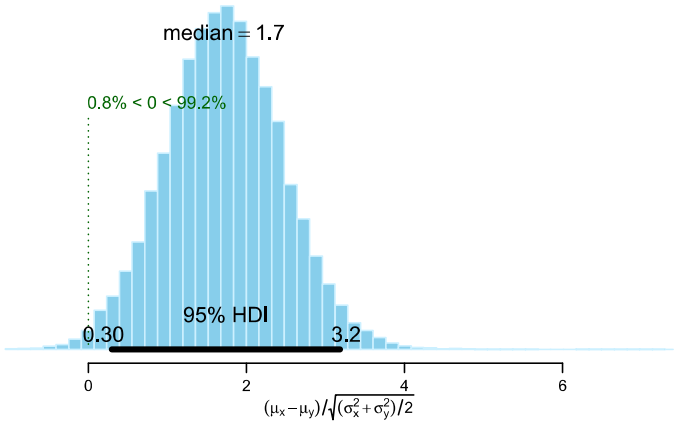

Fertility_Generation2_control Mean
median = 1.6
95% HDI
1.3
1.8
μx

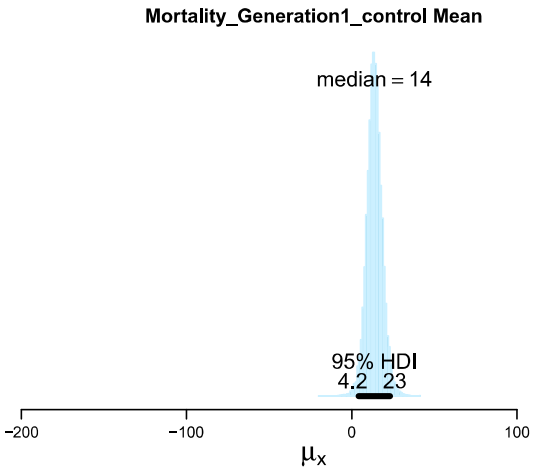

Data Fertility_Generation2_control w. Post. Pred.
N = 10
Probability
Fertility_Generation2_control

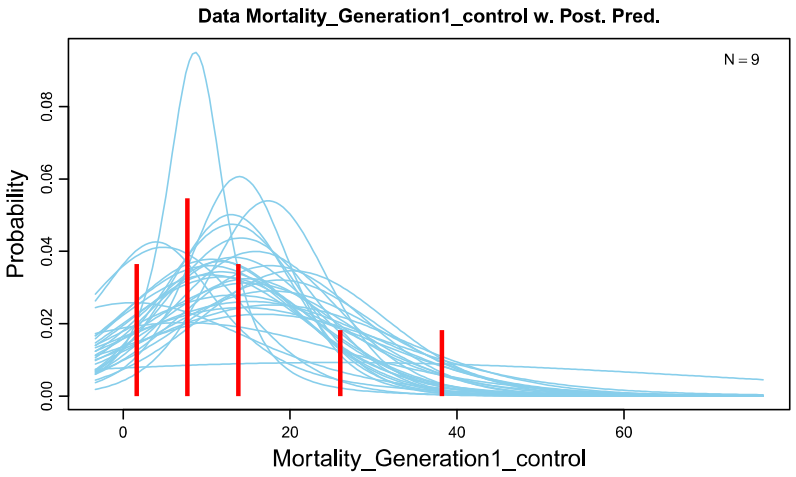

Fertility_Generation2_100 Mean
median = 0.97
95% HDI
0.57
1.3
μy

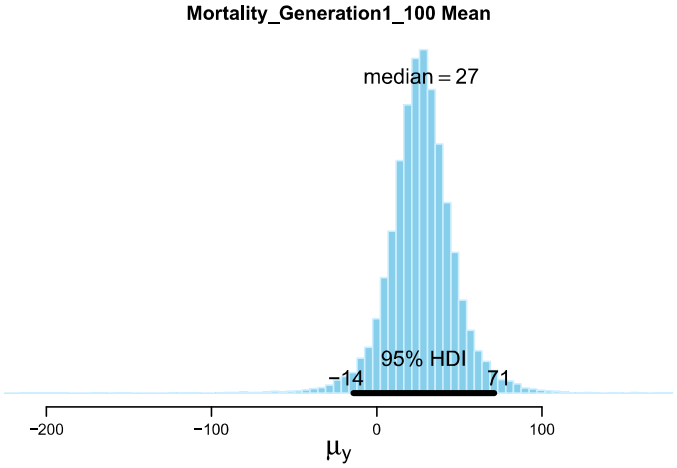

Data Fertility_Generation2_100 w. Post. Pred.
N = 5
Probability
Fertility_Generation2_100

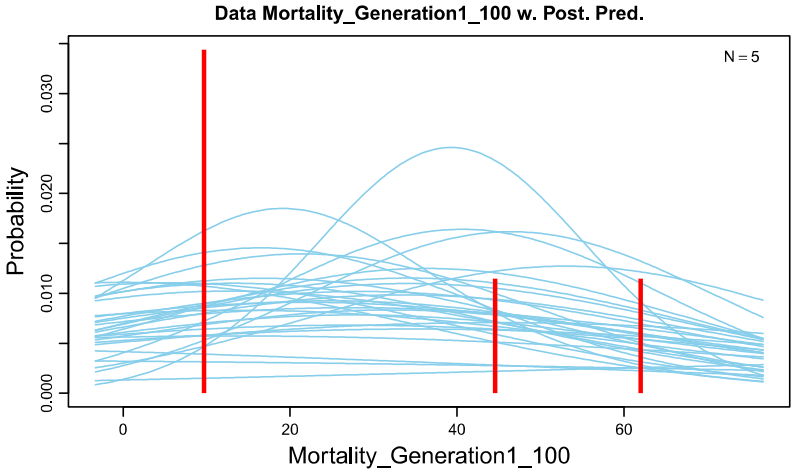

Difference of Means
median = 0.60
0.8% < 0 < 99.2%
95% HDI
0.15
1.1
μx − μy

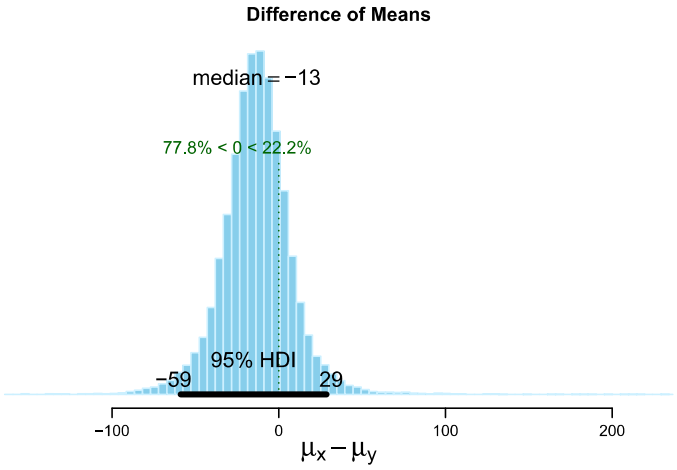

Effect Size
median = 1.7
0.8% < 0 < 99.2%
95% HDI
0.30
3.2
(μx − μy)/√((σx² + σy²)/2

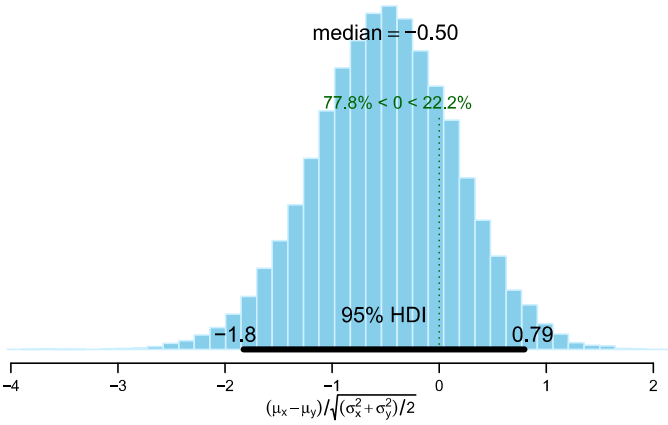

Mortality_Generation1_control Mean
median = 14
95% HDI
4.2 23
−200
−100
0
100
μx

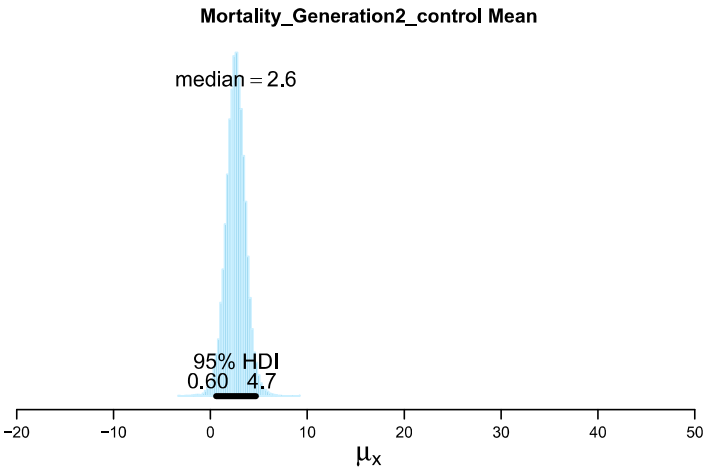

Data Mortality_Generation1_control w. Post. Pred.
N = 9
Probability
0.00
0.02
0.04
0.06
0.08
0
20
40
60
Mortality_Generation1_control

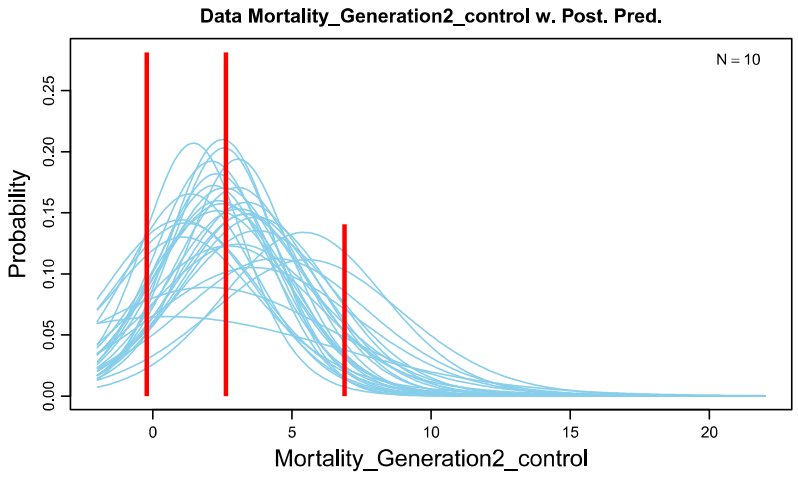

Mortality_Generation1_100 Mean
median = 27
95% HDI
−14
71
−200
−100
0
100
μy

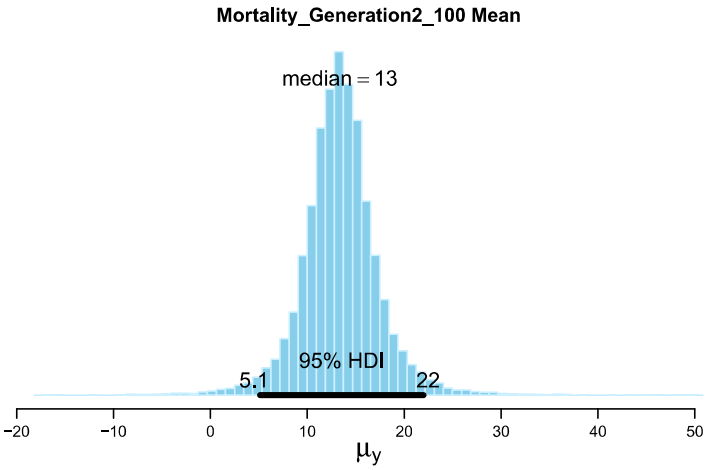

Data Mortality_Generation1_100 w. Post. Pred.
N = 5
Probability
0.000
0.010
0.020
0.030
0
20
40
60
Mortality_Generation1_100

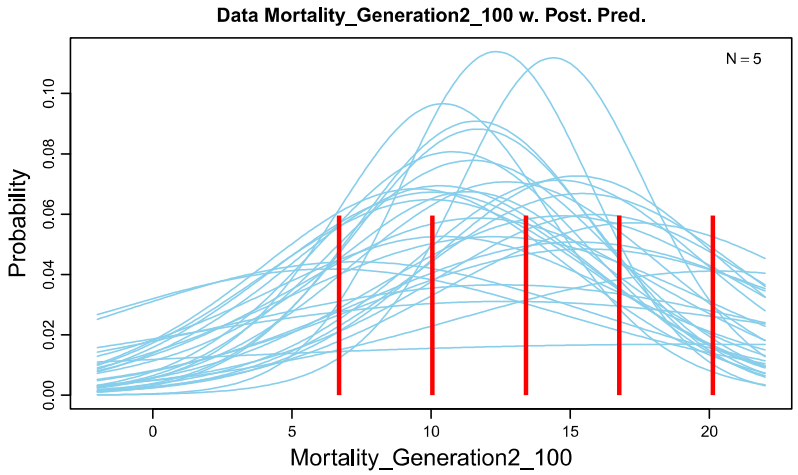

Difference of Means
median = −13
77.8% < 0 < 22.2%
95% HDI
−59
29
−100
0
100
200
μx − μy

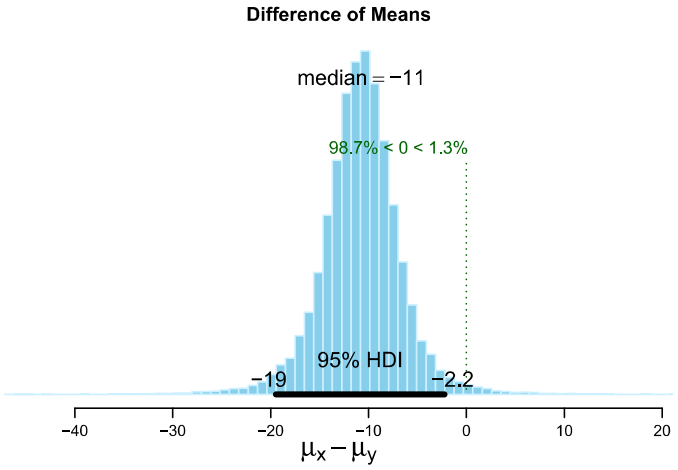

Effect Size
median = −0.50
77.8% < 0 < 22.2%
95% HDI
−1.8
0.79
−4
−3
−2
−1
0
1
2
(μx − μy)/√((σx² + σy²)/2)

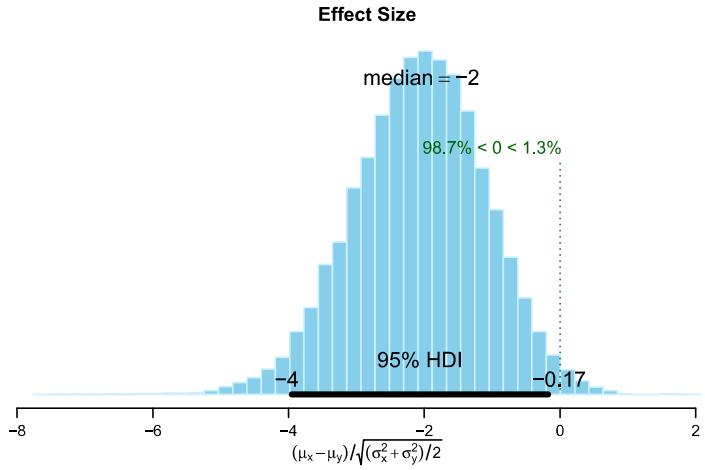

Mortality_Generation2_control Mean
median = 2.6
95% HDI
0.60 4.7
−20 −10 0 10 20 30 40 50
μx

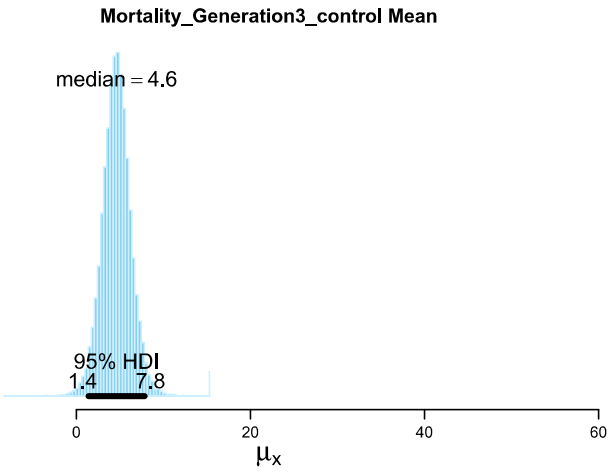

Data Mortality_Generation2_control w. Post. Pred.
N = 10
Probability
0.00 0.05 0.10 0.15 0.20 0.25
0 5 10 15 20
Mortality_Generation2_control

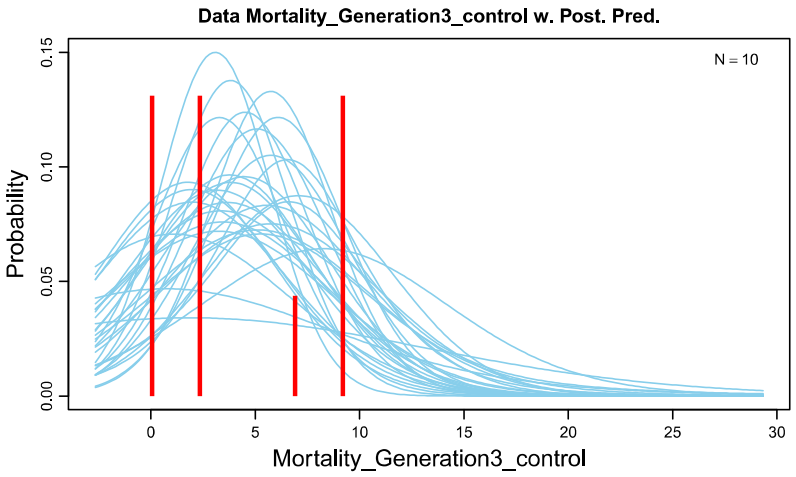

Mortality_Generation2_100 Mean
median = 13
95% HDI
5.1 22
−20 −10 0 10 20 30 40 50
μy

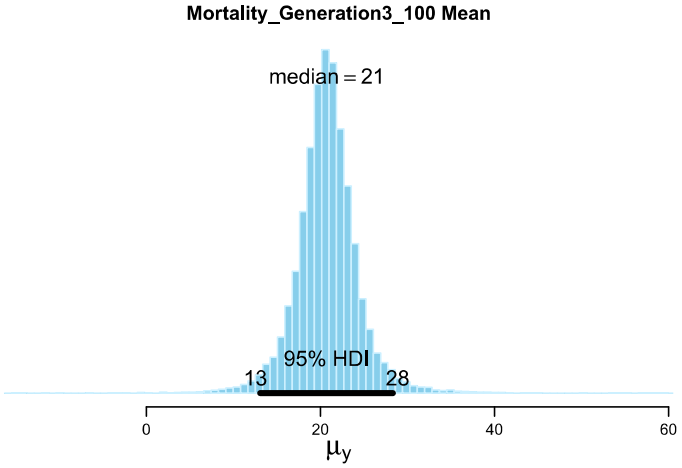

Data Mortality_Generation2_100 w. Post. Pred.
N = 5
Probability
0.00 0.02 0.04 0.06 0.08 0.10
0 5 10 15 20
Mortality_Generation2_100

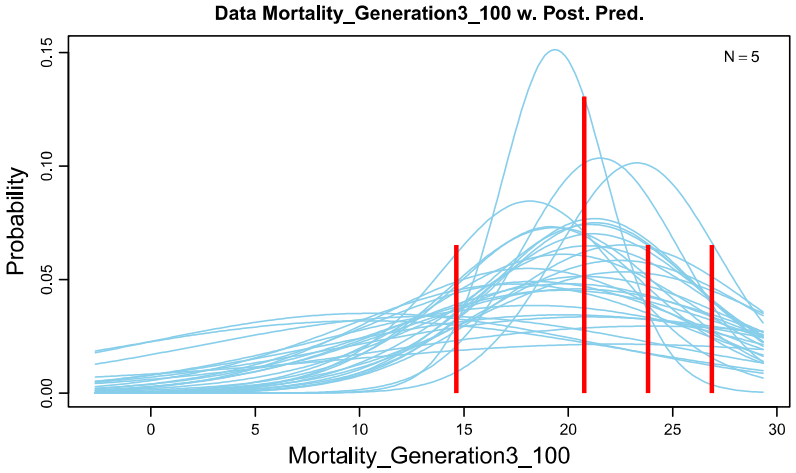

Difference of Means
median = −11
98.7% < 0 < 1.3%
95% HDI
−19 −2.2
−40 −30 −20 −10 0 10 20
μx − μy

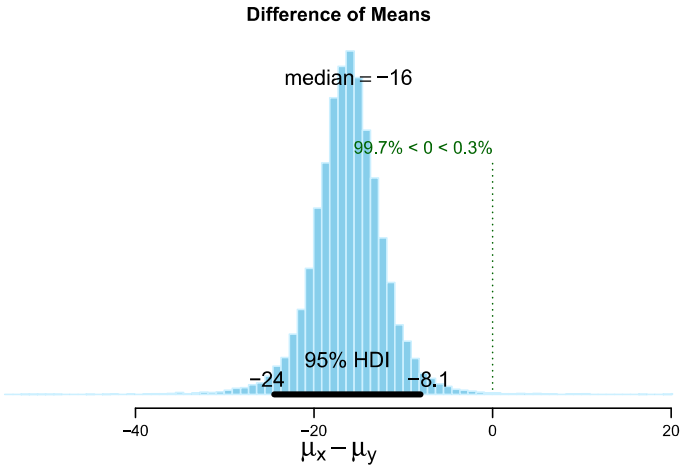

Effect Size
median = −2
98.7% < 0 < 1.3%
95% HDI
−4 −0.17
−8 −6 −4 −2 0 2
(μx − μy)/√((σx² + σy²)/2)

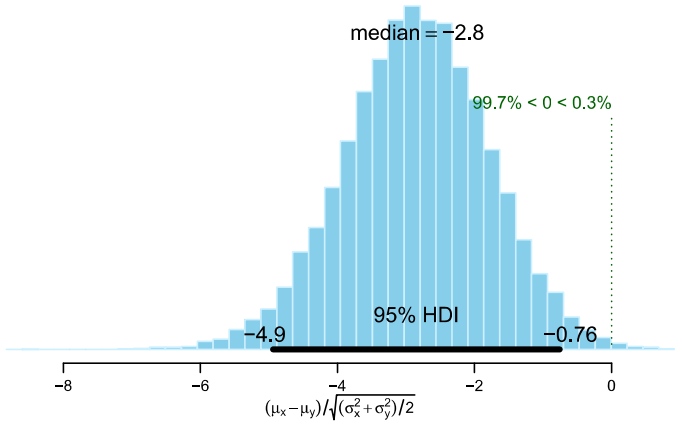

Mortality_Generation3_control Mean
median = 4.6
95% HDI
1.4
7.8
0
20
40
60
μx

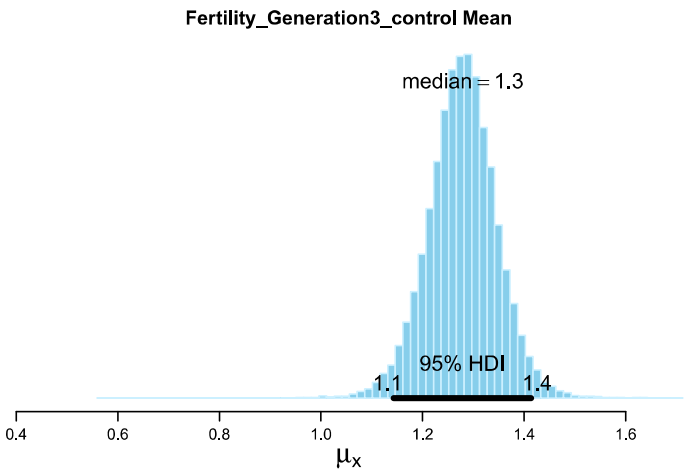

Data Mortality_Generation3_control w. Post. Pred.
N = 10
Probability
0.00
0.05
0.10
0.15
0
5
10
15
20
25
30
Mortality_Generation3_control

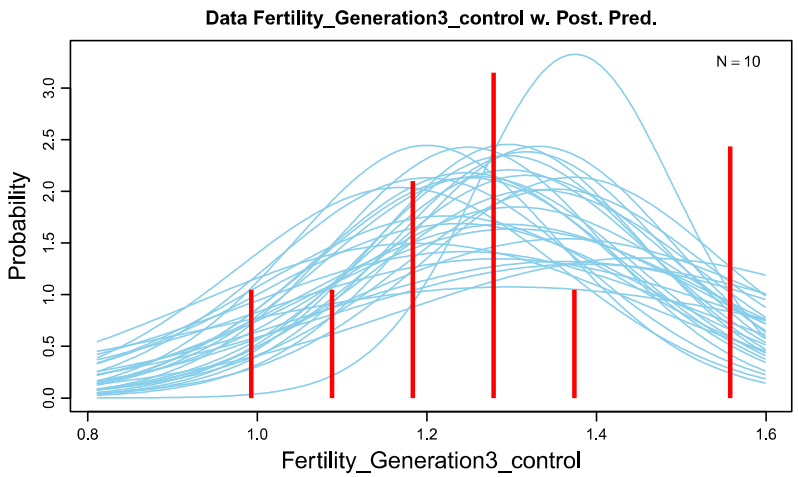

Mortality_Generation3_100 Mean
median = 21
95% HDI
13
28
0
20
40
60
μy

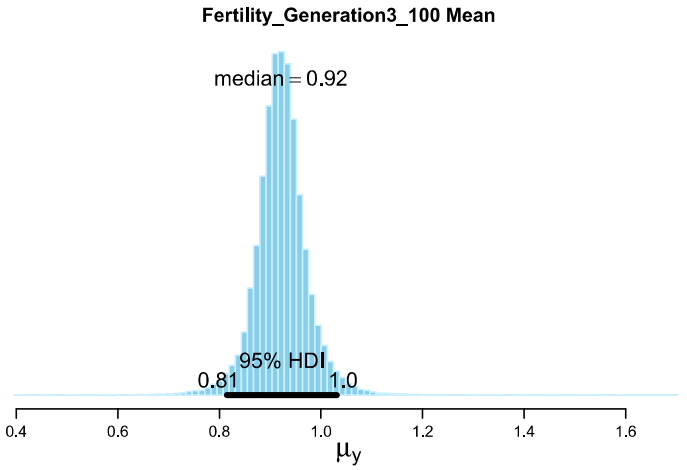

Data Mortality_Generation3_100 w. Post. Pred.
N = 5
Probability
0.00
0.05
0.10
0.15
0
5
10
15
20
25
30
Mortality_Generation3_100

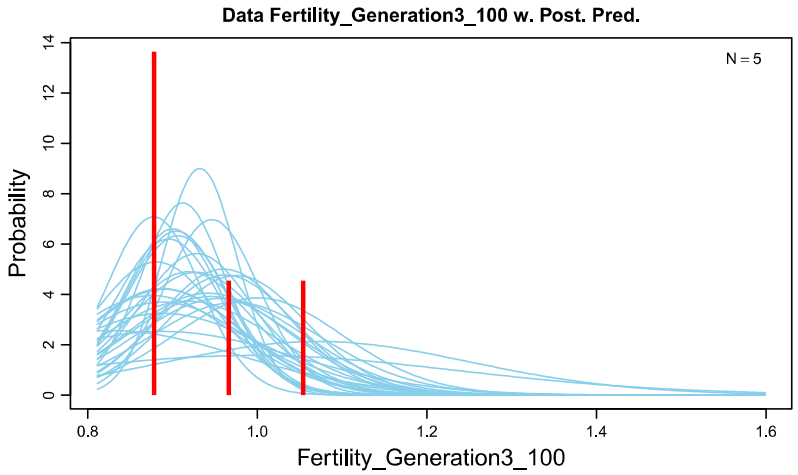

Difference of Means
median = −16
99.7% < 0 < 0.3%
95% HDI
−24
−8.1
−40
−20
0
20
μx − μy

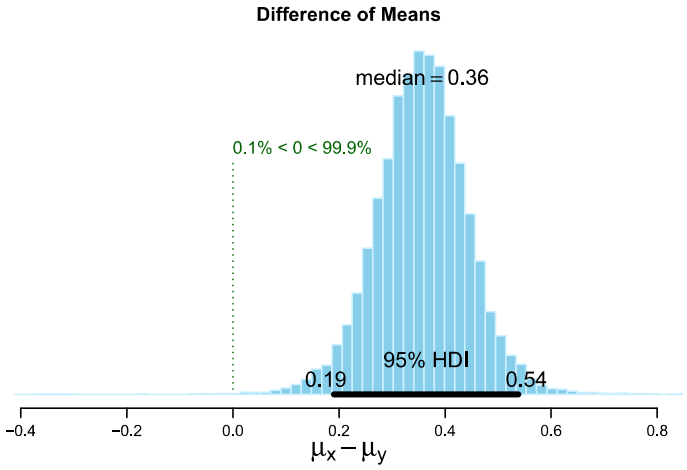

Effect Size
median = −2.8
99.7% < 0 < 0.3%
95% HDI
−4.9
−0.76
−8
−6
−4
−2
0
(μx − μy)/√((σx² + σy²)/2

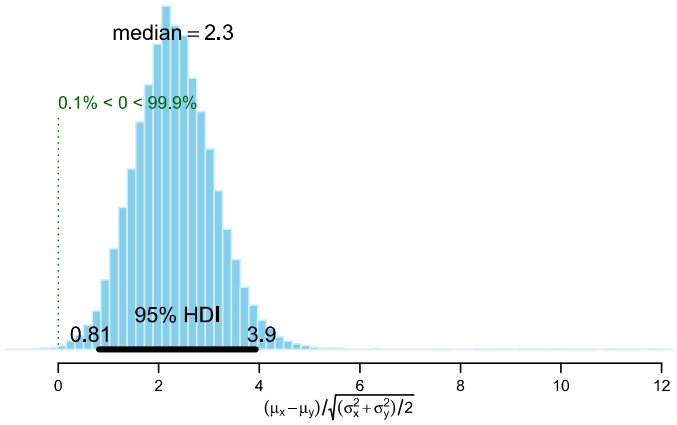

Fertility_Generation3_control Mean
median = 1.3
95% HDI
1.1
1.4
μx

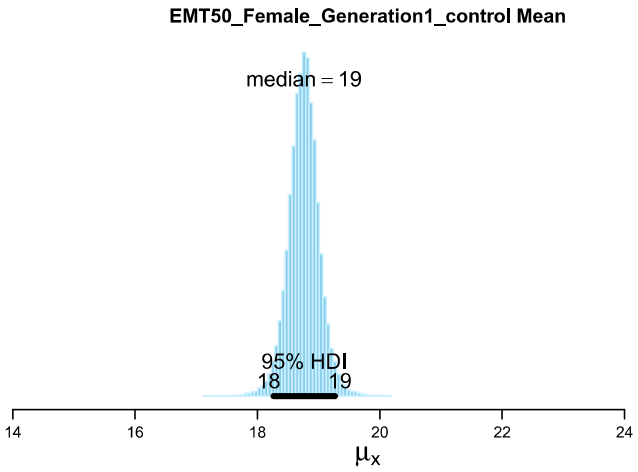

Data Fertility_Generation3_control w. Post. Pred.
N = 10
Probability
Fertility_Generation3_control

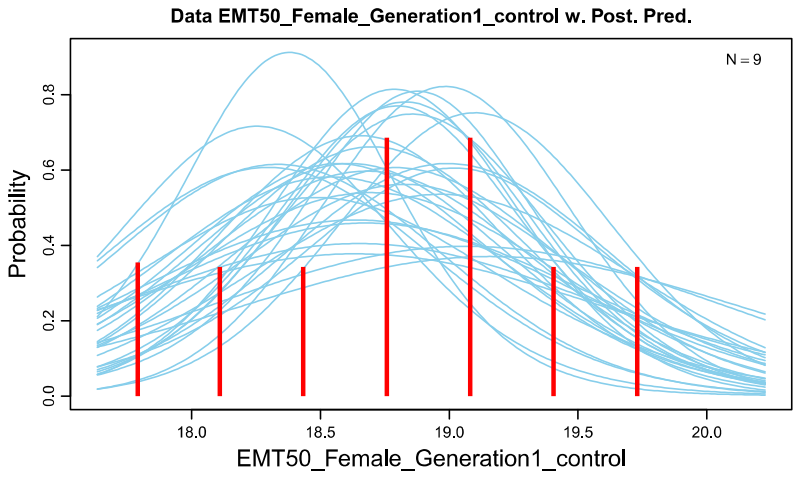

Fertility_Generation3_100 Mean
median = 0.92
95% HDI
0.81
1.0
μy

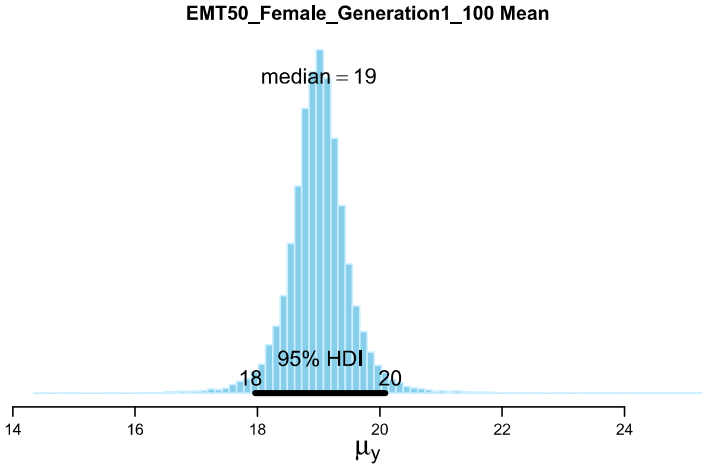

Data Fertility_Generation3_100 w. Post. Pred.
N = 5
Probability
Fertility_Generation3_100

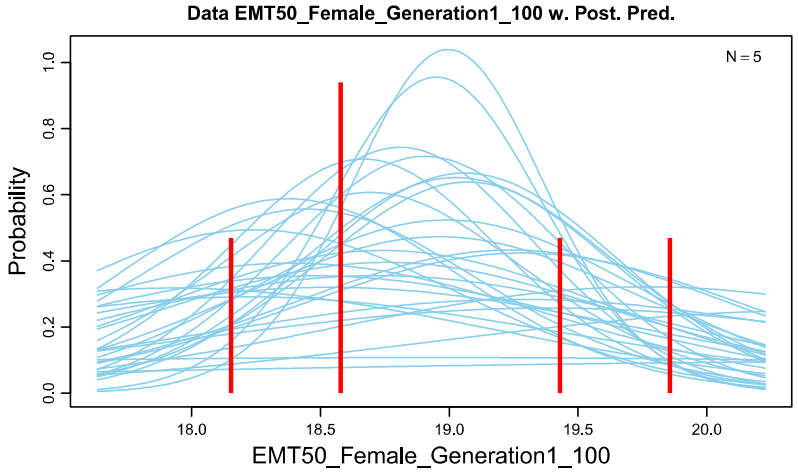

Difference of Means
median = 0.36
0.1% < 0 < 99.9%
95% HDI
0.19
0.54
μx − μy

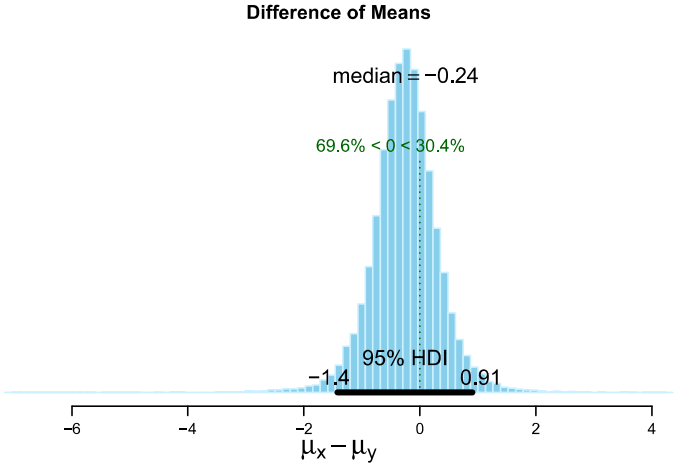

Effect Size
median = 2.3
0.1% < 0 < 99.9%
95% HDI
0.81
3.9

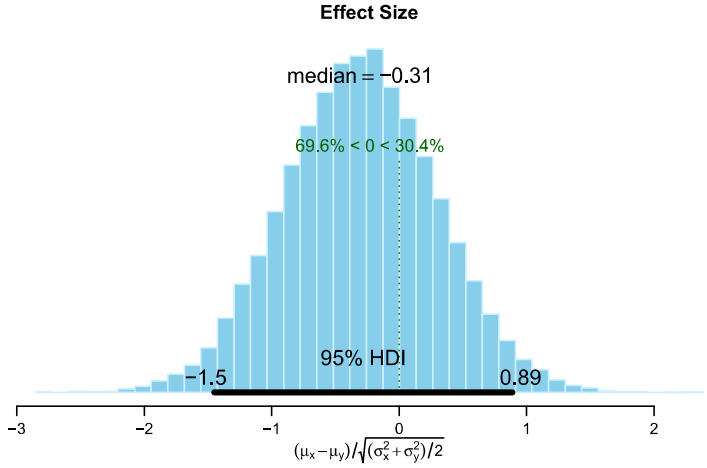

EMT50_Female_Generation1_control Mean
median = 19
95% HDI
18
19
14
16
18
20
22
24
μx

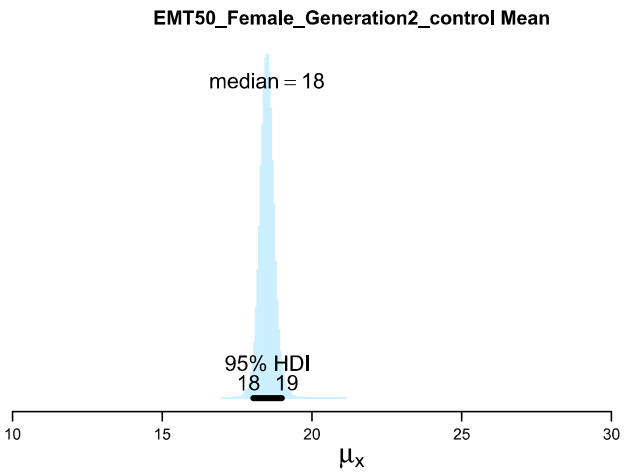

Data EMT50_Female_Generation1_control w. Post. Pred.
N = 9
Probability
0.0
0.2
0.4
0.6
0.8
18.0
18.5
19.0
19.5
20.0
EMT50_Female_Generation1_control

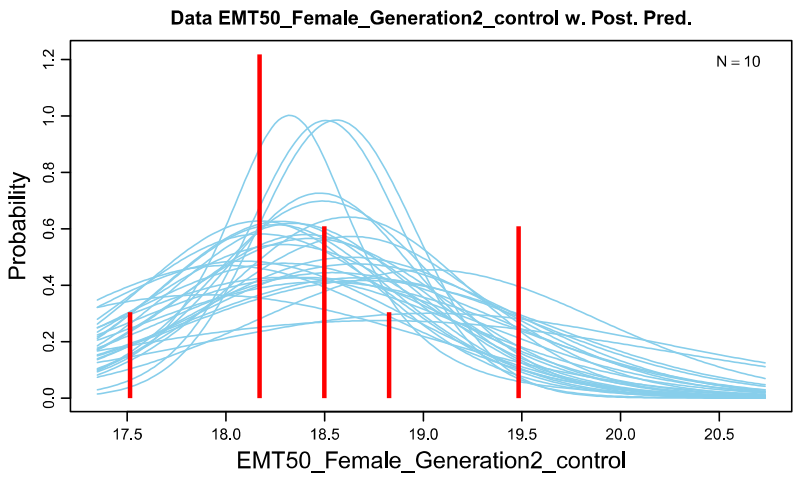

EMT50_Female_Generation1_100 Mean
median = 19
95% HDI
18
20
14
16
18
20
22
24
μy

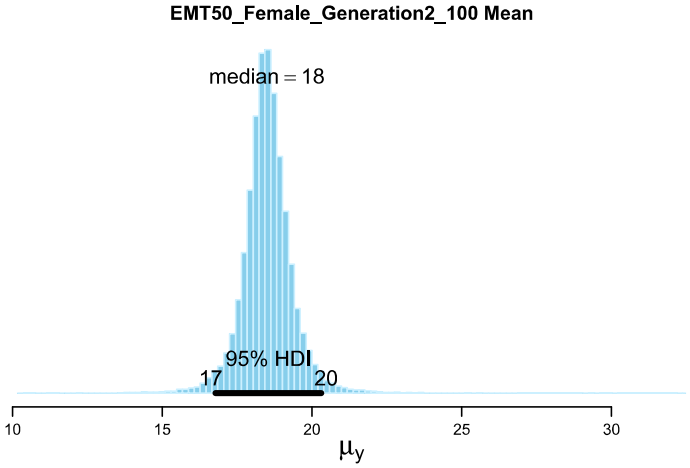

Data EMT50_Female_Generation1_100 w. Post. Pred.
N = 5
Probability
0.0
0.2
0.4
0.6
0.8
1.0
18.0
18.5
19.0
19.5
20.0
EMT50_Female_Generation1_100

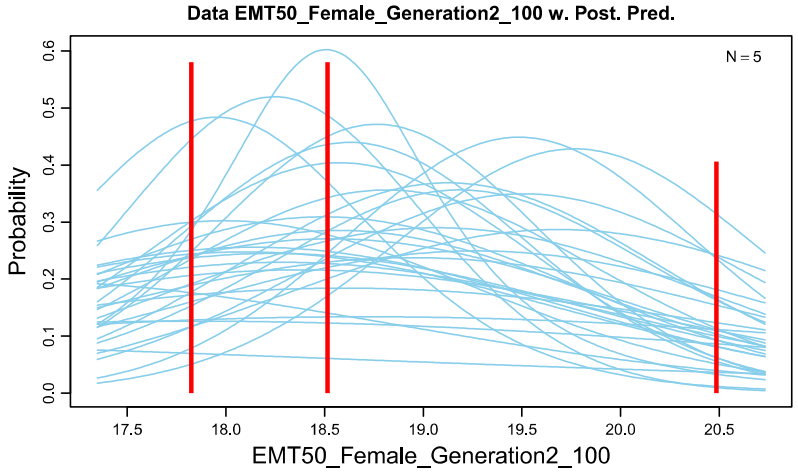

Difference of Means
median = −0.24
69.6% < 0 < 30.4%
95% HDI
−1.4
0.91
−6
−4
−2
0
2
4
μx − μy

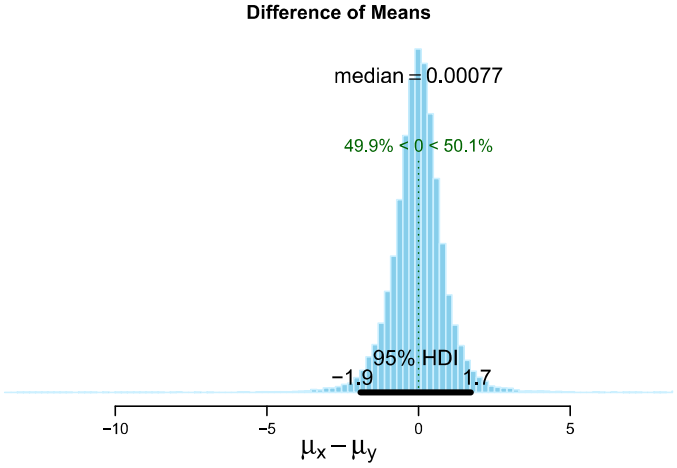

Effect Size
median = −0.31
69.6% < 0 < 30.4%
95% HDI
−1.5
0.89
−3
−2
−1
0
1
2
(μx − μy)/√((σx² + σy²)/2)

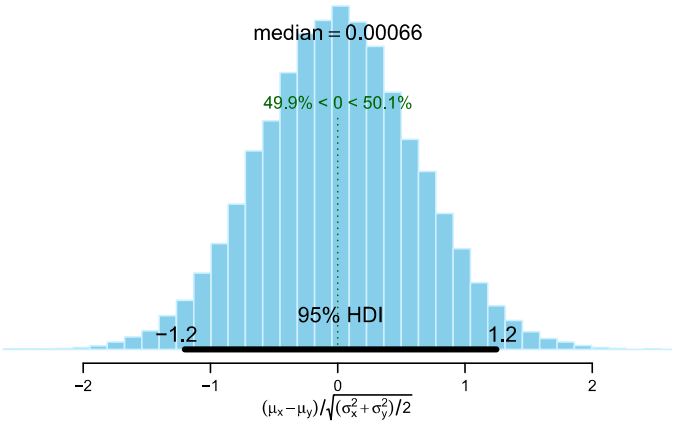

EMT50_Female_Generation2_control Mean
median = 18
95% HDI
18 19
10
15
20
25
30
μx

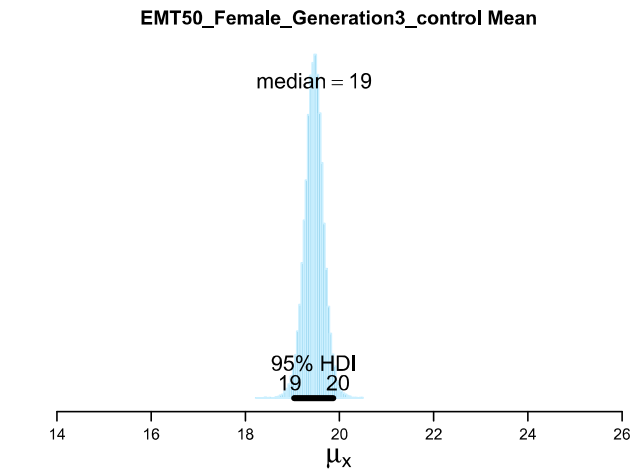

Data EMT50_Female_Generation2_control w. Post. Pred.
N = 10
Probability
0.0
0.2
0.4
0.6
0.8
1.0
1.2
17.5
18.0
18.5
19.0
19.5
20.0
20.5
EMT50_Female_Generation2_control

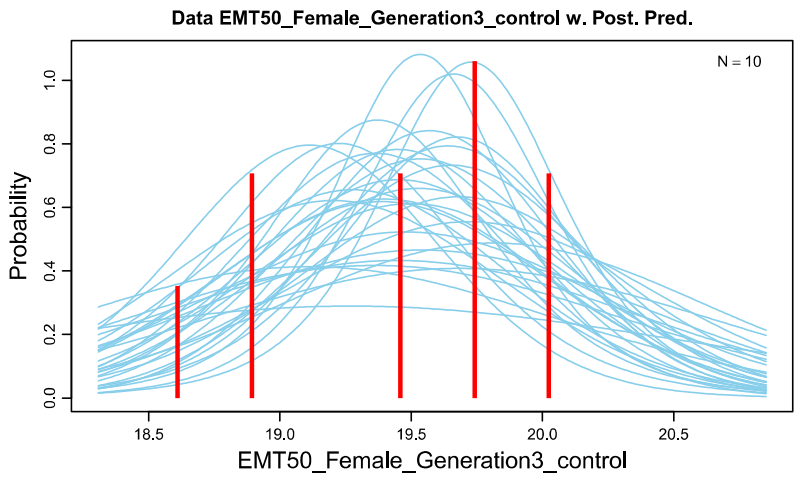

EMT50_Female_Generation2_100 Mean
median = 18
95% HDI
17
20
10
15
20
25
30
μy

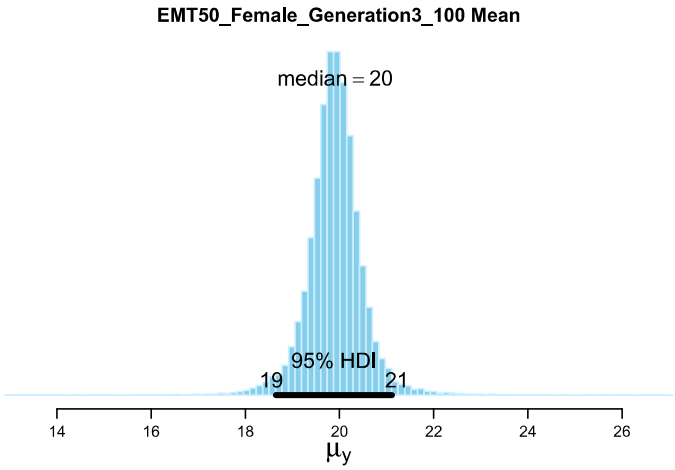

Data EMT50_Female_Generation2_100 w. Post. Pred.
N = 5
Probability
0.0
0.1
0.2
0.3
0.4
0.5
0.6
17.5
18.0
18.5
19.0
19.5
20.0
20.5
EMT50_Female_Generation2_100

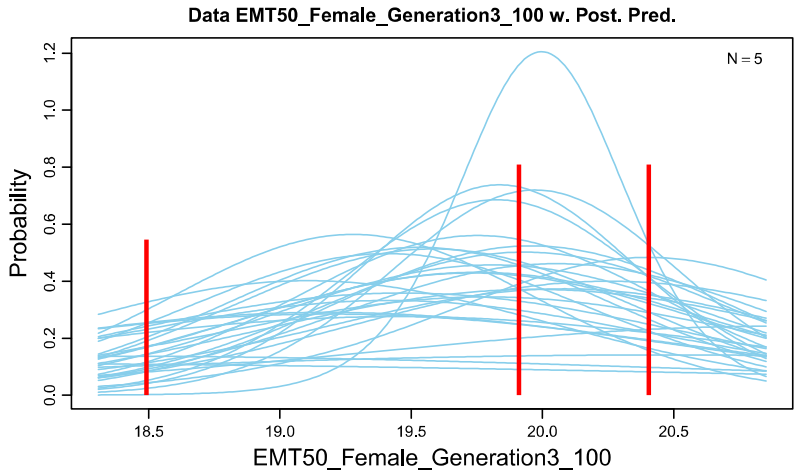

Difference of Means
median = 0.00077
49.9% < 0 < 50.1%
95% HDI
−1.9
1.7
−10
−5
0
5
μx − μy

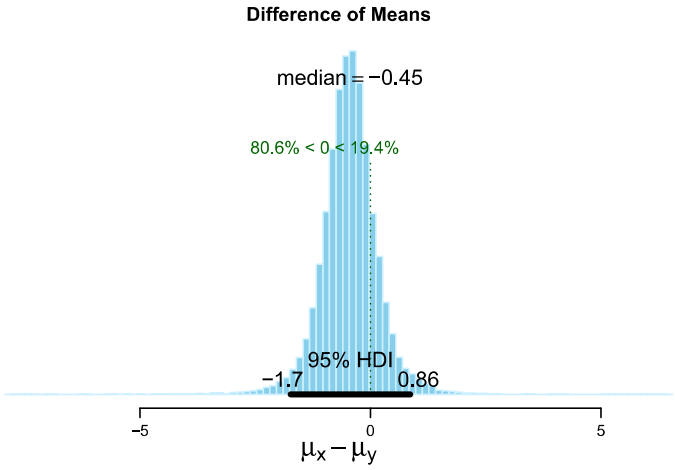

Effect Size
median = 0.00066
49.9% < 0 < 50.1%
95% HDI
−1.2
1.2
−2
−1
0
1
2
(μx − μy)/√((σx² + σy²)/2)

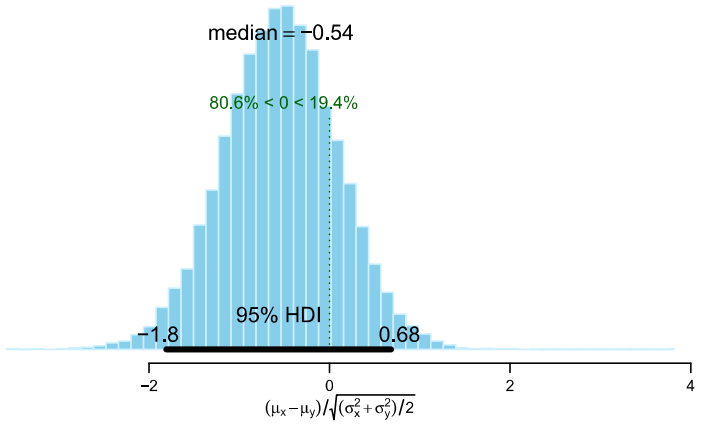

EMT50_Female_Generation3_control Mean
median = 19
95% HDI
19 20
14 16 18 20 22 24 26
μx

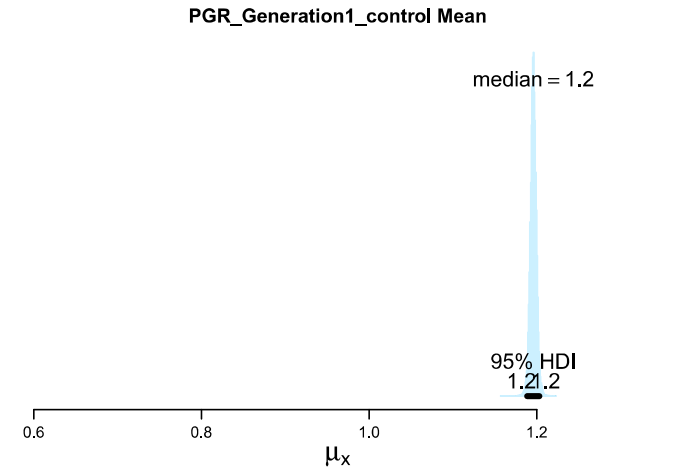

Data EMT50_Female_Generation3_control w. Post. Pred.
N = 10
Probability
0.0 0.2 0.4 0.6 0.8 1.0
18.5 19.0 19.5 20.0 20.5
EMT50_Female_Generation3_control

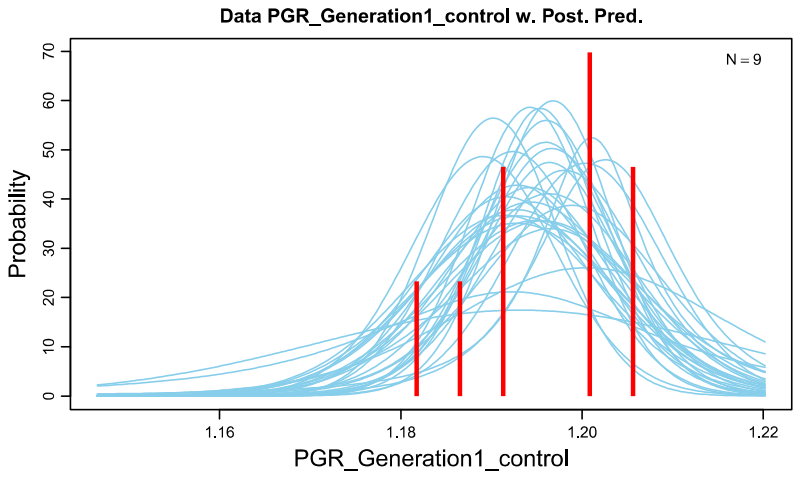

EMT50_Female_Generation3_100 Mean
median = 20
95% HDI
19 21
14 16 18 20 22 24 26
μy

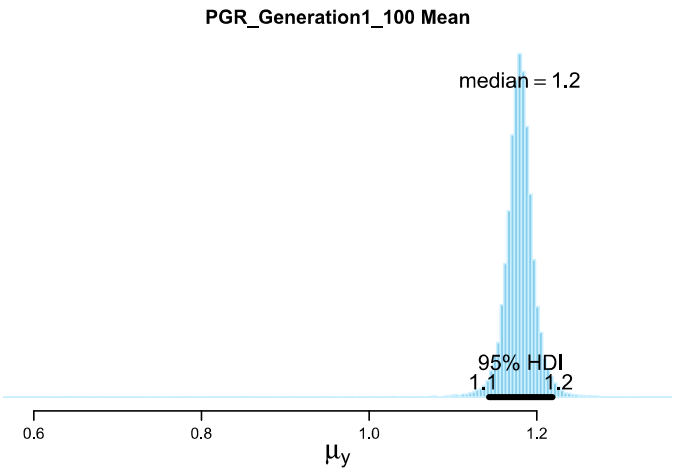

Data EMT50_Female_Generation3_100 w. Post. Pred.
N = 5
Probability
0.0 0.2 0.4 0.6 0.8 1.0 1.2
18.5 19.0 19.5 20.0 20.5
EMT50_Female_Generation3_100

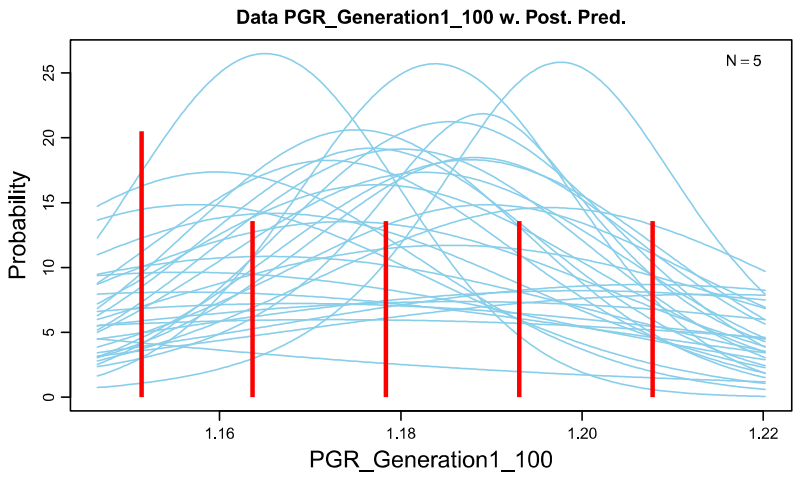

Difference of Means
median = −0.45
80.6% < 0 < 19.4%
95% HDI
−1.7 0.86
−5 0 5
μx − μy

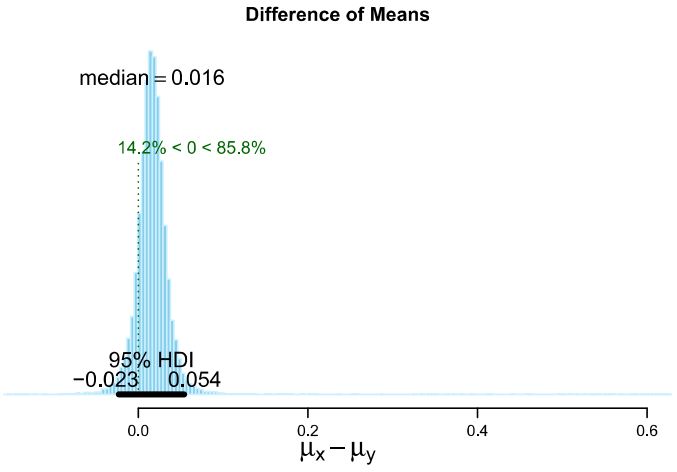

Effect Size
median = −0.54
80.6% < 0 < 19.4%
95% HDI
−1.8 0.68
−2 0 2 4
(μx − μy)/√((σx² + σy²)/2

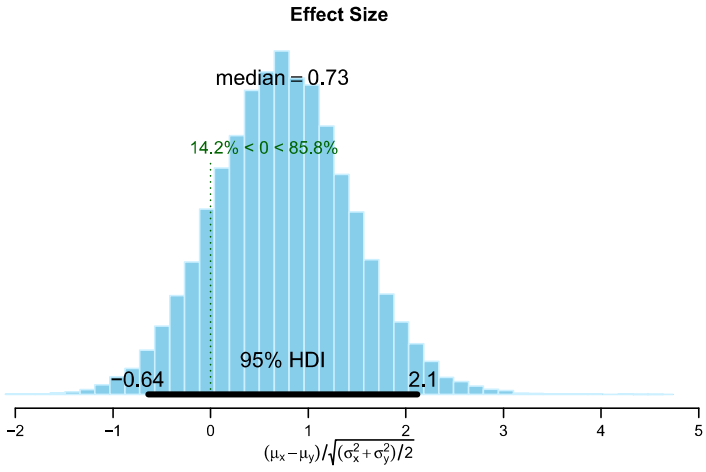

PGR_Generation1_control Mean
median = 1.2
95% HDI
1.2 1.2
0.6
0.8
1.0
1.2
μx

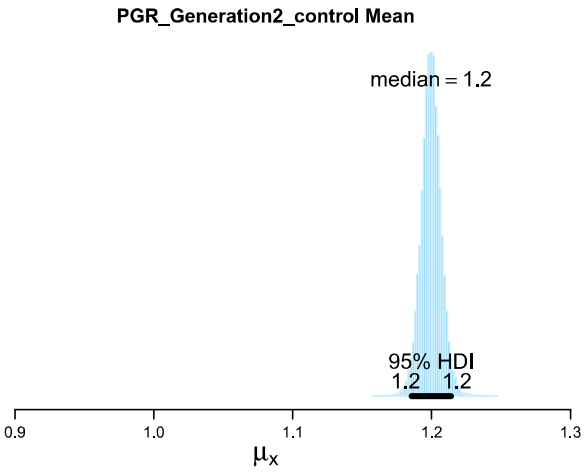

Data PGR_Generation1_control w. Post. Pred.
N = 9
Probability
0
10
20
30
40
50
60
70
1.16
1.18
1.20
1.22
PGR_Generation1_control

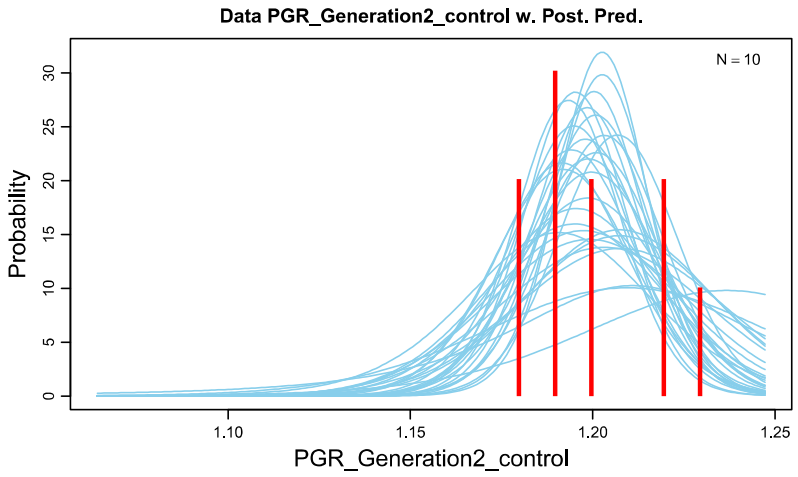

PGR_Generation1_100 Mean
median = 1.2
95% HDI
1.1 1.2
0.6
0.8
1.0
1.2
μy

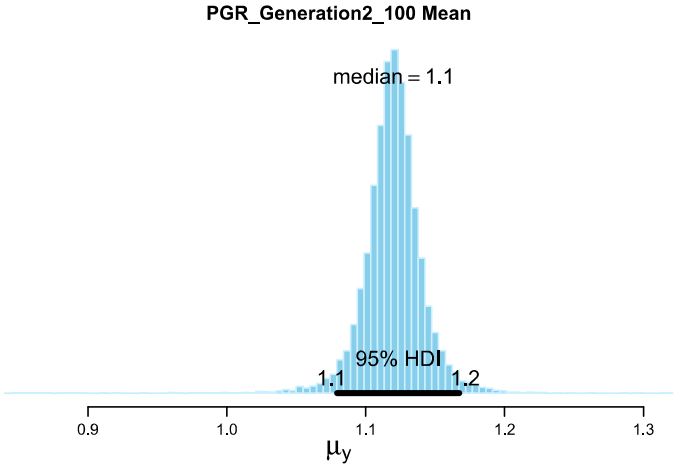

Data PGR_Generation1_100 w. Post. Pred.
N = 5
Probability
0
5
10
15
20
25
1.16
1.18
1.20
1.22
PGR_Generation1_100

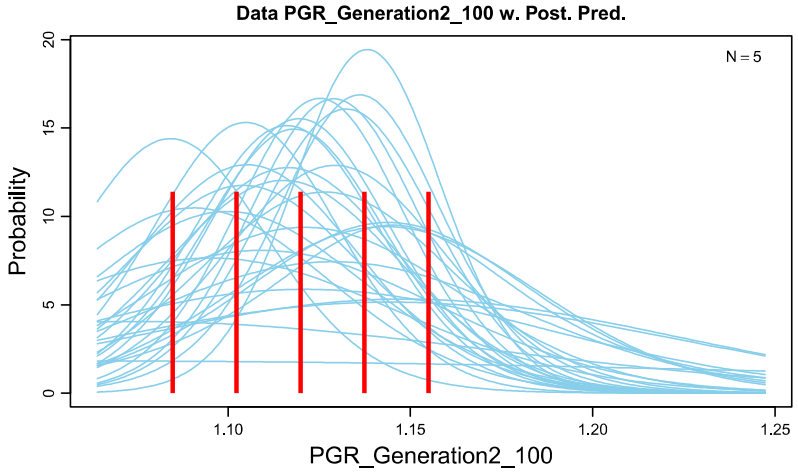

Difference of Means
median = 0.016
14.2% < 0 < 85.8%
95% HDI
−0.023 0.054
0.0
0.2
0.4
0.6
μx − μy

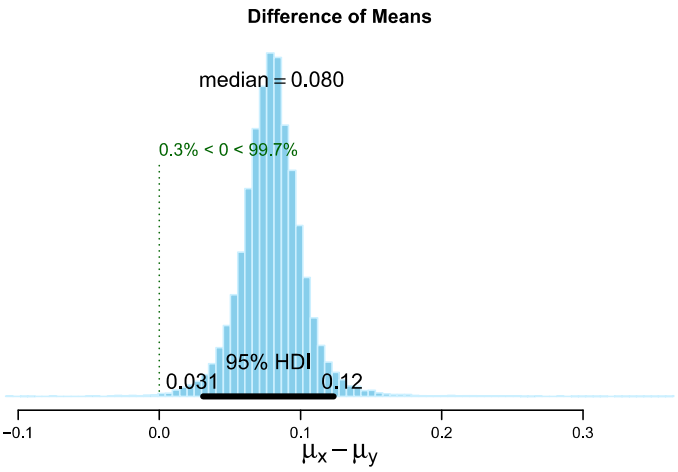

Effect Size
median = 0.73
14.2% < 0 < 85.8%
95% HDI
−0.64
2.1
−2
−1
0
1
2
3
4
5
(μx − μy)/√((σx² + σy²)/2)

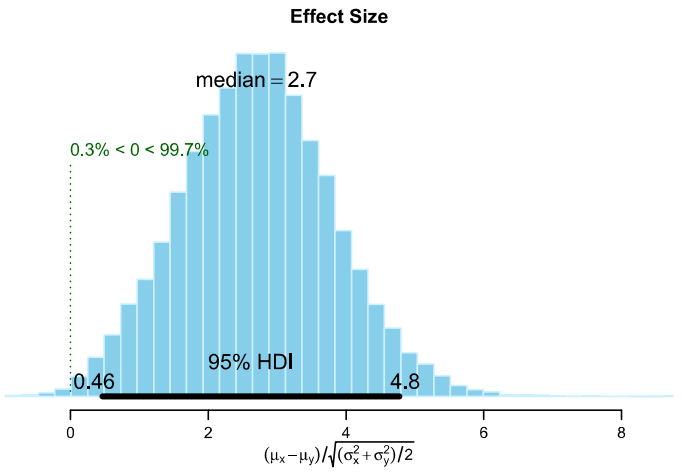

PGR_Generation2_control Mean
median = 1.2
95% HDI
1.2 1.2
0.9 1.0 1.1 1.2 1.3
μx

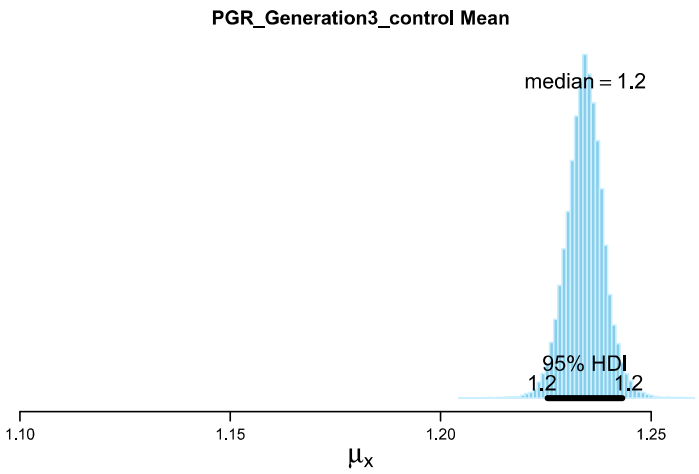

Data PGR_Generation2_control w. Post. Pred.
N = 10
Probability
0 5 10 15 20 25 30
1.10 1.15 1.20 1.25
PGR_Generation2_control

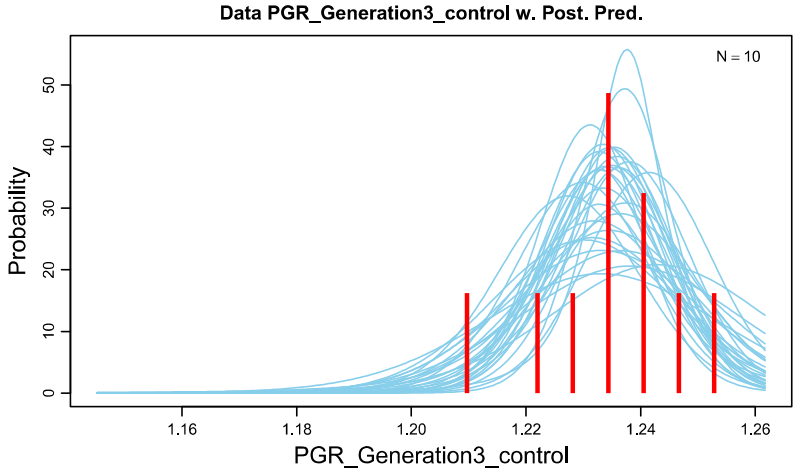

PGR_Generation2_100 Mean
median = 1.1
95% HDI
1.1 1.2
0.9 1.0 1.1 1.2 1.3
μy

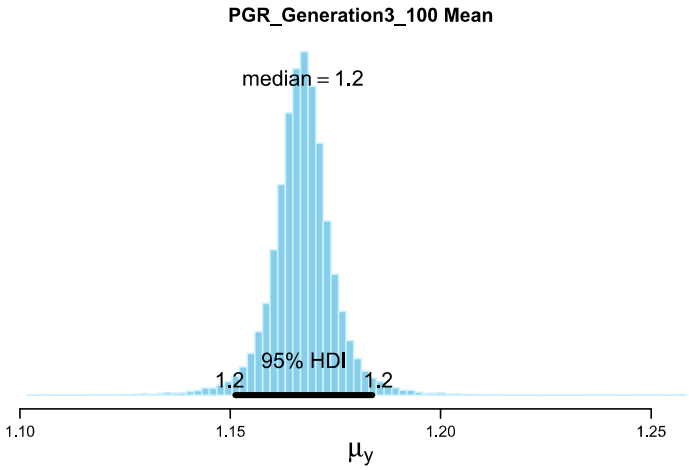

Data PGR_Generation2_100 w. Post. Pred.
N = 5
Probability
0 5 10 15 20
1.10 1.15 1.20 1.25
PGR_Generation2_100

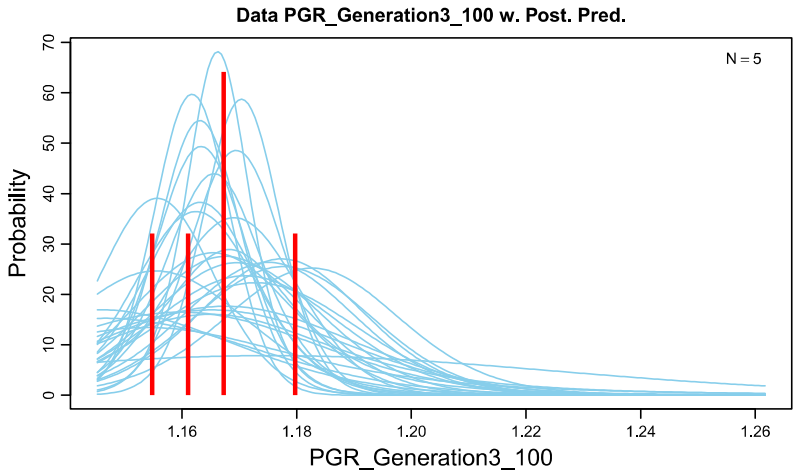

Difference of Means
median = 0.080
0.3% < 0 < 99.7%
95% HDI
0.031 0.12
−0.1 0.0 0.1 0.2 0.3
μx − μy

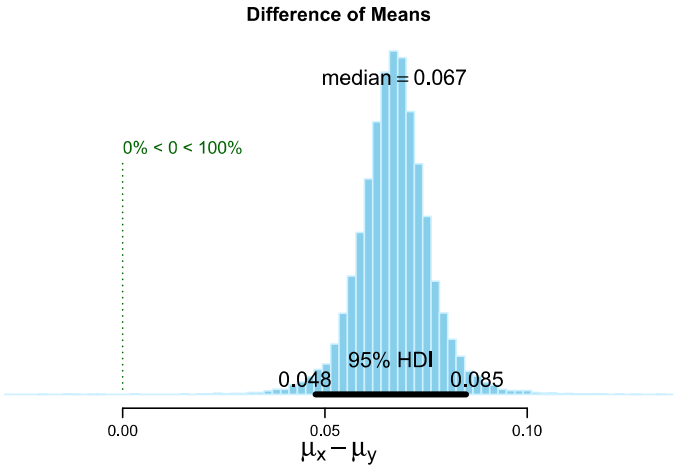

Effect Size
median = 2.7
0.3% < 0 < 99.7%
95% HDI
0.46 4.8
0 2 4 6 8
(μx − μy)/√((σx² + σy²)/2)

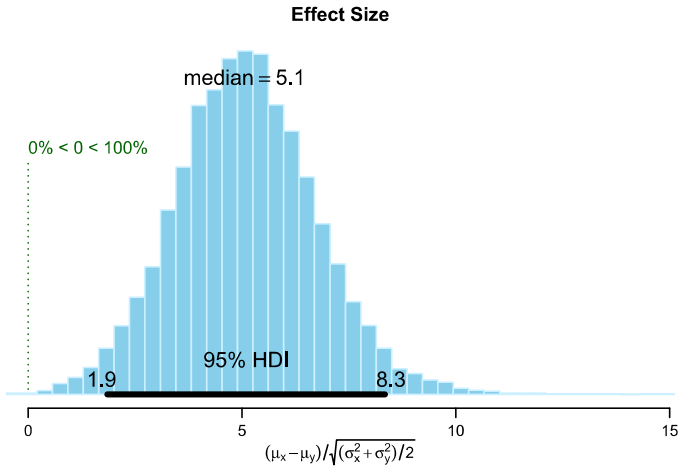

PGR_Generation3_control Mean
median = 1.2
95% HDI
1.2
1.2
1.10
1.15
1.20
1.25
μx

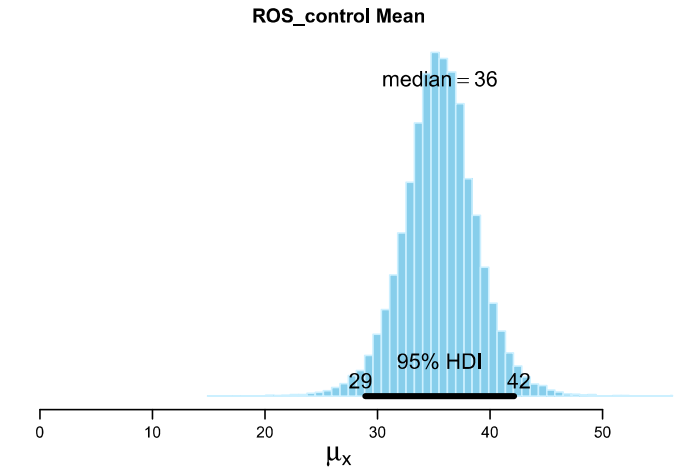

Data PGR_Generation3_control w. Post. Pred.
N = 10
Probability
0
10
20
30
40
50
1.16
1.18
1.20
1.22
1.24
1.26
PGR_Generation3_control

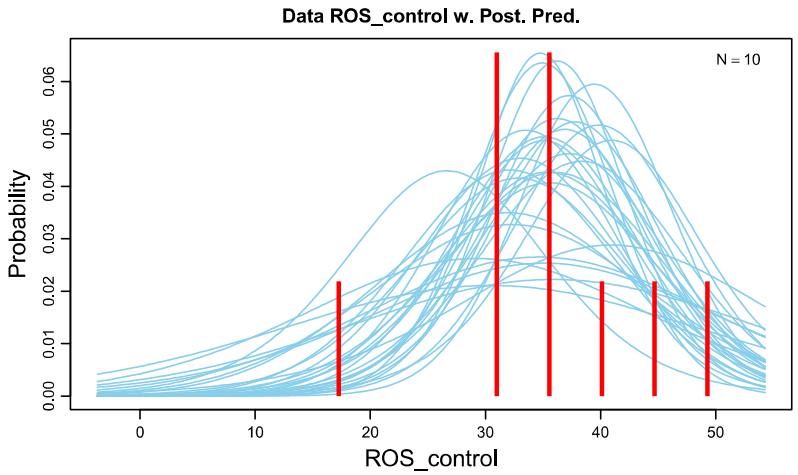

PGR_Generation3_100 Mean
median = 1.2
95% HDI
1.2
1.2
1.10
1.15
1.20
1.25
μy

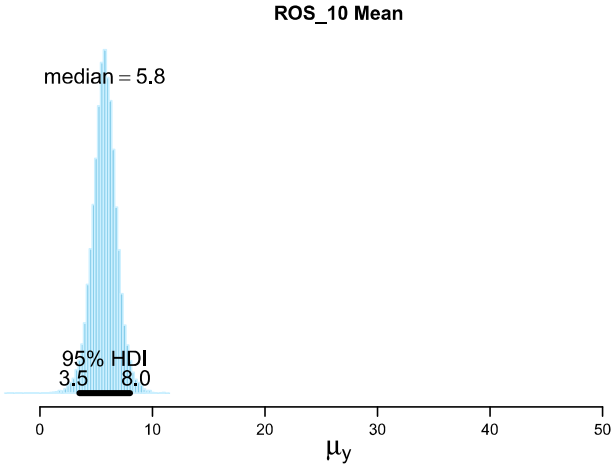

Data PGR_Generation3_100 w. Post. Pred.
N = 5
Probability
0
10
20
30
40
50
60
70
1.16
1.18
1.20
1.22
1.24
1.26
PGR_Generation3_100

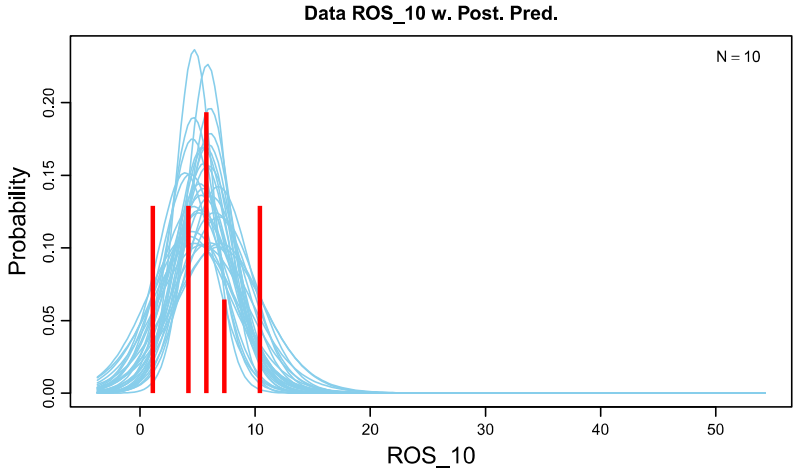

Difference of Means
median = 0.067
0% < 0 < 100%
95% HDI
0.048
0.085
0.00
0.05
0.10
μx − μy

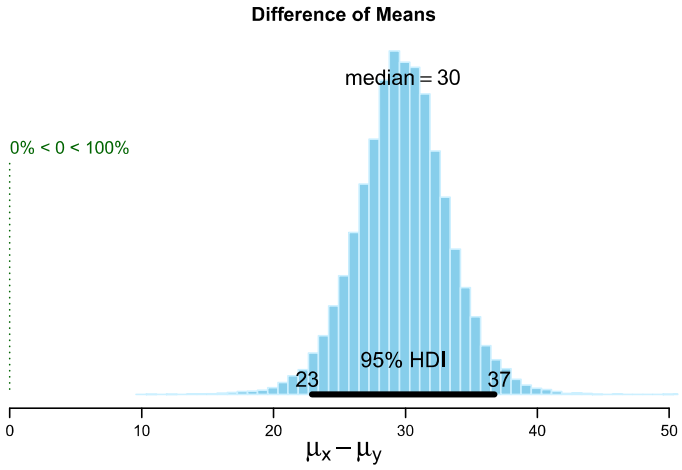

Effect Size
median = 5.1
0% < 0 < 100%
95% HDI
1.9
8.3
0
5
10
15
(μx − μy)/√((σx² + σy²)/2)

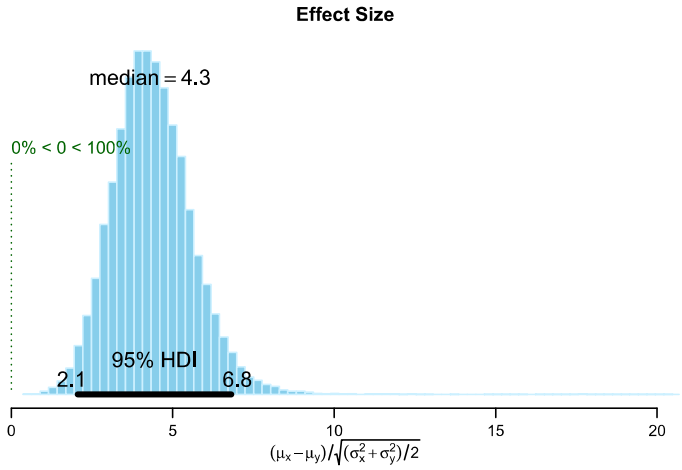

ROS_control Mean
median = 36
95% HDI
29
42
0
10
20
30
40
50
μx

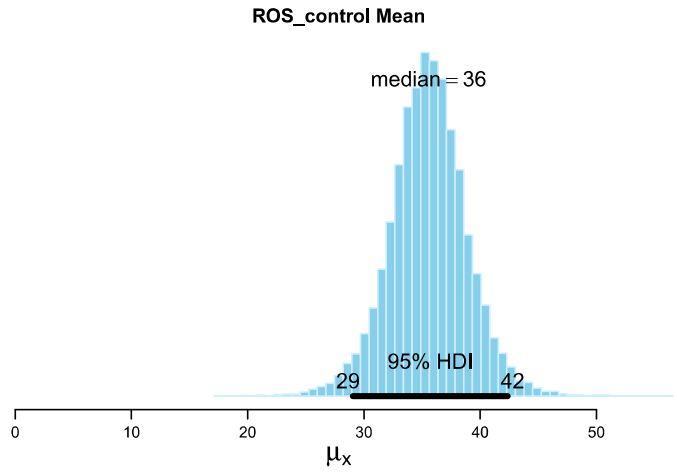

Data ROS_control w. Post. Pred.
N = 10
Probability
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0
10
20
30
40
50
ROS_control

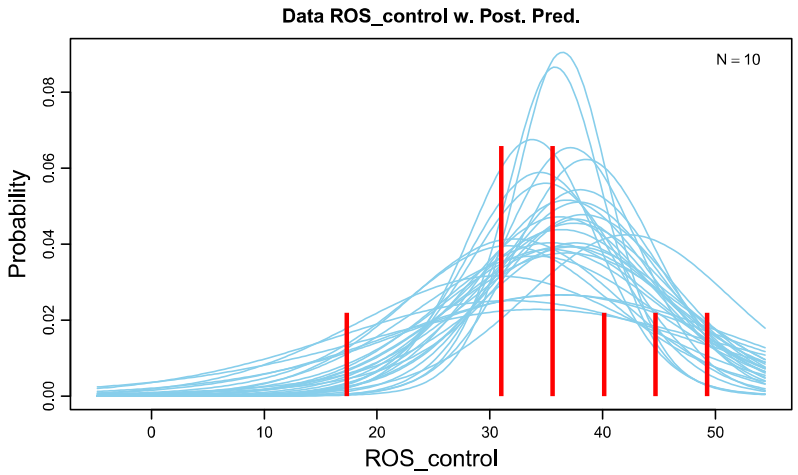

ROS_10 Mean
median = 5.8
95% HDI
3.5
8.0
0
10
20
30
40
50
μy

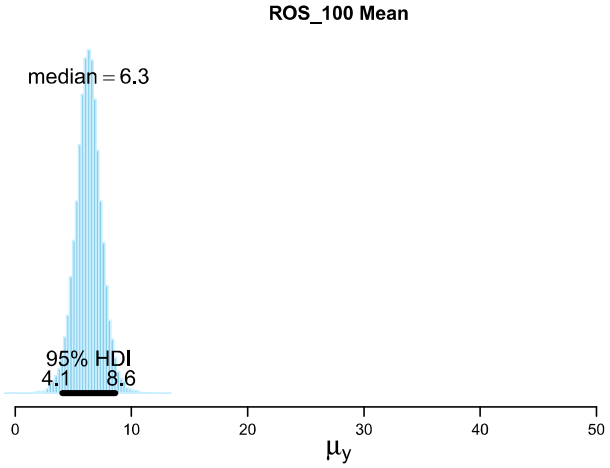

Data ROS_10 w. Post. Pred.
N = 10
Probability
0.00
0.05
0.10
0.15
0.20
0
10
20
30
40
50
ROS_10

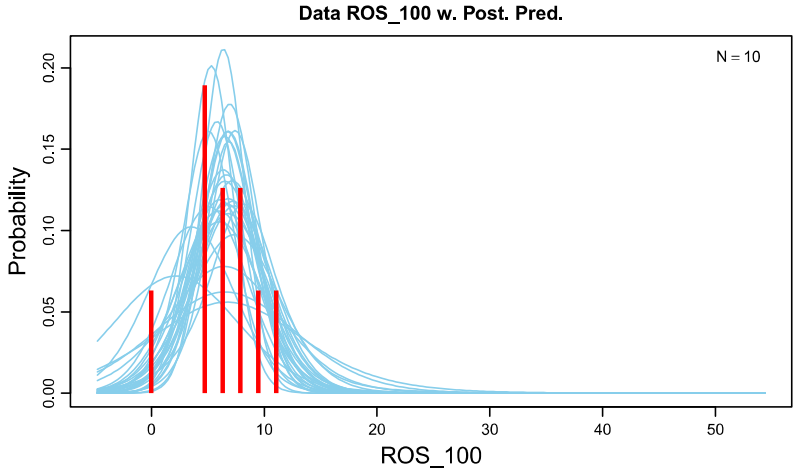

Difference of Means
median = 30
0% < 0 < 100%
95% HDI
23
37
0
10
20
30
40
50
μx − μy

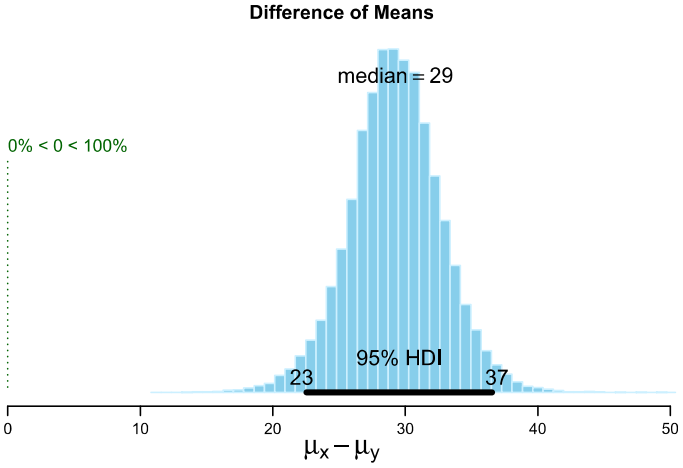

Effect Size
median = 4.3
0% < 0 < 100%
95% HDI
2.1
6.8
0
5
10
15
20
(μx − μy)/√((σx² + σy²)/2

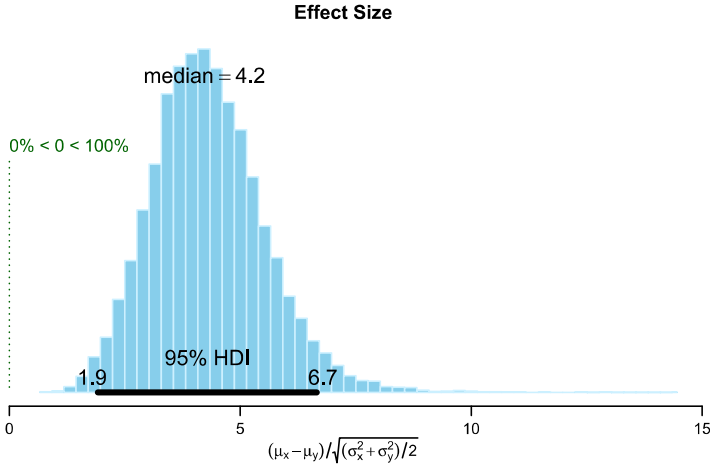

ROS_control Mean
median = 36
95% HDI
29
42
0
10
20
30
40
50
μ_x

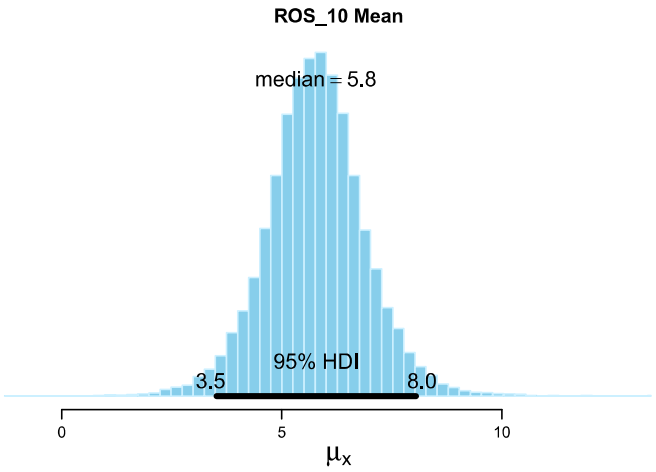

Data ROS_control w. Post. Pred.
N = 10
Probability
0.00
0.02
0.04
0.06
0.08
0
10
20
30
40
50
ROS_control

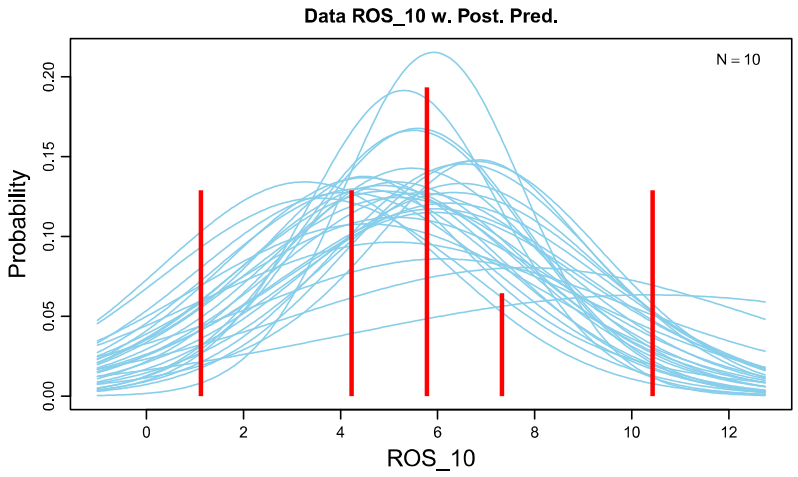

ROS_100 Mean
median = 6.3
95% HDI
4.1
8.6
0
10
20
30
40
50
μ_y

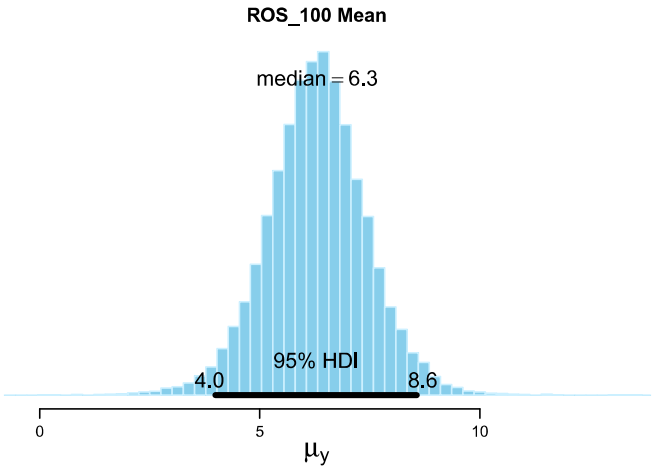

Data ROS_100 w. Post. Pred.
N = 10
Probability
0.00
0.05
0.10
0.15
0.20
0
10
20
30
40
50
ROS_100

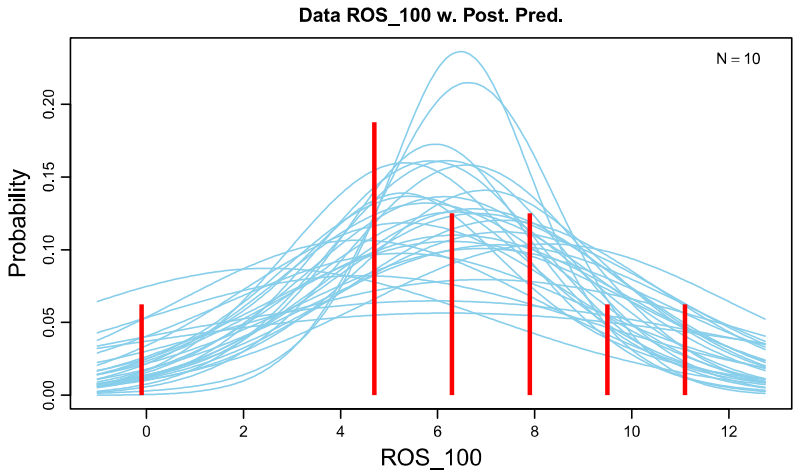

Difference of Means
median = 29
0% < 0 < 100%
95% HDI
23
37
0
10
20
30
40
50
$\mu_x - \mu_y$

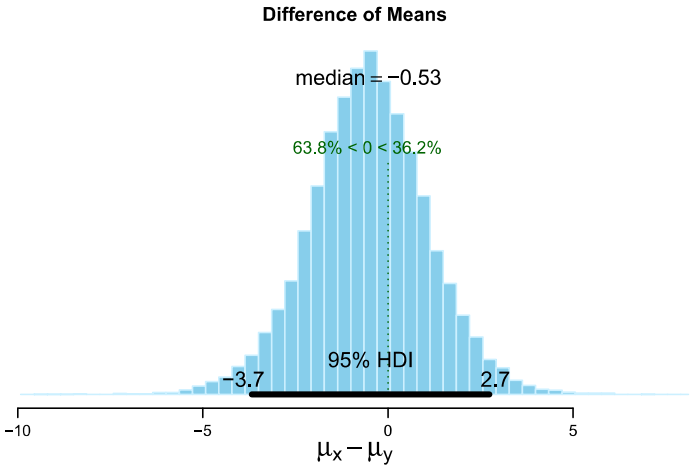

Effect Size
median = 4.2
0% < 0 < 100%
95% HDI
1.9
6.7
0
5
10
15
$(\mu_x - \mu_y)/\sqrt{(\sigma_x^2 + \sigma_y^2)/2}$

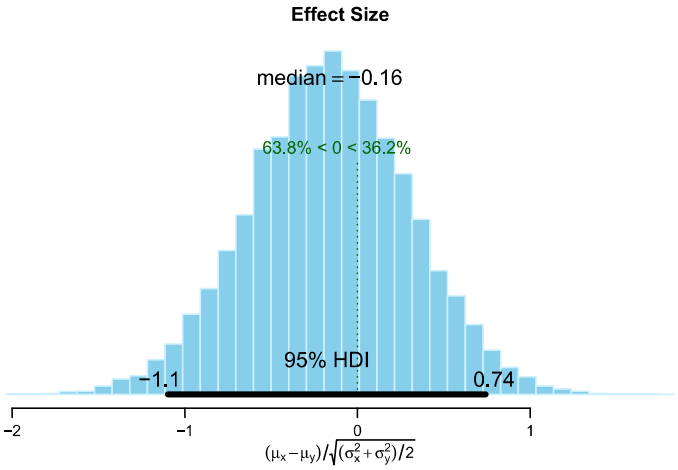

ROS_10 Mean
median = 5.8
95% HDI
3.5
8.0
0
5
10
μx


Data ROS_10 w. Post. Pred.
N = 10
Probability
0.00
0.05
0.10
0.15
0.20
0
2
4
6
8
10
12
ROS_10


ROS_100 Mean
median = 6.3
95% HDI
4.0
8.6
0
5
10
μy


Data ROS_100 w. Post. Pred.
N = 10
Probability
0.00
0.05
0.10
0.15
0.20
0
2
4
6
8
10
12
ROS_100


Difference of Means
median = −0.53
63.8% < 0 < 36.2%
95% HDI
−3.7
2.7
−10
−5
0
5
μx − μy


Effect Size
median = −0.16
63.8% < 0 < 36.2%
95% HDI
−1.1
0.74
−2
−1
0
1
(μx − μy)/√((σx² + σy²)/2

CHAPTER 2

Exposure to complex mixtures of urban sediments containing Tyre and Road Wear Particles (TRWPs) increases the germ-line mutation rate in *Chironomus riparius*

Lorenzo Rigano, Markus Schmitz, Volker Linnemann, Martin Krauss, Henner Hollert, Markus Pfenninger

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Appendix to Chapter 2

Declaration of contributions

Publication title: *Exposure to complex mixtures of urban sediments containing Tyre and Road Wear Particles (TRWPs) increases the germ-line mutation rate in Chironomus riparius*
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Authors and contributions:

- **Lorenzo Rigano**: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.
- **Markus Schmitz**: Writing – original draft, Visualization, Formal analysis, Data curation.
- **Volker Linnemann**: Writing – review & editing, Supervision.
- **Martin Krauss**: Writing – review & editing, Supervision.
- **Henner Hollert**: Writing – review & editing, Supervision.
- **Markus Pfenninger**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review & editing.

Introductory note

The aim of this second chapter was to investigate whether multigenerational exposure of *C. riparius* populations to urban runoff sediment could induce detectable genomic and stress-related responses. Urban runoff sediments represent a complex and chemically diverse matrix, integrating contaminants derived from traffic and urban activities. Due to their ubiquity and ecological relevance, these mixtures constitute an important, yet understudied, source of environmental stress for aquatic ecosystems and a prototype of multiple stressors.

The same MAL-WGS methodology employed in Chapter 1 was applied. Populations were exposed over five generations to two concentrations (0.5% and 10%) of sediments, and mutation rate together with oxidative stress markers (ROS) were measured.

This study addressed the need to move from controlled single-compound experiments to more realistic environmental scenarios. In the context of the thesis, it complements the findings from Chapter 1 by considering mutagenic effects under complex, ecologically representative

conditions, thereby strengthening the broader objective of understanding how multiple stressors shape population resilience in aquatic insects.

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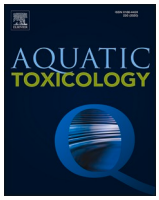

Exposure to complex mixtures of urban sediments containing Tyre and Road Wear Particles (TRWPs) increases the germ-line mutation rate in *Chironomus riparius*

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ABSTRACT

Tyre and road wear particles (TRWPs) are a significant yet often underestimated source of environmental pollution, contributing to the accumulation of microplastics and a complex mixture of contaminants in both terrestrial and aquatic ecosystems. Despite their prevalence, the long-term evolutionary effects of TRWPs, beyond their immediate toxicity, remain largely unknown. In this study, we assessed mutagenicity in the non-biting midge *Chironomus riparius*, upon exposure to urban sediment collected from a runoff sedimentation basin. To assess the extent of mutagenic effects over multiple generations, we combined the urban sediment exposure model with short-term mutation accumulation lines (MALs) and subsequent whole genome sequencing (WGS). The study was conducted over five generations, with urban sediment concentrations of 0.5 % and 10 %. Our results reveal that the exposure to urban sediment significantly increases mutation rates compared to control groups by 50 %, independent of concentration (0.5 % and 10 %). To infer potential causal processes, we conducted a comparative analysis using known mutational spectra from previous studies. This comparison showed that the mutation profiles induced by urban sediment clearly clustered with those caused by Benzo[a]Pyrene (BaP), a known Polycyclic Aromatic Hydrocarbon (PAH). A comprehensive chemical characterization of the sediment confirmed a considerable impact of road runoff and traffic-related contamination, including PAHs of primarily petrogenic origin. This suggests that PAH-like compounds present in urban sediments may play a significant role in the observed mutagenic effects. Our study shows that urban sediments influence mutation rates and alter mutational spectra in exposed organisms, potentially compromising genomic stability and shaping evolutionary trajectories. These genetic changes can have profound long-term effects on population dynamics and ecosystem health, underlining the importance of understanding the evolutionary consequences of environmental pollution. Additionally, we show that comparatively analysing of mutational spectra may provide valuable insights into mutational processes.

1. Introduction

Globalisation is largely driven by an ever-expanding transport network of people and goods, transforming global trade and mobility. However, this progress comes with multiple costs (Sovacool et al., 2021). One of the less recognised consequences of road transport is Tyre and Road Wear Particles (TRWPs). As vehicles travel on asphalt surfaces, the inevitable wear generates constantly particles. These tiny fragments,

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ranging from nanometres to micrometres in size, consist of a mixture of rubber, asphalt components, and other various environmental contaminants. The widespread presence of TRWPs in water bodies, soils and airborne particles highlights their environmental mobility (Baensch-Baltruschat et al., 2021; Sieber et al., 2020; Unice et al., 2019), raising concerns about their ecological impact. In Germany alone, approximately 20,000 tonnes of TRWPs enter water bodies annually, representing one of the largest sources of microplastics released into the environment (Baensch-Baltruschat et al., 2021). Once microplastic particles enter aquatic ecosystems through rainwater runoff or wind, they can harm organisms in these environments (Khosrovyan et al., 2022).

Urban runoff, managed by sedimentation basins strategically placed near busy roads, represents one of the most significant pathways for TRWPs to enter aquatic ecosystems. The main role of sedimentation basins is to separate water and particles from road runoff through sedimentation, allowing purified water to enter nearby water sources and retaining sediment within the basins (Grung et al., 2016; Proteau et al., 2022). However, road runoff often enters water bodies or surrounding areas untreated (Grung et al., 2016), making sediment accumulated in urban runoff sedimentation basins an ideal subject to assess the ecotoxicological effects of TRWPs and urban particulate matter. The sediments investigated in this study represent urban systems well, as confirmed by earlier studies from the same site (Rigano et al., 2024) and similar chemical compositions reported in related research (Maurer et al., 2023; Meland et al., 2024). Variations may result from altered cleaning frequencies of such sites, size and use-case of drained areas of such basins, meteorologic conditions during the collection period, additional or accidental inputs into separate sewer systems, including household chemicals, pharmaceuticals from occasional wastewater inflow, and pesticides from green management, which can alter sediment composition.

The introduction of anthropogenic substances into the environment can adversely affect organisms by lowering their fitness. This shift in environmental conditions caused by stressors can alter the selective pressures acting on organisms, influencing their evolutionary trajectory (Charlesworth, 2009, 1971). The key factor for the evolutionary response to anthropogenic pollution is therefore 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 and Geyer, 2010). Adaptation to adverse changes in the environment by environmental pollutants is widespread and can occur rapidly within a few generations (Barghi et al., 2020; Bulut et al., 2024). Changes in population fitness can influence demography, potentially affecting community structure and triggering eco-evolutionary dynamics (Carroll et al., 2007; Fussmann et al., 2007; Shaw and Geyer, 2010). This highlights the role of anthropogenic stressors as agents of natural selection that can shape evolutionary processes (Otto, 2018).

Another impact of anthropogenic stressors on evolutionary fate is the induction of mutations that, although essential for evolution, in most cases have a negative impact on fitness (Eyre-Walker and Keightley, 2007). Therefore, anthropogenic substances should be considered not only as drivers of immediate lethal and sublethal toxic effects but also as potential agents of natural selection that may trigger profound change in the evolutionary trajectory (Ankley et al., 2010; Brady et al., 2017). However, current ecotoxicological research tends to focus more on the immediate ecological impacts of tested contaminants, rather than their evolutionary consequences. Thus, to comprehensively understand the impact of anthropogenic stress on biodiversity, it is imperative to investigate its evolutionary effects on organisms. Recognizing these long-term impacts is vital for enhancing ecological risk assessments and ensuring that environmental policies address both immediate and evolutionary risks.

In vitro studies have demonstrated that TRWPs can induce cytotoxicity through various biological mechanisms, with inflammation and oxidative stress being the most evident (Fussell et al., 2022). Furthermore, genotoxicity has been reported as another mechanism of toxicity, as DNA damage has been observed after exposure of human lung cells to TRWPs (Bouredji et al., 2023). Although genotoxicity, which refers to damage to DNA in somatic cells, may occur, it does not necessarily result in mutagenicity, meaning it does not always induce mutations in germ line cells that can be inherited and thus have implications for evolutionary processes (Doria and Pfenninger, 2021).

The non-biting midge *Chironomus riparius* is a widely used model in evolutionary ecotoxicology due to its fully sequenced genome (Pettrich et al., 2024; Schmidt et al., 2020), short generation time, and ecological relevance as a freshwater species frequently exposed to urban runoff. These features make it particularly suited for studying mutagenesis and stress responses caused by anthropogenic contaminants. However, its rapid adaptability to chemical stressors (Foucault et al., 2019; Oppold and Pfenninger, 2017) may underestimate impacts on less adaptable species, and its habitat specificity limits direct applicability to marine or terrestrial environments. While working with model organisms inherently has limitations and results cannot be universally generalized (Lopes et al., 2021), *C. riparius* remains an invaluable tool for experimentally exploring the genomic and physiological mechanisms underlying responses to environmental stressors.

In the current study, we investigated the microevolutionary responses of *Chironomus riparius* to urban sediments from an urban runoff sedimentation basin by assessing mutagenicity across multiple generations. The primary objective of the study was to explore how exposure to complex mixtures of urban contaminants influences the mutation rates and mutational spectra in *C. riparius*. To address this hypothesis, we employed a recently introduced mutation rate test (Oppold and Pfenninger, 2017) as an effective ecotoxicological mutagenicity test tool for metazoan organisms (Doria and Pfenninger, 2021). Previous studies, in which *C. riparius* was exposed to Cadmium (Cd) and Benzo[a]pyrene (BaP), substances known for their potential mutagenicity, have shown the robustness of our approach in assessing germline mutagenicity (Bulut et al., 2024; Doria and Pfenninger, 2021). The test consists of a combination of short-term mutation accumulation (MA) lines, whole genome sequencing (WGS), and dedicated data analysis. After exposure to potentially mutagenic substances, whole genome sequencing (WGS) of individuals from these lines can reveal *de novo* mutations compared to the initial parents. By analysing the number and spectrum of these mutations, the mutagenic potential of the tested compound can be investigated. Additionally, an extensive chemical characterisation of the sediment was performed. Since oxidative stress is a known trigger of mutations (Aitken and Krausz, 2001), we measured Reactive Oxygen Species (ROS) levels to evaluate the extent of oxidative stress, using a newly developed method. By combining mutagenicity data with chemical profiles, we investigated the contribution of different contaminants to the observed effects. In addition, we present a comparative analysis of mutational spectra induced in *C. riparius* by different environmental stressors, based on data from previous studies conducted with the same experimental setup.

2. Materials and methods

2.1. Culturing conditions

The culture of *C. riparius* used in the present study came from a local population collected in a small river located in Hasselbach, Hessen, Germany (co-ordinates: 50.167562 N, 9.083542E). This culture was maintained and regularly replenished from the field as an in-house laboratory standard. The cultivation conditions of the stock culture conformed to a modified protocol based on the methodology outlined in OECD guideline N219, as previously described (Foucault et al., 2019).

2.2. Sediment collection and preparation

In February 2023, the sediment was collected from an urban runoff sedimentation basin located in Aachen, Germany (50°48′03.6″N 6°06′29.7″E) receiving runoff via a separate sewer system from a large area in the district of Aachen Soers, including a sports park and a highly frequented road, Bundesstraße B57 Krefelder Straße. The total drained area covers 68 hectares. The B57 serves around 26,000 vehicles per day, according to a 2021 manual count by the Federal Highway Research Institute (BASt). The sampling took place during cleaning of the basin, and samples were obtained from different spots within the basin using a stainless-steel bucket, thoroughly mixed, and stored in a cold room at 4 °C until further use. For further analyses, aliquots were freeze-dried and sieved (stainless steel mesh, 1 mm mesh size) to remove large particles (Shuliakevich et al., 2022).

2.3. Mutation Accumulation Lines (MALs) experimental design

All MALs originated from a single egg rope (F0) raised under optimal conditions to avoid selection. Following the successful reproduction of their offspring (F1), forty-five egg ropes were collected, and the maximum number of larvae possible was used to establish 65 MA lines. Due to the technical limitation of having so many egg ropes at the same time, we maximized their use by splitting some egg ropes into two. The larvae from the hatched clutches were then placed in glass bowls (Ø20×10 cm) containing three increasing concentrations of urban sediment, as a percentage of total weight: (Table 1).

The quartz playground sand used had a granularity of 0–2 mm, was pH neutral, and was washed before use. In the glass bowls containing the urban sediment, sand and urban sediment were mixed. The operation was performed with a laboratory spatula to homogenize the resulting sediment column. To all glass bowls, 1.250 L of medium was added. The medium consisted of purified, deionised water adjusted to a conductivity of 550–650 µS/cm with aquarium sea salt (e.g., TropicMarin®), and a basic pH around 8. Only a single egg-clutch originating from each MA line was chosen to start the following next generation. The test vessels were maintained in a constantly ventilated room at a temperature of 20 ± 1 °C, with a relative humidity of 60 % and a 16:8-hour light/dark cycle. To compensate for water evaporation in the test vessels, demineralized water was constantly supplemented. The replicates were then raised and fed daily with finely ground fish food (Tetramin® Flakes) whose amount was calculated for the respective developmental stage as described elsewhere (Foucault et al., 2019).

Because of the swarm fertilization of *C. riparius* and the thereby following impossibility of determining the parents of specific egg ropes, adult offspring of the first generation were collected and used as a pooled reference to infer the joint genotype of the parents. At the end of the fifth generation, one randomly chosen female was collected for DNA extraction.

2.4. Whole genome sequencing, bioinformatic and statistical analysis

DNA extraction was performed with the Blood and Tissue QUIAGEN Kit following the manufacturer's instructions. To obtain an ancestor to generate a baseline to identify de novo mutations (DNMs) 120 legs, one of each individual from F1, were pooled together to an expected mean coverage of 60X. After five generations, one female from each of the MA-lines was whole-genome sequenced to an expected mean coverage of 30X on the Illumina NovaSeq 6000 platform, following the generation of libraries and their quantification using real-time PCR (Doria et al., 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 for POOL 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 manually validated using IGV. The mutation rate was estimated by dividing the number of mutations per generational passage times the callable sites. To compare mutational rates between treatments, a Bayesian Poisson test was applied using the R package BayesianFirstAid (Bååth, 2014).

Table 1
The three increasing concentrations (0, 0.5 %, and 10 %) of urban sediment tested. One asterisk (*) indicates the quartz playground sand, while two asterisks (**) indicate the urban sediment.

0 (control)	0,5 %	10 %
500 g of quartz playground sand*	497,5 g* + 2.5 g of urban sediment**	450 g* + 50 g**

2.5. Chemical analyses

The sediment was screened for Polycyclic Aromatic Hydrocarbon content (16 EPA PAHs) using gas chromatography coupled with mass spectrometry (GC–MS) (EN17503:2022–02), light and heavy metals using inductively coupled plasma mass spectrometry (ICP-MS) or optical emission spectrometry after microwave assisted aqua regia digestion (EN ISO 17,294–2:2026–08 and EN ISO 11,885:2009–05), as well as mineral oil indices by gas chromatography with flame ionization detection (GC-FID) (EN ISO 9377–2:2000–10) were measured in freeze-dried particles. According to methods published earlier (Rigano et al., 2024), the maximum possible tire wear content was determined based on the measurement of the residual organic carbon ROC_{900} following the temperature-gradient method (EN 17,505:2023–10 former DIN 19,539 (2016–12)), which offers a temperature-dependent differentiation between TOC_{400} (EN 15,936 (2022–02)), remaining organic carbon (ROC_{900}) and total inorganic carbon (TIC_{900}).

Moreover, organic extracts were prepared using an exhaustive ultrasonic-assisted extraction via an eluotropic solvent gradient adapted from previous methods (Hiki and Yamamoto, 2022; Klöckner et al., 2021). A series of two solvents from n-heptane-acetone (1:1 v/v) and methanol was used, covering a broad spectrum of the eluotropic series (Snyder, 1974). 400 mg of sediment (sieved to 1000 µm) was suspended in 10 mL of solvent, sonicated for 15 min. in an ice-cooled water bath to prevent overheating, and then centrifuged at 1900 x *G* for 2 min. After centrifugation, the supernatant was carefully aspirated using a glass Pasteur pipette and transferred to a Büchi round flask. The extraction was repeated once more with n-heptane-acetone mixture and two more times with methanol. After these four extraction steps, the combined extracts were evaporated to almost dryness, redissolved in 1 mL methanol, and filtered using a 0.2 µm PTFE-membrane filter attached to a luer-lock syringe to remove finest aspirated particles. For target-screening on tyre rubber additives and other known and potential polar water pollutants the extracts were diluted 5-fold and spiked with an internal standard mixture containing 40 isotope-labelled compounds before being analysed using liquid chromatography coupled to high-resolution mass spectrometry (LC—HRMS). To this end, a Thermo Ultimate 3000 LC system coupled to a quadrupole-Orbitrap instrument (Thermo QExactive Plus) with electrospray ionisation in positive and negative ion mode was used. For LC separation, a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 µm particle size, Phenomenex) with in-line

filter and pre-column of the same type (5 × 2.1 mm) was employed. The gradient separation used 0.1 % formic acid (eluent A)/methanol containing 0.1 % formic acid (eluent B)/acetonitrile (eluent C) for positive mode and 1 mm ammonium fluoride (A)/methanol containing 1 mM ammonium fluoride (B)/acetonitrile (C) for negative mode. The gradient program started for both modes at 97 % A/3 % B/0 % C, held for 2 min, increasing to 3 % A/97 % B/0 % C in 14 min, and changing to 3 % A/0 % B/97 % C in 4 min, held for 4 min. The eluent was afterwards re-equilibrated to initial conditions in 4.8 mins. The HRMS analysis combined a full scan acquisition (m/z 80–1200, nominal resolving power of 70,000) with six data-independent acquisition scans (m/z 80–182, 178–282, 278–382, 378–482, 478–682, 682–1200) at a nominal resolving power of 35,000.

For quantification, Thermo raw files were converted to mzML files using ProteoWizard (Chambers et al., 2012), followed by peak detection and annotation in MZmine 2.38 (Pluskal et al., 2010) and further processing by the MZquant R package (Finckh et al., 2022). We used an internal standard calibration with target analyte concentrations ranging from 0.2 to 1000 ng/mL and an internal standard concentration of 50 ng/mL.

2.6. ROS detection, image and statistical analysis

First-stage chironomid larvae were subjected to the three increasing concentrations of urban sediment (0, 0.5 % and 10 %). Subsequently, 20 L3 larvae were collected and placed in 24-well plates. Plates were filled with 2.5 ml medium as described before (Foucault et al., 2019). 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 localised 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 10x magnification. The images were taken with AxioVision Rel. v.4.8. For fluorescence images, an HXP 120 C 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 489,043–9901–000) with 1 second 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 analysed by ImageJ Fiji (v. 2.15.0). Images were uploaded to ImageJ as an image sequence and converted to 8-bit grayscale from RGB Colour images to avoid colour difference and only calculate light intensity. The same threshold was applied to all images (Threshold: 23). After setting the threshold, the “measure” function was used. Fluorescence intensities were calculated as the mean values of each image. The fluorescence images were analysed with ImageJ to determine red colour density and intensity. Data from ROS measurements were analysed using the R package *brms* to perform a Bayesian ANOVA, aimed at testing the differences between treatments. The analysis included Bayesian linear regression to model treatment effects, with posterior distributions and credible intervals computed to assess differences in means and their significance.

2.7. Heatmap and dendrogram of different treatment conditions

To compare the mutational spectra of different treatment conditions from previous experiments conducted with the same set-up but different environmental conditions, we constructed a heatmap and dendrogram. These are based on the similarity of mutation rates for different mutational classes of *C. riparius*. Data were collected from 14 experimental conditions, covering six classes of nucleotide substitutions (G<>*T*, *A*<>*T*, C<>G, A<>C, C<>T, G<>*A*). The heatmap, generated with ggplot2, displayed experimental conditions on the y-axis and nucleotide substitutions on the x-axis. A grayscale gradient, ranging from light grey (low values) to black (high values), was used to represent transition and transversion rates, emphasizing differences in mutation rates between conditions. Clustering patterns were emphasized through faceting to clearly show trends. The dendrogram was created using hierarchical clustering with Ward’s method on a distance matrix derived from scaled mutation data, illustrating the similarity between experimental conditions based on their mutational spectra. All data analysis, clustering, and visualization were performed using R (v. 4.2.1).

3. Results

3.1. Mutation rate and spectrum

Mean sequence coverage ranged from 31.8x to 37.9x for the individual MALs lines and 55.7x for the reference pool. The number of callable sites ranged from 137,847,045 bp to 161,044,682 bp. Overall, we identified 27 mutations during 60 generational passages in control, 38 mutations during 70 generational passages in 0.5 % and 42 mutations during 70 generational passages in 10 % (Table 2). For the control, 7 transversion and 20 transition mutations were identified. In 0.5 % urban sediment, 17 transversion, and 21 transition mutations were identified. In 10 % urban sediment, 13 transversion, and 29 transition mutations were identified (Table 3).

The mutation rate estimate for the control was $\mu = 2.79 \times 10^{-9}$ (95 % HDI 1.8×10^{-9} and 4.0×10^{-9}), for 0.5 % of urban sediment was $\mu = 4.11 \times 10^{-9}$ (95 % HDI 2.9×10^{-9} and 5.5×10^{-9}) and for 10 % of urban sediment was $\mu = 4.23 \times 10^{-9}$ (95 % HDI 3.1×10^{-9} and 5.6×10^{-9}). The 0.5 %/control rate ratio was 1.5, with 93.9 % certainty this ratio being larger than 1. The 10 %/control rate ratio was 1.5 (95.5 % certainty). The 0.5 %/10 % rate ratio was 0.97 (55 %<1 < 45 %) (Fig. 1).

3.2. Chemical characterisation of urban sediment

The chemical characterisation of the sediment from the investigated lamellar basin revealed a broad spectrum of anthropogenic and urban and traffic-related contamination (Fig. 2). The total organic carbon content of the investigated sediment was 124 g/Kg sediment dry weight, of which a maximum measured tyre rubber content (TWP_{max}) was 5.2 g/kg, resulting in a total share of 0.52 % tyre rubber. Based on TWP_{max} measurements in road runoff from highly frequented roads in Aachen city of 11.5 % TWP_{max}, we can estimate a contribution of 4.5 to 8.5 % road runoff particles to the overall urban particulate share in the sampled lamella basin, which corresponds to the share of the drained area that contributes runoff mandatory for treatment (4.4 ha/7 % of the area). Road salts (Ca, Na, K, Mg) contributed a total of 46.12 g/Kg dw. Further, metals contributed largely to the composition of the

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	N. of callable sites	N. of SNM	Mean µ Rate
Control	33.1	1.6E+08	27	2.79E-09
0.5 % urban sediment	31.8	1.4E+08	38	4.11E-09
10 % urban sediment	37.9	1.5E+08	42	4.23E-09

Table 3
Mutation spectrum of *C. riparius* after 5 generations of urban sediments exposure.

Treatment	Transversions		Transitions			
	G<>*T*	*A*<>*T*	C<>G	A<>C	C<>T	G<>*A*
Control	2	5	1	3	9	7
0.5 % urban sediment	6	11	3	1	7	10
10 % urban sediment	5	8	2	9	7	11

investigated matrix with heavy metals sharing 21.67 g/Kg dw and Aluminum (Al) sharing 13.07 g/Kg dw. Heavy metal contamination was largely dominated by Iron (Fe) with 19.5 g/Kg dw, followed by Zinc (Zn) (1.74 g/Kg dw) and Copper (Cu) (144 mg/Kg dw).

The screening of the 16 EPA PAHs revealed 4 mg PAHs per kg of sediment of which about 80 % were shared by high molecular weight PAHs, such as Benzo[b]fluoranthene (1200 μg/Kg), Benzo[g,h,i]perylene (1090 μg/Kg), and Benz[a]anthracene (633 μg/Kg). Additionally, 983 μg/Kg mineral oils were measured in the sediment. The measured PAH content differs from a previously reported sampling of the investigated site. The sediment in this study contained a share of 4.0 mg/Kg dw PAHs, whereas sediment sampled earlier contained 2.9 mg/Kg dw. The fluoranthene to pyrene ratio is 0 as these were below the LOQ (0.55 in Rigano et al. (2024)), the indeno[1,2,3,c,d]pyrene to benzo[g,h,i] perylene ratio was 0.14, comparable to our previous study. The considerable lower share of low molecular weight PAHs and resulting altered composition hints to a stronger contribution of PAHs of petrogenic origin (Tobiszewski and Namieśnik, 2012a).

The LC—HRMS/MS target screening on polar chemicals and tyre rubber additives revealed a strong contribution of known plastic and rubber additives. Of the total 124 detected synthetic chemicals, 49 were labelled as polymer additives, 23 as personal care and household chemicals, 19 as pesticides, 14 as biocides, and 12 as food and beverage related. Additionally, three PFAS compounds, three intermediate compounds, and one industrial dye were detected. Of the top 50 most abundant substances detected (by mass), 31 were identified as tyre rubber additives, composed mainly of phosphate-esters (38.7 mg/Kg dw, $n = 8$) used as flame retardants, phthalates (10.1 mg/Kg dw, $n = 4$) used as plasticisers, benzothiazole derivates (2,1 mg/Kg dw, $n = 3$) used as vulcanization agents, and bisphenols (2.6 mg/Kg dw, $n = 2$), also used in tyre manufacturing. Additionally, the ubiquitous tyre rubber additives N'N-diphenyl guanidine (3.1 mg/Kg dw), and the oxidation product 6PPD-quinone (112 μg/Kg dw) were detected.

3.3. Reactive Oxygen Species (ROS) measurements

The fluorescence intensity we measured is not the actual ROS amount within the organism, but the current amount of reagent entered in the organism and oxidised by binding to ROS, which is the remaining amount after the organism's antioxidant system scavenges ROS.

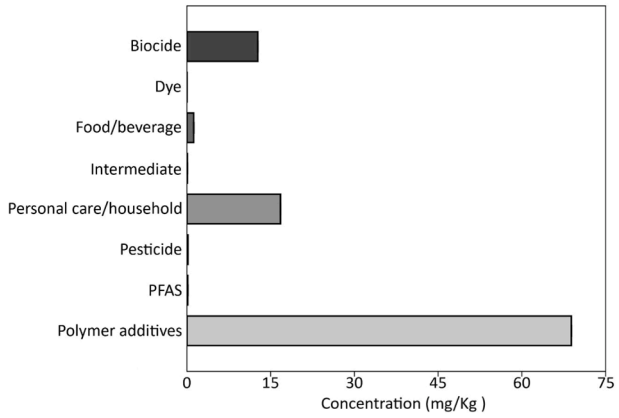

Fig. 2. Tyre rubber additives and wastewater chemicals LC—HRMS target screening in lamella basin sludge organic extracts. The sum of concentrations is indicated in mg/kg sediment equivalent.

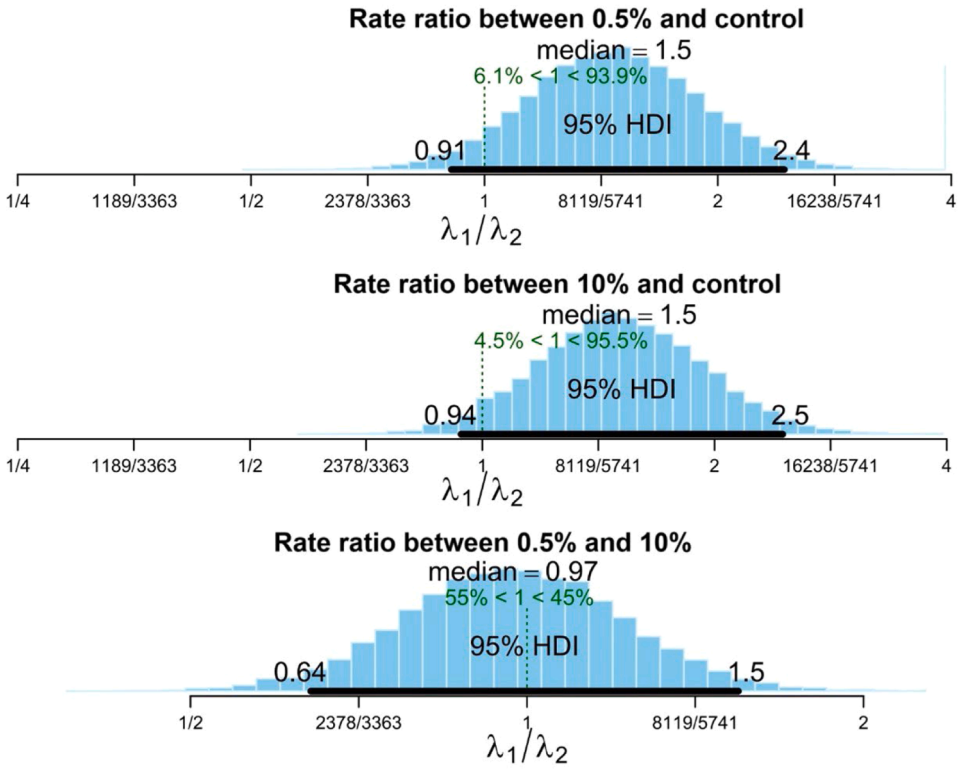

Fig. 1. Posterior distributions for rate ratios between treatment groups.

A difference of means of −7.21 was found between control and 0.5 % (95 % HDI, between −11.96 and 2.45), with a posterior probability of 98.8 %, that control was smaller than 0,5 %. A difference of means of 2.64 was found between control and 10 % (95 % HDI, between 1.92 and 7.35), with a posterior probability of 100 %, that control was larger than 10 %. A difference of means of 9.85 was found between 0.5 % and 10 % (95 % HDI, between 5.00 and 14.71), with a posterior probability of 100 %, that 0.5 % was larger than 10 % (Fig. 3).

3.4. Heatmap and dendrogram of different treatment conditions

The dendrogram results show that the control groups cluster separately from the other treatments. Among the treatments, the results show that, apart from the highest temperature of 26 °C, which clusters with Cd, the intermediate temperatures (16 °C to 23 °C) cluster together. The lowest temperature, 12 °C, clusters with the highest concentration of BaP (100 µg/L) and the highest concentration of urban sediment (10 %). In contrast, the two lowest concentrations of urban sediment (0.5 %) and BaP (10 µg/L) cluster exclusively together (Fig. 4).

4. Discussion

While the substantial direct toxic effects of urban sediments containing TRWPs on *C. riparius* were recently evaluated (Rigano et al., 2024), few multigenerational studies have been conducted to date. Our study investigated a microevolutionary response of the same species to this stressor by directly assessing mutagenicity across multiple generations. This focus is underscored by the growing recognition of mutagenicity as a crucial endpoint in ecotoxicology, particularly in metazoans. Measuring genetic impacts is essential to fully understand the long-term ecological effects of contaminant exposure (Bulut et al., 2024; Doria and Pfenninger, 2021).

Both tested urban sediment concentrations (0.5 % and 10 %) are estimated to cause a 1.47- and 1.51-fold increase in mutation rates, respectively, compared to the control. This about 50 % increase in mutagenicity highlights the potential of urban runoff sediments to cause lasting genetic changes, even at very low concentrations. Furthermore, given the pervasive presence of TRWPs in urban environments, such concentrations are likely common in surface waters (Baensch-Baltruschat et al., 2021; Mattsson et al., 2023; Zhang et al., 2014). The ecological relevance of *C. riparius* as a model organism in ecotoxicology suggests that our findings are significant not only for this species but may also be broadly applicable to other aquatic organisms. Consequently, sediment-dwelling freshwater biota in surface waters exposed to urban runoff likely experience persistent mutagenic effects, gradually altering the genetic composition of local populations.

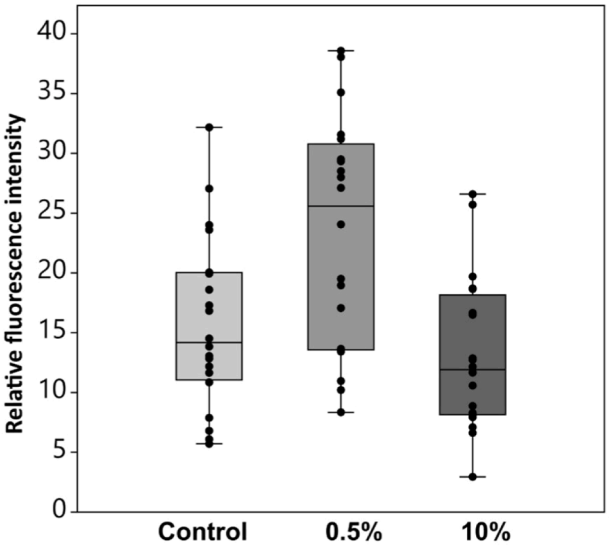

Fig. 3. ROS measurement: comparison of relative fluorescence intensity between control, 0.5 % and 10 %.

Although mutations fuel evolution, most of them have deleterious effects (Crow, 2000). High mutation rates can, therefore, reduce individual fitness on average and pose a long-term risk to population stability. The accumulation of mutations can interfere with critical biological processes such as reproduction, development, and survival, ultimately threatening overall population viability (Charlesworth, 2009; Rigano et al., 2024). Over time, accumulating mutations may paradoxically reduce genetic diversity (Lynch et al., 2016). This loss of diversity further compromises the population's ability to adapt to environmental changes, increasing the likelihood of population decline or extinction (Lynch et al., 2016; Markert et al., 2010). However, we found no difference in mutation rate between the two concentrations, and thus no dose-response relationship.

The chemical characterization of the investigated sediment revealed a broad spectrum of chemical contamination related to traffic and urban impact. The measured tire rubber content was approximately 5 g/kg of dry sediment, with an estimated road runoff contribution of 4.5–8.5 %, comparable to previous measurements from this sampling site (Rigano et al., 2024). The levels of salts and metals reflect the sediment accumulation period in the sedimentation basin, which spans late autumn and early winter, including freezing conditions. Road salts, the primary contributors to the overall contaminant load, not only induce osmotic stress but also accelerate the corrosion of vehicle parts, increasing the release of metals into road runoff. This effect is evidenced by the high concentrations of Fe and Al found in the matrix (Ebrahimi Gardeshi et al., 2024; Hintz and Relyea, 2019; Schuler and Relyea, 2018). Additionally, elevated concentrations of Pb, Cd, and Cu were detected, all linked to brake wear (Hwang et al., 2016; Rocha Vogel et al., 2024), with Cu being considered acutely toxic to aquatic wildlife (Brix et al., 2022) and phytotoxic in the mg/L range (Cruz et al., 2022). Furthermore, Cu toxicity in fish and invertebrates is closely linked to pH value and the salinity of the surrounding medium, suggesting that road salt loads may amplify the toxic effects of Cu and other metal contaminants in the environment (Grosell et al., 2007). However, little is known about the possible effects of co-exposure to Cd, Pb, and other metals (Balali-Mood et al., 2021; Liao et al., 2021; Wu et al., 2012; Yuan et al., 2014). Zinc, in its oxidized form, zinc oxide (ZnO), is associated with oxidative stress responses in *C. riparius* and is commonly found in high concentrations as an additive in tire rubber (Gopalakrishnan Nair and Chung, 2015). The analysis of PAH revealed a dominant contribution of High Molecular Weight (HMW) PAHs, suggesting a significant contribution from petrogenic sources (Tobiszewski and Namieśnik, 2012b). Furthermore, the presence of HMW PAHs, coupled with detected mineral oils, indicates a significant dioxin-like activity and mutagenic potential (Brinkmann et al., 2014; Grung et al., 2016; Shuliakevich et al., 2022). HMW PAHs may activate oxidative stress pathways, which could lead to mutagenic changes as a form of cellular adaptation to these toxic environmental conditions (Arambourou et al., 2019; Finckh et al., 2022). Concerning tire rubber additives, the detected organo-phosphate flame retardant Tris(1-chloro-2-propyl) phosphate (TCPP, 20 mg/kg dw) is known to cause DNA damage and act as a mutagen (Saquib et al., 2021). Furthermore, other conditions of pH, temperature, or salinity, could influence the mutagenic effects of sediments, e.g. by altering the bioavailability and toxicity of contaminants (Cipullo et al., 2018; Eggleton and Thomas, 2004).

Previous studies have shown that ROS can cause oxidative damage to both chromosomal DNA and free nucleotides, leading to genetic alterations (Aitken and Krausz, 2001; Dröge, 2002; Sakai et al., 2006). Additionally, elevated ROS levels are often used as indicators of increased mutation rates (Aitken and Krausz, 2001). Our ROS analysis revealed, however, that at 0.5 %, oxidative stress level increased significantly, while at 10 %, ROS level was comparable to the control.

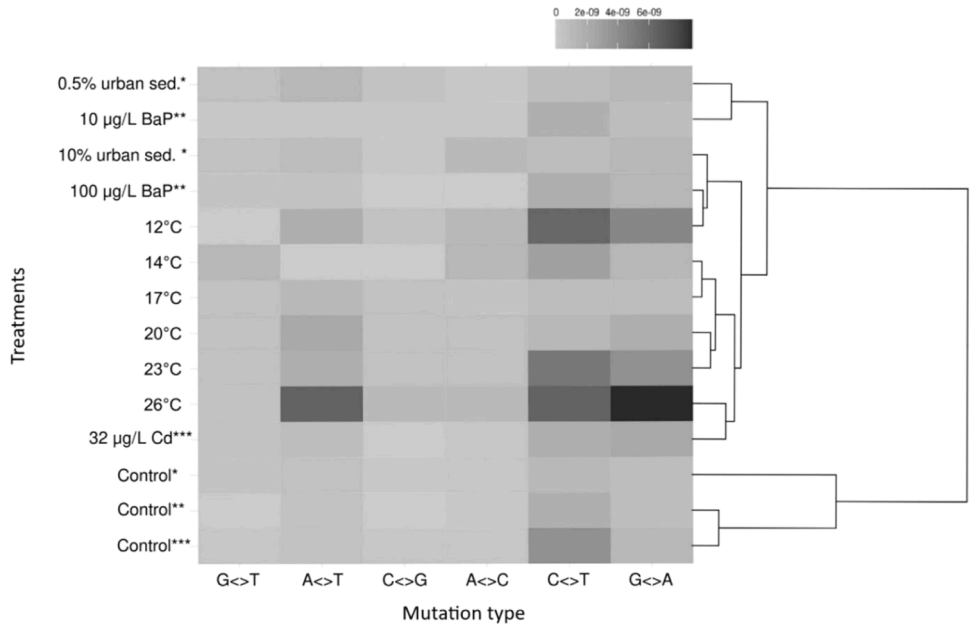

Fig. 4. Heatmap and dendrogram of the mutational spectra induced on *C. riparius* by different treatment conditions.

Although it remains unclear why a lower concentration exerted higher oxidative stress, this pattern suggests that oxidative stress might primarily drive mutagenicity at lower concentrations, but not at higher ones. This interpretation is supported by the clustering of the respective mutation spectra in two distinct groups (Fig. 4). While the spectrum induced by 0.5 % urban sediment closely resembled that induced by 10 µg/L BaP, the spectrum induced by 10 % urban sediment was like that induced by 100 µg/L BaP and low temperatures (12 °C). This pattern suggested that different underlying mechanisms caused the mutations observed at the two concentrations of urban sediment and PAHs. PAHs, which are abundant in complex mixtures like urban sediments (Rigano et al., 2024), are known to induce oxidative stress and DNA damage. Furthermore, BaP is considered a key marker of PAH exposure (Bukowska et al., 2022). However, a recent study discovered that pure BaP exposure actually reduced oxidative stress levels irrespective of the concentration, rather than inducing them (Bulut et al., 2024). However, this might be different in complex mixtures.

Thus, while oxidative stress cannot be excluded as the primary driver of mutagenicity at 0.5 %, at higher concentrations like 10 %, organisms may counteract ROS effects by activating detoxification pathways. These pathways employ cellular defences to neutralize ROS, such as the activation of Superoxide Dismutase (SOD) enzymes and catalase, converting them into less harmful by-products (Fukai and Ushio-Fukai, 2011). Despite the activation of these defence mechanisms, PAH-like compounds in urban sediments may still chemically modify the mutation spectrum, causing it to resemble that induced by 100 µg/L BaP. Alternatively, the observed consumption of urban sediment as a food source by the midges may explain the differences among concentrations. At 0.5 %, the organic sediment content appeared to be mostly consumed by the larvae, whereas this was not the case at 10 %. These organic molecules may capture most of the emerging ROS (Mir et al., 2024). Additionally, the composition of the organic matter in urban sediments may vary in a way that affects how ROS are neutralized or modulated, adding another layer of complexity, thereby externally quenching some of the mutagenic potential (Baptista Neto et al., 2016).

Exposure to low temperatures is necessarily associated with prolonged developmental times in insects due to reduced metabolic activity and slower cell cycle progression (Régnière et al., 2012). Likewise, the developmental time of *C. riparius* is significantly longer at 12 °C (Oppold and Pfenninger, 2017) and this might induce a higher mutation rate (Waldvogel and Pfenninger, 2021). The similarity of mutational spectra between low temperatures and 10 % urban sediment suggested that the latter might have caused an increase in developmental time. However, previous studies have shown that exposure to BaP and urban sediments, especially at a concentration of 10 %, does not prolong developmental time (Bulut et al., 2024; Rigano et al., 2024). This suggests that the similarities in mutation spectra observed at 10 %, 100 µg/L BaP, and 12 °C could result from either an unidentified common mechanism or coincidental effects.

Although heavy metals are a key component of urban sediments (Rigano et al., 2024, this study), the mutation spectrum did not resemble those of Cadmium (Doria and Pfenninger, 2021), which induces a mutation spectrum similar to those of very high temperatures (26 °C). This is consistent with studies showing that heavy metals may act through mechanisms distinct from PAHs, including oxidative stress and interference with DNA repair processes (Chen et al., 2022). While investigating the effects of the interactions between the multiple compounds present in urban sediments would be valuable, the objective of our study is to evaluate the synergistic effects of this complex mixture. Moreover, focusing solely on individual compounds would not be relevant for environmental risk assessment, as these substances typically coexist in the environment. Furthermore, linking mutational spectra to their causative agents is still in its early stages, and as such, the spectral clustering presented in this study represents a preliminary effort to advance the limited existing knowledge on the subject.

The changes we observed in the mutational spectrum may have broader implications for genomic stability. The mutation spectrum plays a crucial role in shaping the AT content of genomes. Transitions and transversions from GC to AT, particularly in regions with high GC content, often lead to an overall increase in AT content. Normally, this shift is counterbalanced by GC-biased gene conversion (gBGC) during recombination, which preferentially preserves G and C bases (Capra et al., 2013; Mugal et al., 2015). However, the introduction of mutagenic agents can disrupt this delicate equilibrium. In GC-rich genomic regions, changes in the mutation spectrum may result in a higher frequency of deleterious mutations and, therefore, reduced recombination efficiency (Capra et al., 2013). These changes could affect not only mutation rates but also the overall base composition of the genome, leading to long-term evolutionary consequences for populations exposed to these environmental stressors. Furthermore, the long-term effects of the

mutations identified in our study could be studied through multigenerational experiments, with the aim of assessing their impact on fitness in *C. riparius*. Such experiments could also demonstrate whether certain mutations confer selective advantages or disadvantages under different environmental conditions. They could then provide valuable insights into the mechanisms of natural selection and the processes of adaptive evolution driven by environmental stressors. Our findings highlight the importance of considering mutagenicity not only as a research focus but also as a critical endpoint in regulatory ecotoxicology, where its implementation could significantly enhance the evaluation of long-term environmental risks.

5. Conclusions

The result of our study demonstrates that urban sediments containing TRWPs significantly increased the mutation rate of *C. riparius* by 50 %, regardless of concentration. However, this was not the only mutagenic effect of urban runoff sediment. The observed changes of mutation spectra observed closely align with those induced by BaP, highlighting the putative role of PAH-like compounds in these genetic changes, which align with the considerable presence of PAHs, mineral oils, and tire rubber additives that have similar modes of action. Our results indicated that urban sediments not only impacted individual fitness but also reshaped genetic diversity and evolutionary dynamics. This highlights the need for multigenerational studies to better understand and manage the long-term effects of complex mixtures of pollutants and urban impacts on natural populations and entire ecosystems.

CRediT authorship contribution statement

Lorenzo Rigano: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Markus Schmitz:** Writing – original draft, Visualization, Formal analysis, Data curation. **Volker Linnemann:** Writing – review & editing, Supervision. **Martin Krauss:** Writing – review & editing, Supervision. **Henner Hollert:** Writing – review & editing, Supervision. **Markus Pfenninger:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107292.

Data availability

Data will be made available on request.

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CHAPTER 3

Mind your tyres: The ecotoxicological impact of urban sediments on an aquatic organism

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Appendix to Chapter 3

Declaration of contributions

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Authors and contributions:

- **Lorenzo Rigano**: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.
- **Markus Schmitz**: Writing – original draft, Visualization, Formal analysis, Data curation.
- **Henner Hollert**: Writing – review & editing, Supervision.
- **Volker Linnemann**: Writing – review & editing, Supervision.
- **Martin Krauss**: Writing – review & editing, Supervision, Formal analysis, Data curation.
- **Markus Pfenninger**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review & editing.

Introductory note

The third chapter of this thesis addresses the acute ecotoxicological effects of urban runoff sediments. While Chapters 1 and 2 focused primarily on mutation rate responses, this study examined short-term life cycle and physiological endpoints in response to a chemically complex matrix, thereby providing a more immediate view of organismal fitness under exposure.

The goal was to evaluate the impact of a gradient of urban runoff sediment concentrations on *C. riparius* within a single generation. Standard ecotoxicological traits such as mortality, mean emergence time (EmT50), fertility, larval size, and population growth rate (PGR), together with oxidative stress markers (ROS), were measured.

This short-term assessment not only offered insight into the acute consequences of exposure to complex mixtures but also served to define exposure levels for subsequent long-term investigations. By integrating organismal and physiological responses with the genomic

approaches presented in other chapters, this study contributes to a comprehensive understanding of the ecological risks posed by urban runoff sediments.

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Mind your tyres: The ecotoxicological impact of urban sediments on an aquatic organism

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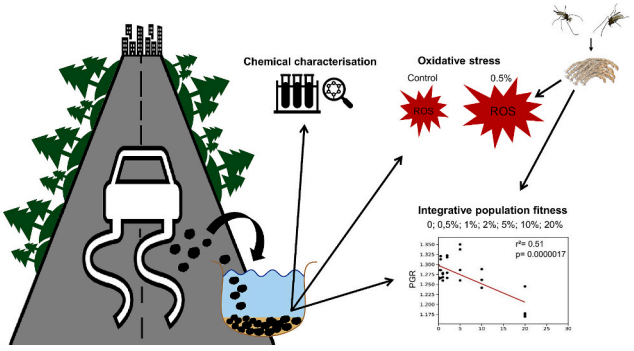
HIGHLIGHTS

- Exposure to urban sediments containing TWPs induces oxidative stress in *C. riparius* larvae.
- Exposure to urban sediments containing TRWPs is detrimental for the fitness of *C. riparius*.
- The discharge of TRWPs represents a potential hazard to freshwater ecosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of tyre and road wear particles (TRWP) in the environment is an underestimated threat due to their potential impact on ecosystems and human health. However, their mode of action and potential impacts on aquatic ecosystems remain largely unknown. In the present study, we adopted a sediment exposure scenario to investigate the influence of sediment coming from an urban runoff sedimentation basin on the life cycle of *Chironomus riparius*. Targeted broad-spectrum chemical analysis helped to characterise the urban sediments and confirmed the significant contribution of contaminants from traffic (e.g. tyre wear contribution, Polycyclic Aromatic Hydrocarbons [PAHs], metals, tyre rubber additives). First-stage chironomid larvae were subjected to increasing concentrations of urban whole sediment. The results showed that exposure to this urban sediment influenced all measured endpoints. In vivo quantification of ROS showed that larvae exposed to the lowest

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concentration of contaminated sediment exhibited increased fluorescence. The contaminated sediment conditions increased mortality by almost 30 %, but this effect was surprisingly not concentration-dependent. Fertility decreased significantly and concentration-dependently. The results of the Mean Emergence Time (EmT50) and larval size showed an optimality curve. Furthermore, as a consequence of the effects on fitness, the Population Growth Rate (PGR) exhibited a significant decrease, which was concentration-dependent. Therefore, after a single generation, PGR calculation can be adopted as a sensitive tool to monitor pollution caused by complex matrices, i.e. composed of several contaminants. Our research highlights the importance of effective management of road runoff and underlines the need for further investigation to better understand the toxicity of TRWPs.

1. Introduction

The continuous expansion of modern transport infrastructure and the resulting increase in road traffic are well-known factors contributing to environmental degradation. However, among what is generated on the roads during driving processes, little attention is paid to emissions that are not discharged into the air. As vehicles drive along the road surface, tyres, composed mainly of synthetic polymers and enriched with various additives, are subject to constant wear, but the road surface itself is also subject to constant degradation. As a result, abrasion causes the production of tiny particles, known as Tyre and Road Wear Particles (TRWPs), ranging in size from nanometers to micrometers, which are easily spread into the surrounding environment by wind and rain (Baensch-Baltruschat et al., 2021).

The presence of TRWPs in water bodies, soils, and airborne particles, which underline the wide mobility of these particles, is well documented (Baensch-Baltruschat et al., 2021; Sieber et al., 2020; Unice et al., 2019). TRWPs alone are responsible for approximately one- third of the annual microplastic emissions in Germany (330,000 t/a), of which ~20,000 t reach water bodies (Baensch-Baltruschat et al., 2021). However, attention has only recently been paid to studying the potential impacts of TRWPs pollution on the environment. Furthermore, there is a significant knowledge gap regarding the transformations that TRWPs undergo once in the environment and the effects that mobilisation from the road to aquatic ecosystems, e.g. through rainfall, may have on biota. Urban runoff sedimentation basins are artificial ponds typically located near busy roads. They separate water and particles from road runoff through sedimentation, allowing the clarified water to be channeled into connected water bodies while retaining the sediment (Grung et al., 2016). However, road runoff is often released untreated into water bodies or nearby road areas (Grung et al., 2016), making sediment accumulated within urban basins a suitable model for the ecotoxicological assessment of urban and tyre and road wear-impacted particulate matter.

Current data strongly indicate potential risks posed by these particles and their leachates, which include heavy metals, polycyclic aromatic hydrocarbons (PAHs), benzothiazoles, and resin acids (Caballero-Carretero et al., 2024). A recent study revealed that exposure of *Chironomus riparius* to tyre rubber particles (TRP) and tyre rubber leachates (TRL), induces more pronounced molecular effects, such as alterations in gene expression related to stress response, when exposed to TRLs compared to TRPs (Caballero-Carretero et al., 2024). In particular, in response to leachates, downregulation of the glutathione-*s*-transferase (GST) family gene GSTd3 and downregulation of the catalase gene were evidenced, indicating a more pronounced impact on detoxification processes and the antioxidant response than with particles alone. Furthermore, in the case of leachate exposure, an impact on the endocrine system was observed through the upregulation of the insulin receptor (InR), suggesting a potential alteration of the insulin pathway. This study shows the importance of considering not only the toxicity of individual rubber particles but also the impact of its leachates when assessing the potential effects of tyre rubber contamination in freshwater ecosystems. In this context, the importance of studies investigating the effects of TRWPs particles from real environmental samples and at environmentally relevant conditions was highlighted (Wagner et al., 2018). Furthermore, urban sediments are influenced not only by contaminants transported by tyres and road surfaces but also by other human activities.

The larvae of non-biting midge *Chironomus riparius* are widely used as an ecologically bioindicator in environmental toxicology. *C. riparius* is an EPA-approved test organism and an OECD-approved standard model organism (EPA - Environmental Protection Agency, 1996; Test no. 233, 2010). This species belongs to the family of Chironomidae (Diptera), which are among the most abundant species within benthic macroinvertebrates and among the most abundant biota in a wide variety of freshwater ecosystems. Moreover, fitness endpoints like survival tests and developmental parameters are still the most used and more reliable ones to evaluate toxicity responses. In this study, we analysed urban sediments from an urban runoff sedimentation basin under relevant environmental conditions for their effect on *C. riparius*. In contrast to many other bioassays, the *Chironomus* assay can also be performed as a whole-sediment assay, which reflects the bioavailable toxicity (Feiler et al., 2013).

In this study, we tested the hypothesis that TRWPs in the sediment negatively affect the fitness of *C. riparius*. Measurement of tyre wear contribution and targeted chemical analysis of polycyclic PAHs, mineral oil content, metals and tyre rubber additives supported the characterization of the sediment. First instar larvae were exposed over their development to different concentrations of urban sediment. To assess negative fitness effects and a concentration effect, established fitness component parameters such as mortality and development rate, sex ratio and fertility were measured, and integrated to an estimate of the population growth rate, an inclusive fitness measure. In addition, larval size was calculated as a measure of larval development. Furthermore, as contaminants are known to induce oxidative stress (Valavanidis et al., 2006), we measured oxidative stress through the in vivo quantification of reactive oxygen species (ROS).

The aim of our research is to contribute to a broader understanding of urban anthropogenic disturbance on the environment and, consequently, the potential repercussions for ecosystem health. Hence, we investigated the influence of urban sediments from an urban runoff sedimentation basin on *C. riparius*, focusing on their effect on oxidative stress as mediators and population fitness, respectively their components as endpoints.

2. Materials and methods

2.1. Culturing conditions

The culture of *C. riparius* used in the present study came from a local population collected in a small river located in Hasselbach, Hessen, Germany (co-ordinates: 50.167562N, 9.083542E). In order to reliably identify *C. riparius* and separate individuals from the cryptic sister species *C. piger* and potential hybrids (Pedrosa et al., 2017), wild-collected larvae were identified by DNA-barcoding, through the combination of mitochondrial COI coding with sequencing of the nuclear marker L44 (Foucault et al., 2019). The laboratory strain used thus contains *C. riparius* individuals only. The culture was maintained and regularly replenished from the field as an in-house laboratory standard. The cultivation conditions of the stock culture conformed to a modified protocol based on the methodology outlined in OECD guideline N219, as previously described (Foucault et al., 2019).

2.2. Sediment collection and preparation

In October 2021, the sediment was collected from an urban runoff sedimentation basin located in Aachen, Germany (50°48′03.6″N 6°06′29.7″E) receiving runoff via a separate sewer system from a large area in the district of Aachen Soers, including a sports park and a highly frequented road, Bundesstraße B57 Krefelder Straße. The total drained area covers 68 ha, including 42 ha of effective runoff area mandatory for treatment. The B57 serves about 26.000 vehicles a day (BASt, manual counting; 2021). The sampling took place during cleaning of the basin, and samples were obtained from different spots within the basin using a stainless-steel bucket, thoroughly mixed and transferred to 2 L acetone-cleaned glass bottles. The samples were returned to Frankfurt and stored in a cold room at 4 °C until further use. For further analyses, aliquots were freeze-dried and sieved (stainless steel mesh, 1 mm mesh size) to remove large particles at the Goethe University, Frankfurt am Main.

2.3. Chemical analyses

The maximum tyre abrasion in percent (TGA via the ROC content (DIN EN 17505:2024-04)) and a tyre-specific external calibration was measured and the sediment was screened for polycyclic aromatic hydrocarbon content (16 EPA PAHs) using GC–MS (DIN 38407-39:2011-09), heavy metals using aqua regia digestion (DIN EN 16174:2012-11), ICP-MS (DIN-EN ISO 16171:2016) and LAWA AQS-Merkblatt P-3/6, as well as mineral oil indices (DIN EN ISO 9377-2:2001-07) in freeze-dried particles by the Institute of Environmental Engineering at the RWTH Aachen University. Quality control measures were performed according to guidelines and are given in the supplementary data (SD). Methodic details, including quality control measures are also given in the SD.

Moreover, organic extracts were prepared using an exhaustive ultrasonic-assisted extraction via an elutropic solvent gradient (Klöckner et al., 2021). A series of solvents from n-heptane over n-heptane-acetone (1:1 *v*/v), acetone, acetone-methanol (1:1 v/v) and methanol was used, covering a broad spectrum of the elutropic series (Snyder, 1974). 1.5 g of sediment was suspended in 20 mL of solvent, sonicated for 1 h in an ice-cooled water bath to prevent overheating and then centrifuged at 1250 x G for 10 min. After each solvent step, the supernatant was carefully aspirated using a glass Pasteur pipette and transferred to a Büchi round flask. After extraction, the combined extracts were evaporated to almost dryness and redissolved in 1 mL n-heptane-acetone mixture (1:1 v/v). For target-screening on tyre rubber additives and wastewater pollutants, the extracts were filtered using a 0.2 µm PTFE membrane, evaporated and transferred to methanol, and diluted 5-fold before being analysed using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS).

For target screening analysis by LC-HRMS, a Thermo Ultimate 3000 LC system coupled to a quadrupole-Orbitrap instrument (Thermo QExactive Plus) with electrospray ionisation in positive and negative ion mode was used and compounds were quantified against reference standards by internal calibration (Finckh et al., 2022). Details on the method and quality control are given in the sd.

For quality control, a process control was prepared from analytical clean quartz sand (Büchi, Switzerland) ground in a mortar. It was extracted alongside the samples and treated the same way throughout the whole process. Furthermore, a solvent blank was prepared from the same batch of Methanol used for the final extracts. Blanks were analysed alongside the samples, and the received chemical data was blank-corrected.

2.4. ROS detection and image analysis

First-stage chironomid larvae were subjected to increasing concentrations of contaminated whole sediment. For the Control (0 % urban sediment) and 0.5 % urban sediment, 20 L3 larvae were collected and placed in 24-well plates. Plates were filled with 2.5 mL medium as described in Foucault et al., 2019. 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 localised 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 lx 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: 423013-9010-000). 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 analysed by ImageJ Fiji (v. 2.15.0). Images were uploaded to ImageJ as an image sequence and converted to 8-bit grayscale from RGB Colour images to avoid colour 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 organism, but the current amount of reagent entered in the organism and oxidised by binding to ROS, which is the remaining amount after the organism's antioxidant system scavenges ROS.

2.5. Life-cycle toxicity test and larval size

The life cycle test was performed according to the specifications outlined in OECD guideline 233 (Test no. 233, 2010), with minor adjustments. The test vessels were subjected to the same conditions as the stock culture of *C. riparius*, maintaining a constantly ventilated room at a temperature of 20 ± 1 °C, a relative humidity of 60 % and a 16:8-hour light/dark cycle. To compensate for water evaporation in the test vessels, demineralized water was constantly supplemented. Conductivity was maintained between 550 and 650 mS/cm and pH around 8. The experiment was designed in seven different increasing concentrations of urban sediment, as a percentage of total weight, each containing four replicates for biological analysis. Four days prior to the start of the experiment, 10 freshly laid egg ropes were separated from stock culture into 6-well plates (3 mL of medium per well) (Foucault et al., 2019). One day prior to the start of the experiment, 28 glass bowls (Ø20 × 10 cm) were filled (Table 1).

The quartz playground sand* used had a granularity of 0–2 mm, was pH neutral, and was washed before use. In the glass bowls containing the urban sediment, the sand and the urban sediment were mixed, so that the resulting sediment column was homogenised. In all the glass bowls, 1.250 L of medium were added, and then aeration was applied for 24 h.

Table 1
The seven increasing concentrations of urban sediment tested.

0 (Control)	0,5 %	1 %	2 %	5 %	10 %	20 %
500 g of quartz playground sand*	497,5 g* + 2.5 g of urban sediment**	495 g* + 5 g**	490 g* + 10 g**	475 g* + 25 g**	450 g* + 50 g**	400 g* + 100 g**

All the experiments were initiated by adding 50 first instar larvae in each of the bowls while aeration was suspended for 6 h to allow the animals to reach the sediment. Following the OECD 233 guideline (Test no. 233, 2010), the exposure of the animals happened on the same day, and the larvae were introduced into the test vessels, and the test duration was set at a maximum of 28 days. The replicates were then raised and fed daily with finely ground fish food (Tetramin® Flakes) whose amount calculated for the respective developmental stage as described elsewhere (Foucault et al., 2019).

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 was counted, and the egg numbers were determined according to (Foucault et al., 2019). 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).

On the 12th day of exposure, 10 larvae per treatment (0, 0.5 %, 2 %, 5 %, 10 %, 20 %) were taken from the sediment and transferred in 6-well plates (3 mL medium) to observe their developmental status. Each larva was photographed, and the images were subjected to analysis using ImageJ® (version 1.54). Utilizing the “Freehand' tool”, the larvae's size was determined by measuring the area they occupied in the images (in pixels).

2.6. Data analysis

Microsoft Excel® (MS Excel®) was used to enter the raw data and determine fitness parameters. For statistical analysis, the program PAST® (version 4.15) was used, utilizing the ‘Nonlinear fit’ function to run a bivariate linear regression (Mortality, Fertility, and PGR) and a second-order polynomial function (EmT50 and Larval size) against the percentages of TRWPs in the sediment, respectively. Control vs. treatment data from ROS measurements were analysed using the Bayesian *t*-test implemented in the R package BayesianFirstAid (Baath, 2014).

3. Results

3.1. Chemical characterization of urban sediment

Measured tyre wear contribution amounted to a maximum possible tire wear content (TWP_{max}) of 0.44 %, equaling 4.4 g/kg sediment. In relation to the measured tyre wear concentration of a highly frequented road in Aachen city of 11.5 % TWP_{max} (publication in preparation), we can derive a possible contribution of 4–7 % road runoff to the overall urban runoff share of the sedimentation basin. Chemical analysis revealed high concentrations of PAHs, mineral oils, metals, and tyre rubber additives, as well as a variety of anthropogenic micropollutants. In total, all detected substances and elements summed up to 106 g/kg dry weight with road salts (Na, Ca, Mg, K) contributing about 48 % and metals (Cd, Cu, Cr, Ni, Pb, Zn, Fe, As, Hg) contributing about 26 % to the total load. Zn was present with a concentration of 2.4 g/kg dry weight (dw) contributing 2 % of the analysed material and indicating a considerable impact by tyre wear. 16 EPA Polycyclic aromatic hydrocarbons (PAHs) summed up to 2.9 mg/kg dry weight almost exclusively shared by high molecular weight PAHs, indicating a strong impact by traffic exhaust and combustion origin (Tobiszewski and Namieśnik, 2012). Recent studies highlighted that traffic-borne air particulate matter carries considerable loads of PAHs and traffic-related metal contamination, which we assume to contribute via urban surface runoff to the overall contamination load of the sample in addition to road runoff (Jandacka and Durcanska, 2021; Nasrabadi et al., 2022). Further, the mineral oil index revealed a total oil concentration of 0.6 %, equaling 660 mg/kg dry weight, which also confirms the strong traffic impact of the urban and road runoff particulate matter retained in the sedimentation basin. The target screening on tyre and road wear and wastewater chemicals measured after organic extraction of the particles detected a total of 55 contaminants with a concentration of more than 1 μg/kg dry weight, summing up to 14.6 mg/kg dry weight or 0.014 % of the total material (Fig. 1). The most abundant compounds were benzothiazole (4.6 mg/kg dw) and 2-benzothiazolesulfonic acid (2.1 mg/kg dw), which are both transformation products of benzothiazole-derived tyre rubber additives. Summing up all benzothiazole derivatives, this group of compounds shares 59 % by mass of all contaminants detected in the tyre rubber compounds target screening. Benzothiazole derivatives are largely used as vulcanization accelerators, thus underlining the tyre wear impact of the used material (Feltracco et al., 2023). Further, industrial compounds such as *N*-phenyl-1-naphthylamine (PNA, 2 mg/kg dw), N-isopropyl-*N*-phenyl-*p*-phenylenediamine (IPPD, 897 μg/kg dw), 1,3-Diphenylguanidine (DPG, 476 μg/kg dw), and hexa(methoxymethyl)melamine (HMMM, 176 μg/kg dw) were present besides plastic additives and a broad spectrum of tyre-related and industrial use compounds. Although related to industrial use, PNA, IPPD, DPG and HMMM can also be related to tyre and vehicle wear (ECHA, 2022; Seiwert et al., 2020). Pesticides like mepiquat (27 μg/kg dw) and prosulfocarb (14 μg/kg dw), the fragrance ISO E super (19 μg/kg dw) and the sweetener cyclamate (575 μg/kg dw) reflect the broad catchment area of the retention basin including various land use besides draining the frequented B57 Krefelder Straße (Fig. 1).

3.2. Reactive oxygen species measurements

The fluorescence images were analysed with ImageJ to determine the red colour density and intensity. A difference of means of −2.8 was found between control and 0.5 % (95 % HDI, between −4.1 and − 1.6), with posterior probability of 100 %, that control was smaller than 0,5 %. This evidence had a large effect size of significantly higher fluorescence intensity in 0.5 % sediment (Cohen's d = −1.6) (Fig. 2).

3.3. Life-cycle traits and larval size

Urban sediment had no demonstrable negative dose effect on adult emergence, showing a slight, but not significant linear increase in mortality over the seven individual concentrations tested ($r^2 = 0.16$; $p = 0.08$, Fig. 3a). However, contrasting 0 % of urban sediment as control against all treatments showed a substantial difference in mortality (0.092 vs. 0.377, Mann-Whitney U = 10, $p = 0.01$), while the difference in mortality among the urban sediment treatments was rather small (range of mortality fraction 0.33 (at 2 % urban sediment) – 0.43 (at 20 % urban sediment)). Fertility showed a strong concentration-dependent decline. Every additional percent of contaminated urban sediment decreased the number of fertile eggs per parent by 7.5 (95 % c.i. -4.1 - -10.0) or 3.2 % of the mean initial value of 233 eggs. This decrease was highly significant ($r^2 = 0.36$; $p = 0.0007$, Fig. 3b). The Emt50 showed an optimum curve, with significantly later emergence at the lowest and highest concentrations. A second-order polynomial function described the data, therefore, decisively better than a linear model (delta AICc = 35.4). The fitted function (Emt50 = 0.032 * urban sediment concentration^2–0.555 * urban sediment concentration + 20.3) had its minimum at a concentration of 8.7 % ($r^2 = 0.30$; $p = 0.01$) (Fig. 3c). Larval size also showed an optimum response curve (larval size = −13.5 * urban sediment concentration + 280.0 * urban sediment concentration + 1454, delta AICc compared to linear model = 1.61 * 10^7), with a maximum at 10.4 % urban sediment ($r^2 = 0.28$; $p = 0.00007$) (Fig. 3d). Correspondingly, there was no relation between the mean larval size and the mean fertility ($r = 0.21$, $p = 0.69$). The daily population growth rate decreased linearly by 0.005 per additional percent of urban sediment,

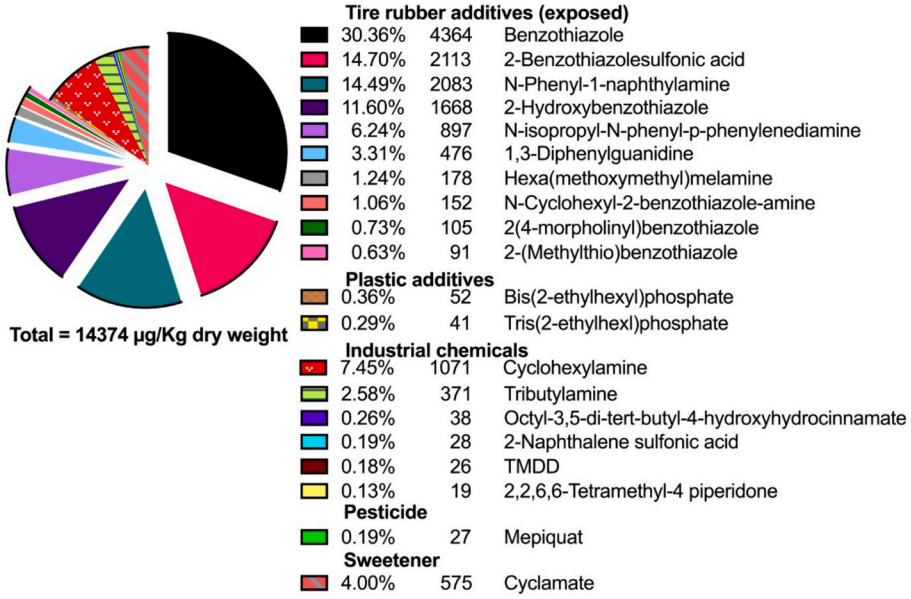

Fig. 1. Chemical composition of the dust sample measured after organic extraction. The top 20 abundant chemicals out of 55 detected compounds are displayed. Values indicate the total share among detected compounds in % and the sediment concentration in µg/Kg dry weight. 59 % of the total mass of contaminants is shared by benzothiazoles, compounds used as vulcanization accelerators.

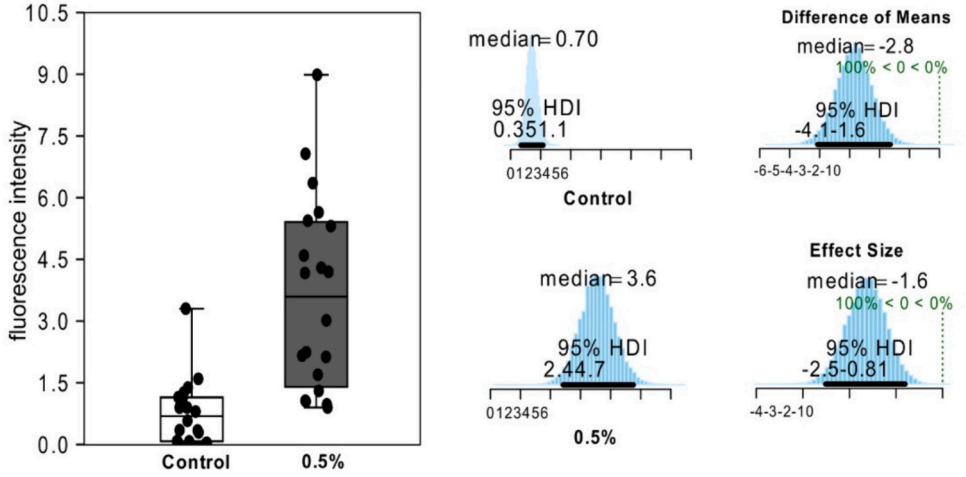

Fig. 2. Comparison of relative fluorescence intensity between control and 0.5 % of urban sediment. The histograms in the middle of the figure show the Bayesian posterior estimates for the medians in control (above) and 0.5 % of urban sediment (below). The difference of means (upper right) has a 100 % posterior probability of being smaller in the control than in the treatment. The huge median effect size has likewise a posterior probability of being different from zero (lower right).

from an average of 130 % in the control sediment to 121 % per day with 20 % of urban sediment ($r^2 = 0.51$; $p = 0.0000017$) (Fig. 3e).

4. Discussion

The chemical screening of our urban sediment revealed a highly complex mixture with typical features of road runoff contamination, including high levels of PAHs, metals, mineral oils, tyre rubber and synthetic chemicals including tyre rubber additives and plasticisers (Aatmeeyata and Sharma, 2010; Hwang et al., 2019, Hwang et al., 2016; Maurer et al., 2023; Wagner et al., 2018). This mixture elicited complex and generally adverse effects on midge larvae and adults. In the direct assessment of ROS activity, larvae exposed to the contaminated sediment showed increased fluorescence, as a consequence of oxidative stress-induced or directly exerted by the contaminants. ROS are a common product of xenobiotic metabolism, and pollutants exert part of their toxicity through the formation of ROS (Lackner, 1998). Furthermore, it was highlighted earlier that microplastics negatively impacted chironomids by imparting the antioxidant-detoxifying processes (Silva et al., 2021). Also here, oxidative stress, measured as in vivo ROS concentration, was one of the exposure effects that negatively imparted fitness. The contaminated sediment conditions also increased mortality by almost 30 %, but this effect was surprisingly not concentration dependent. Increased mortality is a common consequence of exposure to various contaminants (Doria and Pfenninger, 2021; Nowak et al., 2008), but the lack of concentration dependence could be the result of distinct factors. Firstly, when subjected to harmful substances, organisms may experience rapid saturation of toxic effects, implying that an increase in the concentration of contaminants does not inevitably lead to a further increase in mortality (Preston, 2002). Furthermore, once a certain threshold of exposure is reached, the organism can activate various responses at the cellular level, such as the production of detoxifying and antioxidant enzymes (Armstrong, 1987; Kültz, 2005). These acclimative responses enable the organism to withstand higher

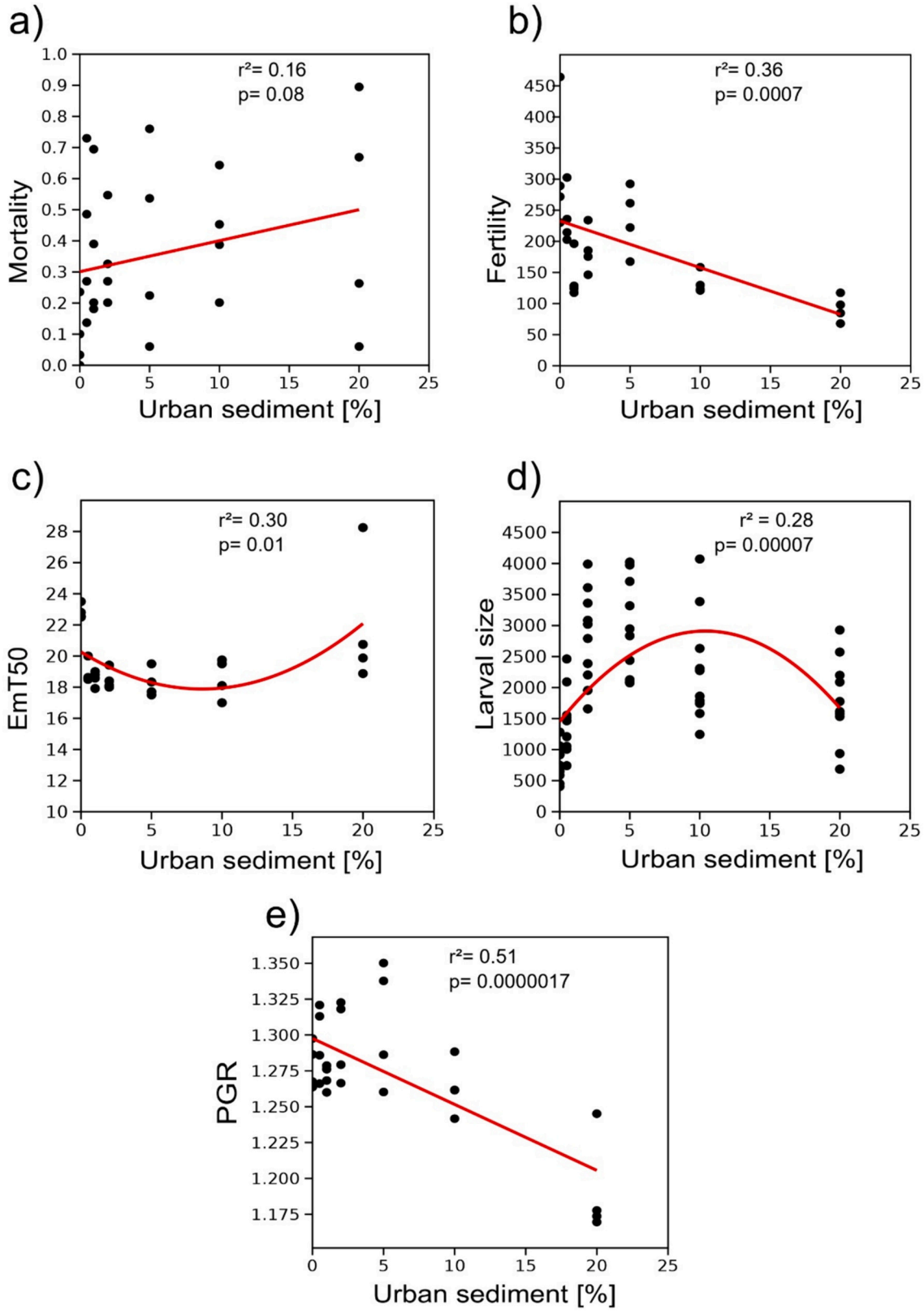

Fig. 3. *C. riparius* effects on a) Mortality, b) Fertility, c) EmT50, d) Larval size and e) PGR after exposure to control and urban sediments.

levels of contamination-induced stress, thus contributing to a reduction in mortality. Secondly, at the higher concentrations of urban sediments, the negative effects could manifest at the sub-lethal level rather than as a direct lethal effect (Preston, 2002). This is the case with fertility, which decreased significantly and concentration-dependently, confirming the toxic effect of these urban sediments. Furthermore, the observed decrease in fertility was determined by a reduction in the number of fertile eggs per female. Finally, considering a substantial degree of

genetic variation in the tested laboratory population, it is possible that some individuals had genotypes better suited to tolerate the negative effects of urban sediments. This would explain the almost constant percentage of survivors compared to the control (0 % TRWPs), which served as an appropriate reference population.

The negative effect on fertility might have been caused by endocrine disruption caused by respectively acting substances in the contaminated sediment. Past studies linked PAHs-urban sediments to anti-androgenic and estrogenic effects (Eriksson et al., 2022; Shuliakevich et al., 2022). While plasticisers have been recognized as endocrine disruptors not only for wildlife but for humans as well for a long time (Sumpter and Johnson, 2005), little is yet known about the complex composition of tyre rubber additives. Capolupo et al. (2020) observed a strong reduction in mussel gamete fertilization after exposure to crumb tyre rubber leachates. Endocrine disruption occurs when contaminants interfere with the normal functioning of hormonal systems within an organism (Bergman et al., 2013). Endocrine disrupting substances mimic or block hormonal signals, leading to dysregulation of key processes involved in reproductive physiology (Monneret, 2017). Thus, a potential consequence of endocrine disruption, induced by contaminants in aquatic biota, is the altered development of the gonads, resulting in reproductive failure and reduced fertility (Marlatt et al., 2022). Benzothiazoles, the most abundant synthetic chemicals detected in our matrix, have been linked to a variety of adverse effects. The detected derivatives were reported to act on the estrogen and the aryl-hydrocarbon receptor in the lower mg/L concentration (Noguerol et al., 2006), as well as interfere with the thyroid system and affect cell viability in higher mg/L concentrations (Liao et al., 2018).

The results of the mean emergence time showed an optimality curve, suggesting that counteracting forces are at work. We hypothesize that at lower concentrations, the observed accelerated development effect may be attributed to increased food intake, due to the presence of organic matter in the contaminated sediment. Indeed, the size of the larvae followed the same pattern. After an initial increase, the size dropped again at higher concentrations. It has been shown that the amount of food ingested by an organism can modulate its response to toxic sediment (Bridges et al., 1997; Ristola et al., 1999). The nutritional status of an organism can determine its resilience to chemical exposure, thus influencing the susceptibility of individuals and populations, depending on food availability (Postma et al., 1994; Ristola et al., 1999). In general, larger organism size is associated with increased reproductive success, as larger individuals may have greater energy reserves and resources to invest in reproduction (Andersson and Iwasa, 1996). At higher concentrations of contaminated sediment, however, the toxic action is exacerbated and can no longer be mitigated by the positive effects of increased food availability on size and development.

As a consequence of the observed effects on fitness, the Population Growth Rate (PGR) exhibited a significant decrease, which was concentration dependent. The significant lower PGR value compared to the control, particularly at higher concentrations of contaminated sediment (10 % and 20 %), can be attributed to the observed decline in fertility. Despite the confirmation of the negative influence of this contaminated sediment as a stress factor for *C. riparius*, the PGR remained above the critical threshold of 1 d^{-1}, because the high number of eggs in egg ropes can support population growth (Doria et al., 2022; Khosrovyan et al., 2022). However, even a small drop in PGR can affect the population dynamics of the species and, with knock-on effects, the entire ecosystem. For instance, a population growing at 8 % less per day would reach only half the size of an undisturbed growing population after just 14 days. Moreover, since chironomids are a link between realms, they may transfer the pollution or their effect from freshwater to terrestrial ecosystems (Roodt et al., 2022).

C. riparius is known for its ability to adapt to environmental stressors (Doria et al., 2022), unlike many other freshwater organisms that may exhibit limited adaptability to contaminants and fluctuating environmental conditions. Therefore, the discharge of TRWP impacted runoff into freshwater environments poses a potential hazard to sensitive organisms, as it introduces a multitude of chemicals and pollutants, which can also accumulate in body tissues, causing cascading effects on freshwater ecosystems. To verify an adaptation potential of *C. riparius* to these sediments, studies exposing larvae over more than one generation and comparing it to an untreated reference population as control would be necessary.

5. Conclusions

With this single generation waterborne exposures of urban sediment containing TWRPs we have shown that this complex mixture negatively affects the fitness of the model organism *C. riparius*. The chemical screening of the used urban runoff sediments revealed a very complex contamination with strong traffic and vehicle influence with contamination levels comparable to previous reports. The possible persistence of reproductive disorders across generations represents a challenge for the long-term viability and adaptability of affected populations. Therefore, there is a need to study adaptive responses in different multigenerational experimental models, investigating the possibility that the observed toxicity of urban sediments exerts transgenerational effects. Moreover, a set of effect-based mechanistic bioanalytical toxicity assays should support the ecotoxicological screening to disentangle the various toxicological effects of such complex environmental matrices.

CRediT authorship contribution statement

Lorenzo Rigano: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Markus Schmitz:** Writing – original draft, Visualization, Formal analysis, Data curation. **Henner Hollert:** Writing – review & editing, Supervision. **Volker Linnemann:** Writing – review & editing, Supervision. **Martin Krauss:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Markus Pfenninger:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175597.

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Supplementary data

Mind your tyres: The ecotoxicological impact of urban sediments on an aquatic organism

Supplemental Information Sample preparation and Chemical Analytics Lamella Basin Aachen Krefelder Straße

2.3 Chemical analyses

The sediment was screened for polycyclic aromatic hydrocarbon content (16 EPA PAHs) using GC-MS according to DIN 38407-39 (2011-09), Heavy Metals using aqua regia digestion following DIN EN 16174 and ICP-MS according to DIN-EN ISO 16171 (2016) and LAWA AQS – Merkblatt P-3/6, as well as mineral oil indices according to DIN EN ISO 9377-2 (2001-07) measured by the Institute of Environmental Engineering at the RWTH Aachen University in freeze-dried particles (see SI).

ICP-MS and ICP-OES analytics:

Digestion:
DIN EN 16174:
Sludge, treated biowaste and soil - Digestion of aqua regia soluble fractions of elements; German version EN 16174:2012

ICP-MS:
DIN EN 16171: Sludge, treated biowaste and soil - Determination of elements by inductively coupled plasma mass spectrometry (ICP-MS); German version EN 16171:2016
LAWA AQS – Merkblatt P-3/6 Element determination with ICP-MS (mass spectrometry with inductively coupled plasma), May 2002

QC:

- QC standards: 20 and 200 µg/L with a measuring range of 0.1 to 1000 µg/L, requirement according to standard: +/- 10% deviation, ISA laboratory: < +/- 10%
- Calibration with blank value solution and 4 standards
- Blank value (ultrapure water) over the entire procedure
- Certified standard (20/200 µg/L), requirement according to standard: +/- 10% deviation
- Certified standard over the entire procedure (100 µg/L), requirement according to standard: +/- 10% deviation, ISA laboratory: < +/- 10%
- Measurement of dilutions for testing matrix influences, tolerable deviations depending on the concentration: 10 - 20%, in the ISA laboratory: < +/- 10%
- Measurement with 4 internal standards

ICP-OES
DIN EN 16170:
Sludge, treated biowaste and soil - Determination of elements using inductively coupled plasma optical emission spectrometry (ICP-OES); German version EN 16170:2016
LAWA AQS – Merkblatt P-3/1 Determination of elements in water using ICP-OES (optical emission spectrometry with inductively coupled plasma), October 2018

QC:
- QC standards: 0.2 and 2 mg/L with a measuring range of 0.001 to 10 mg/L, requirement according to standard: +/- 10% deviation, ISA laboratory: < +/- 10%
- Calibration with blank value solution and 4 calibration standards
- Blank value (ultrapure water) over the entire procedure
- Certified standard over the entire procedure (1 mg/L)Requirement according to standard: +/- 10% deviation, ISA laboratory: < +/- 10%
- Measurement of dilutions for testing matrix influences, tolerable deviations depending on the concentration: 15%, in the ISA laboratory: < +/- 10%
- Measurement on 3 different element wavelengths

Mineral Oil Index:

DIN EN ISO 9377-2 (2001-07)
EN: Water quality - Determination of hydrocarbon oil index - Part 2: Method using solvent extraction and gas chromatography (ISO 9377-2:2000); German version EN ISO 9377-2:2000

QC:
- Two standards (0.5 mg/mL and 5 mg/mL with a measuring range of 0.1 to 10 mg/mL) as direct injection.
- Standard material: Neochema mineral oil standard according to H53, concentration C, n-heptane, 14690-50000HP5
- Blank value (ultrapure water) over the entire procedure, requirement according to standard: < 0.1 mg/mL, measured values in the ISA laboratory: <0.05 mg/mL
- Standard (1 mg/L) over the entire procedure, requirement according to standard: >80% recovery, measured values in the ISA laboratory: >90% recovery
- Standard material: Neochema mineral oil standard according to H53, concentration B, acetone, 14690-20000AC5

- Daily calibration by direct injection (0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10.0 mg/mL), r2>0.999

16 EPA PAHs:

DIN 38407-39 (2011-09)
EN: German standard methods for the examination of water, waste water and sludge - Jointly determinable substances (group F) - Part 39: Determination of selected polycyclic aromatic hydrocarbons (PAH) - Method using gas chromatography with mass spectrometric detection (GC-MS) (F 39)

- QC:
- Blank value (ultrapure water) over the entire procedure, requirement in the ISA laboratory: <2 ng/L
- Two standards (20 ng/L and 800 ng/L) over the entire procedure, requirement in the ISA laboratory: >90% recovery
- Standard material: Neochema PAH-Mix 16, concentration C, acetone, 14020-100AC5
-

Daily calibration by direct injection (10; 20; 50; 100; 200; 500; 1000 ng/mL), r2>0.999

The ratio of indicator-PAHs was used to receive information about emission-sources of detected PAHs according to (Tobiszewski and Namieśnik, 2012). Ratios of fluoranthene to pyrene and indeno(1,2,3,c,d)pyrene to benzo(g,h,i)perylene were calculated according to Equation 1) and plotted in a 2-dimensional grid as shown in Figure SI_01.

$$y = \frac{C(fluoranthene)}{C(fluoranthene) + C(pyrene)}$$

$$x = \frac{\mathrm{C(indeno(1,2,3,c,d)pyrene)}}{\mathrm{C(indeno(1,2,3,c,d)pyrene)} + \mathrm{C(benzo(g,h,i)perylene)}}$$

Equation 1): Calculation of indicator PAH-ratios to estimate PAH emission sources according to Tobiszewski and Namieśnik, 2012.

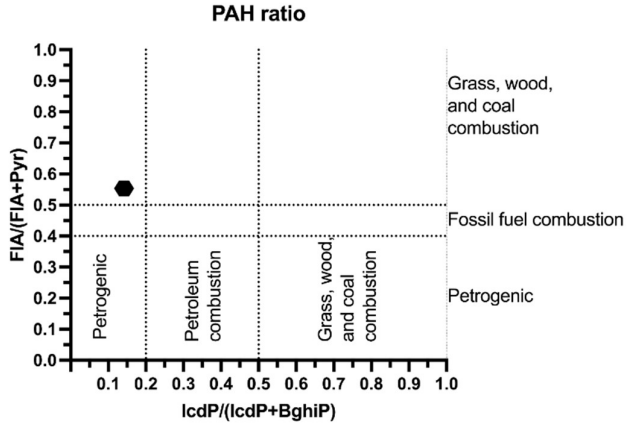

Figure SI_01: PAH ratios as described in the methods section: fluoranthene - pyrene ratio (FlA/(FlA + Pyr) (Y-axis) versus Indeno(1,2,3,c,d)pyren - Benzo(g,h,i)perylene (IcdP/(IcdP + BghiP) (X-axis) as indicators for emission sources (Tobiszewski and Namieśnik, 2012). The

black hexagon indicates the Classification of the PAH composition according to both ratio factors according to the origin of the combustion products

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Tire Rubber Content Measurement

The maximum tyre abrasion in percent was determined using TGM via the ROC content and a tyre-specific external calibration.

Determination of ROC900 according to the temperature gradient method - DIN EN 17505 (2024-04):

(EN) Soil and waste characterization- Temperature dependent differentation of total carbon (TOC400, ROC, TIC900)

QC: Standard for system control: 2.0 % TOC400; 2.0 % ROC; 2.0 % TIC900 à recovery between 85 % and 115 %
Blank value (empty crucible) à < half the limit of quantification

Table SI_0 1:Measurement Results Tire Rubber Content (TRC): Tire Rubber Content was quantified using Thermogravimetric analysis via Remaining Organic Carbon (ROC) content and a reference tire rubber material. Ratios to known tire rubber content of other investigated road sides allow for an estimation of road runoff impact oft he analysed material.

Sample No.	Project	Sample	mg C absolut from ROC	maximum possible tire rubber content %		
2023-04739	Roadtox	GU_RoadTox_LamKla_SPM_20211019	<0,1	0,44		
					Ratio	
Ratio to Lammellae Basin Particles: Share of Road Runoff Particulate Matter					TRC(Road)/TRC(LamellaBasin)	
B57 Urban Road Runoff Average				11,66581602	4%	
L23 Country Road Runoff Average				6,055896568	7%	
BAB4 Motorway Runoff Average				10,91747405	4%	

Chemical LC-MS/MS Target Screening on Tire rubber contaminants in organic extracts:

Organic extracts were prepared using an exhaustive ultrasonic-assisted extraction via an elutropic solvent gradient (Klöckner et al., 2021). A series of solvents from n-heptane over n-heptane-acetone (1:1 v/v), acetone, acetone-methanol (1:1 v/v) and methanol was used, covering a broad spectrum of the elutropic series (Snyder, 1974). 1.5 g of sediment was suspended in 20 mL of solvent, sonicated for 1h in an ice-cooled water bath to prevent overheating and then centrifuged at 1250 x G for 10 minutes. After each solvent step, the supernatant was carefully aspirated using a glass Pasteur pipette and transferred to a Büchi round flask. After extraction the combined extracts were evaporated to almost dryness and redissolved in 1 mL n-heptane-acetone mixture (1:1 v/v). For target-screening on tire rubber additives and wastewater pollutants the extracts were filtered using a 0.2 µm PTFE membrane and transferred to methanol and diluted 5-fold before being analysed using Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS).

For LC-HRMS analysis a Thermo Ultimate 3000 LC system coupled to a quadrupole-Orbitrap instrument (Thermo QExactive Plus) with electrospray ionisation in positive and negative ion mode was used. For LC separation a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 µm particle size) equipped with in-line filter and pre-column of the same type (5x 2.1 mm) was employed. The gradient separation uses 0.1 % formic acid (eluent A) / methanol containing 0.1% formic acid (eluent B) / acetonitrile (eluent C) for positive mode and 1 mm ammonium fluoride (A) / methanol containing 1 mM ammonium fluoride (B) / acetonitrile (C) for negative mode. The gradient program started for both modes at 97% A / 3% B / 0% C, held for 2 minutes, increasing to 3% A / 97% B / 0% C in 14 minutes, and changing to 3% A /0% B / 97% C in 4 minutes, held for 4 minutes. The eluent was afterwards re-equilibrated to initial conditions in 4.8 minutes. The HRMS analysis combined a full scan acquisition (m/z 80-1200, nominal resolving power of 70,000) with six data independent acquisition scans (m/z 80-182, 178-282, 278-382, 378-482, 478-682, 682-1200) at a nominal resolving power of 35,000.

For quantification, Thermo raw files were converted to mzML files using ProteoWizard (Chambers et al., 2012), followed by peak detection and annotation in MZmine 2.38 (Pluskal et al., 2010) and further processing by the MZquant R package as detailed in Finckh et al. (2022). We used an internal standard calibration using respective isotope-labelled compounds listed in Table SI_02. For calibration, target analyte concentrations ranged from 0.2 to 1000 ng/mL and the internal standard concentration was 50 ng/mL in vial.

Table SI_02: Isotope-labelled compounds used for internal standard calibration.

Name	Formula	LC-HRMS mode	Adduct	m/z	RT [min]
Mono-isobutylphthalate-D4	$C_{12}H_{10}D_4O_4$	ESI_neg	M-H$^-$	225.1070	10.6
4-Nitrophenol-D4	$C_6H_1D_4N_1O_3$	ESI_neg	M-H$^-$	142.0448	5.7
Triclosan-D3	$C_{12}H_4D_3Cl_3O_2$	ESI_neg	M-H$^-$	289.9627	13.4
Mecoprop-D3	$C_{10}H_8D_3Cl_1O_3$	ESI_neg	M-H$^-$	216.0512	11.5
Diclofenac-D4	$C_{14}H_7D_4Cl_2N_1O_2$	ESI_neg	M-H$^-$	298.0345	12.5
Laurylsulfate-D25	$C_{12}H_1D_{25}O_4S$	ESI_neg	M-H$^-$	290.3048	24.4
Bezafibrate-D4	$C_{19}H_{16}D_4Cl_1N_1O_4$	ESI_neg	M-H$^-$	364.1259	11.6

Acesulfame-D4	$C_4H_1D_4N_1O_{14}S$	ESI_neg	$M\text{-}H^-$	166.0118	1.5
Hydrochlorothiazide-^{13}C6	$C_1{}^{13}C_6H_8Cl_1N_3O_4S_2$	ESI_neg	$M\text{-}H^-$	301.9773	1.7
Bentazone-D6	$C_{10}H_6D_6N_2O_3S_1$	ESI_neg	$M\text{-}H^-$	245.0872	9.7
Cyclamate-D11	$C_6H_2D_{11}N_1O_3S_1$	ESI_neg	$M\text{-}H^-$	189.1234	4.1
Mono-isobutylphthalate-D4_	$C_{12}H_{10}D_4O_4$	ESI_pos	$M+H^+$	227.1216	10.6
Creatinine-D3	$C_4H_4D_3N_3O_1$	ESI_pos	$M+H^+$	117.0850	0.6
Diazinon-D10	$C_{12}H_{11}D_{10}N_2O_3P_1S_1$	ESI_pos	$M+H^+$	315.1711	12.8
Benzophenone-3-D5	$C_{14}H_7D_5O_3$	ESI_pos	$M+H^+$	234.1173	12.3
p-Toluene-sulfonamide-D4	$C_7H_5D_4NO_2S_1$	ESI_pos	$M+NH_4^+$	193.0933	5.0
Cotinine-D3	$C_{10}H_9D_3N_2O_1$	ESI_pos	$M+H^+$	180.1211	0.9
Diglyme-D6	$C_6H_8D_6O_3$	ESI_pos	$M+H^+$	141.1392	2.4
Chlormequat-D9	$C_5H_4D_9Cl_1N_1$	ESI_pos	M^+	131.1296	0.7
Carbamazepine-D10	$C_{15}H_2D_{10}N_2O_1$	ESI_pos	$M+H^+$	247.1650	9.8
Atrazine-^{13}C3	$C_5{}^{13}C_3H_{14}Cl_1N_5$	ESI_pos	$M+H^+$	219.1111	10.2
Benzotriazole-D4	$C_6H_1D_4N_3$	ESI_pos	$M+H^+$	124.0807	3.9
Carbendazim-D4	$C_9H_5D_4N_3O_2$	ESI_pos	$M+H^+$	196.1019	2.2
Tri-n-butylphosphate-D27	$C_{12}D_{27}O_4P_1$	ESI_pos	$M+H^+$	294.3414	13.3
DEET-D7	$C_{12}H_{10}D_7N_1O_1$	ESI_pos	$M+H^+$	199.1822	10.4
Metolachlor-D6	$C_{15}H_{16}D_6Cl_1N_1O_2$	ESI_pos	$M+H^+$	290.1788	12.2
Isoproturon-D3	$C_{12}H_{15}D_3N_2O_1$	ESI_pos	$M+H^+$	210.1680	10.4
Diclofenac-D4	$C_{14}H_7D_4Cl_2N_1O_2$	ESI_pos	$M+H^+$	300.0491	12.5
Caffeine-D3	$C_8H_7D_3N_4O_2$	ESI_pos	$M+H^+$	198.1065	5.6
Clarithromycin-D3	$C_{38}H_{66}D_3N_1O_{13}$	ESI_pos	$M+H^+$	751.5030	10.3
Desisopropylatrazine-D5	$C_5H_3D_5Cl_1N_5$	ESI_pos	$M+H^+$	179.0855	4.6
Decyltrimethylammonium-D30	$C_{13}D_{30}N_1$	ESI_pos	M^+	230.4256	9.4
Atenolol-D7	$C_{14}H_{15}D_7N_2O_3$	ESI_pos	$M+H^+$	274.2143	1.1
Progesterone-D9	$C_{21}H_{21}D_9O_2$	ESI_pos	$M+H^+$	324.2883	12.6
Verapamil-D6	$C_{27}H_{32}D_6N_2O_4$	ESI_pos	$M+H^+$	461.3281	9.0
Bezafibrate-D4	$C_{19}H_{16}D_4Cl_1N_1O_4$	ESI_pos	M+H+	366.1405	11.6
Sulfamethoxazole-D4	$C_{10}H_7D_4N_3O_3S_1$	ESI_pos	M+H+	258.0845	6.4
Tebuconazole-D9	$C_{16}H_{13}D_9Cl_1N_3O_1$	ESI_pos	M+H+	317.2089	12.6
Imidacloprid-D4	$C_9H_6D_4Cl_1N_5O_2$	ESI_pos	M+H+	260.0847	6.6

For quality control, a process-control was prepared from analytical clean quartz sand (Büchi, Switzerland) ground in a mortar. It was extracted alongside the samples and treated the same way throughout the whole process. Furthermore, a solvent blank was prepared from the same batch of methanol used for the final extracts. Blanks were analyzed alongside the samples, and the concentration data was blank-corrected.

Moreover, internal standards were used to assess the stability of accurate measured masses and retention times during analysis. The mass accuracy was in all cases < 5 ppm and the retention times of all analytes were stable within 0.25 minutes.

Table SI_03: Summary Data on whole contamination profile of investigated sludge matrix. Analyses were performed in freeze dried material (PAHs, metals, and mineral oils, TOC, TIC, ROC, tire rubber) and in organic extracts (tire rubber additives).

Tire rubber additives and wastewater chemicals measured in organic extract using LC-HRMS/MS		
Name	**µg/Kg SEQ**	**Share of total contaminant load**
Benzothiazole	4364,06	0,004081%
2-Benzothiazolesulfonic acid	2112,93	0,001976%
N-Phenyl-1-naphthylamine	2082,66	0,001948%
2-Hydroxybenzothiazole	1667,70	0,001560%
Cyclohexylamine	1070,77	0,001001%
N-isopropyl-N-phenyl-p-phenylenediamine	896,96	0,000839%
Cyclamate	574,90	0,000538%
1,3-Diphenylguanidine	475,96	0,000445%
Tributylamine	370,62	0,000347%
Hexa(methoxymethyl)melamine	177,81	0,000166%
N-Cyclohexyl-2-benzothiazole-amine	152,17	0,000142%
2(4-morpholinyl)benzothiazole	105,39	0,000099%
2-(Methylthio)benzothiazole	91,43	0,000085%
Bis(2-ethylhexyl)phosphate	51,63	0,000048%
Tris(2-ethylhexl)phosphate	41,01	0,000038%
Octyl-3,5-di-tert-butyl-4-hydroxyhydrocinnamate	37,92	0,000035%
TMDD	25,88	0,000024%
2-Naphthalene sulfonic acid	27,72	0,000026%
Mepiquat	27,36	0,000026%
2,2,6,6-Tetramethyl-4 piperidone	19,11	0,000018%
Benzidine	21,76	0,000020%
ISO E Super	18,69	0,000017%
Prosulfocarb	14,20	0,000013%
Lauramidopropylbetaine	7,78	0,000007%
Di-n-butyl phosphate	9,54	0,000009%
Benzenesulfonic acid	9,76	0,000009%
Sulfamethazine	9,35	0,000009%
Azobenzene	7,73	0,000007%
Gabapentin	7,50	0,000007%
m-Xylene-4-sulfonic acid	7,24	0,000007%
2-Methylbenzothiazole	6,77	0,000006%
Pendimethalin	5,39	0,000005%
Tonalide	4,65	0,000004%
Quinoline	4,40	0,000004%
Clonidine	4,30	0,000004%
Octyl-methoxycinnamate	4,20	0,000004%
Lauryl diethanolamide	2,74	0,000003%
DEET	3,78	0,000004%
10,11-Dihydro-10-hydroxycarbamazepine	3,75	0,000004%
Decylsulfate	3,35	0,000003%
Metazachlor OA	3,33	0,000003%
Celestolide	2,80	0,000003%
Benzyldimethyldodecylammonium	1,29	0,000001%
Acridone	2,53	0,000002%
Diphenylphosphate	2,51	0,000002%
Tricresylphosphate	2,47	0,000002%
Carbamazepine	2,36	0,000002%
1-Butyl-3-methyl-imidazolium	0,87	0,000001%
Dicyclohexylphthalate	2,01	0,000002%
Bifonazol	1,89	0,000002%
Triphenylphosphate	1,58	0,000001%
Didecyldimethylammonium	0,77	0,000001%
Phenylbenzimidazole sulfonic acid	1,59	0,000001%
1-Ethyl-3-methyl-imidazolium	1,77	0,000002%
Propiconazole	1,71	0,000002%
Sum chemicals	14560,34	0,013616%
Sum total contamination (µg/Kg SEQ)	106937402,44	
in g/Kg SEQ	106,9374024	

PAHs and MKW measured in naive, freeze-dried dust using GC-MS		
Name	**µg/Kg SEQ**	**Share of total contaminant load**
Low molecular weight PAHS		
Naphthalin	31	0,000029%
Acenaphthylen	6,9	0,000006%
Acenaphthen	7,7	0,000007%
Fluoren	11	0,000010%
Phenanthren	210	0,000196%
Anthracen	75	0,000070%
High molecular weight PAHS		
Fluoranthen	620	0,000580%
Pyren	500	0,000468%
Benz(a)anthracen	180	0,000168%
Chrysen	0,5	0,000000%
Benzo(b)fluoranthen	460	0,000430%
Benzo(k)fluoranthen	180	0,000168%
Benzo(a)pyren	280	0,000262%
Indeno(1,2,3,c,d)pyren	43	0,000040%
Dibenz(a,h)anthracen	34	0,000032%
Benzo(g,h,i)perylen	260	0,000243%
SUM PAHs	2899,1	0,002711%
MKW	660000	0,617183%

Metals and Elements measured in naive, freeze-dried dust using ICP-MS		
Name	**µg/Kg SEQ**	**Share of total contaminant load**
Cd	3100	0,002899%
Cr	82700	0,077335%
Cu	160000	0,149620%
Ni	41500	0,038808%
Pb	174000	0,162712%
Zn	2400000	2,244304%
Ca	38800000	36,282909%
Mg	8580000	8,023385%
K	4190000	3,918180%
Na	103000	0,096318%
Fe	25400000	23,752213%
As	14200	0,013279%
S	4100000	3,834019%
Ti	997000	0,932321%
Hg	173	0,000162%
Se	1270	0,001188%
Mn	883000	0,825717%
P	1630000	1,524256%
Al	18700000	17,486866%
Sum Heavy Metals	28275673	26,441331%
Sum Road Salts	51673000	48,320792%

Organic Carbon & Tire Rubber Content measured in naive, freeze-dried dust	
Parameter	**value**
TOC_{400} (%C)	9,0
ROC (%C)	< 0,1
TIC_{900} (%C)	2,7
TOC-Solid (g/kg C_{mT})	96,1
max. possible tire rubber content (%)	0,44
tire rubber (g/Kg SEQ)	4,4

CHAPTER 4

Beyond acute toxicity: evolutionary response by rapid polygenic adaptation to a complex environmental stressor in *Chironomus riparius*

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Appendix to Chapter 4

Declaration of contributions

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Authors and contributions:

- **Lorenzo Rigano**: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.
- **Markus Schmitz**: Writing – original draft, Visualization.
- **Henner Hollert**: Writing – review & editing, Supervision.
- **Markus Pfenninger**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review & editing.

Introductory note

The fourth chapter of this thesis builds on the previous studies by focusing on the potential for adaptive evolutionary change under chronic exposure to urban runoff sediments.

To address this question, I combined an evolutionary life-cycle experiment with an Evolve and Resequence (E&R) approach. *C. riparius* populations were exposed to two concentrations of sediments (0.5% and 10%) over seven generations. Life-history traits were monitored to capture fitness changes, while genomic analyses tracked allele frequency changes to identify signatures of selection.

In the context of the thesis, this chapter connects phenotypic responses with their genomic basis, showing how complex environmental mixtures can act as selective forces shaping evolutionary trajectories. It complements the earlier chapters by linking mutation rate analyses and acute toxicity with adaptive responses, thereby advancing the overall objective of integrating mechanistic insights into ecotoxicological risk assessment under realistic environmental conditions.

Beyond acute toxicity: evolutionary response by rapid polygenic adaptation to a complex environmental stressor in *Chironomus riparius*

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Abstract

Anthropogenic stressors, such as pollution, habitat degradation, and climate change, are altering selective pressures on natural populations, but the evolutionary consequences of chronic exposure to complex mixtures of contaminants remain poorly understood. Addressing this knowledge gap is critical to the emerging field of evolutionary ecotoxicology, which aims to understand how long-term exposure to environmental contaminants shapes adaptive evolution and genome-wide variation. In this study, we employed urban runoff sediment as complex and environmentally realistic model stressor to investigate how multigenerational exposure affects fitness and potentially drives genomic adaptation in the freshwater midge *Chironomus riparius*. We combined an evolutionary life-cycle test with the Evolve and Resequence (E&R) approach, exposing replicate populations over seven generations to three treatments: an uncontaminated control and two concentrations of urban runoff sediment (0.5% and 10%). Key fitness traits, including mortality, mean emergence time (EmT50), fertility, and population growth rate (PGR), were measured, while allele frequency changes (AFC) were tracked to identify genomic signatures of selection. The results revealed distinct and non-linear fitness responses across treatments, including transgenerational effects, recovery of performance, and evidence of life-history trade-offs. Candidate haplotypes were enriched for genes involved in membrane transport, metabolism, and gene regulation, suggesting selection on general stress-response pathways consistent with polygenic adaptation. Signals of selection were also detected in control populations, underscoring the evolutionary influence of laboratory conditions. Overall, our findings demonstrate how evolutionary ecotoxicology can reveal both the potential and the constraints of rapid adaptation to realistic environmental stressors and highlight the importance of integrating evolutionary perspectives into ecological risk assessment.

Key-words: evolutionary ecotoxicology, urban pollution, whole sediment testing, polygenic adaptation

1. Introduction

The increasing influence of anthropogenic stressors on the environment has reshaped selective pressures on natural populations, with pollutants, habitat destruction, and climate change recognized as key drivers of evolutionary change (Briski et al., 2025). These environmental pressures directly contribute to biodiversity loss and generate multifaceted selective forces that alter environmental conditions across multiple scales. As a result, exposed populations may undergo changes in key fitness traits, including growth, reproduction, and survival (Doria and Pfenninger, 2021; Rigano et al., 2024), ultimately restructuring population dynamics and ecosystem stability (McCann, 2000; Pennekamp et al., 2018).

Despite increasing recognition of chemical pollution as a major threat to biodiversity, research on its ecological and evolutionary consequences often remains compartmentalized. Ecotoxicological studies tend to focus on organismal-level effects or specific molecular mechanisms, while ecological research more commonly addresses large-scale patterns of biodiversity change, often without explicitly considering chemical stressors. This disciplinary separation has limited our ability to assess and predict pollution impacts at higher levels of biological organization. Bridging this gap requires integrative approaches that connect chemical exposure to molecular and phenotypic responses, and link these to ecological consequences at the population, community, and ecosystem levels across time and generations (Sylvester et al., 2023). In this context, the emerging field of evolutionary ecotoxicology combines ecotoxicology and evolutionary biology to understand how long-term exposure to pollutants influences evolutionary processes and shapes how natural populations adapt over time (Brady et al., 2017; Straub et al., 2020).

Adaptive responses can be observed as partial restoration of fitness, while their genetic basis can be inferred through genomic signatures of positive selection (Biswas and Akey, 2006). Positive selection occurs when beneficial alleles increase in frequency due to fitness advantages they confer, leaving detectable genomic patterns, such as changes in allele frequencies at and around selected loci (Manel et al., 2016; Nielsen, 2005). However, stressors in the environment, such as pollutants, rarely act in isolation. Instead, they interact in complex, non-linear ways, often generating unpredictable ecological and evolutionary responses (Rillig et al., 2019). Therefore, the interpretation of allele frequency change (AFC) can be challenging because simultaneous action of multiple selective pressures and non-adaptive processes, such as genetic drift, hitchhiking, and background selection, can hinder the association between a putative selective agent and the observed genomic response (Volis et al., 2005). In this context, integrating phenotypic and genomic responses becomes essential, as genomic data reveal the evolutionary potential and targets of selection, while life-history traits capture the realized fitness consequences.

Studies on single contaminants such as cadmium or microplastics have shown that evolutionary responses can vary widely, from partial fitness recovery to long-term population decline, depending on the type of stressor, exposure conditions, and genetic background of the affected population (Doria et al., 2025, 2021; Doria and Pfenninger, 2021; Khosrovyan et al., 2022). However, capturing ecologically realistic exposure scenarios means considering the simultaneous presence of multiple chemical stressors. In this study, we used sediments from urban runoff as such a scenario, reflecting a complex mixture of contaminants whose composition has been described in detail elsewhere (Rigano et al., 2025).

Urban runoff sediments accumulate in sedimentation basins that collect runoff from urban areas, particularly near high-traffic roads (Proteau et al., 2022). These sediments form complex matrices containing a wide range of contaminants, including tyre and road wear particles

(TRWPs), heavy metals, microplastics and polycyclic aromatic hydrocarbons (PAHs) (Baptista Neto et al., 2016; Proteau et al., 2022; Rigano et al., 2025). Previous studies have shown that exposure to urban runoff sediments can increase mortality by up to 30%, reduce fertility, and decrease population growth rate (PGR) in *Chironomus riparius*, even after a single generation (Rigano et al., 2024). Chronic exposure over multiple generations has also been shown to increase germline mutation rates by approximately 50%, independent of concentration, with mutational spectra resembling those induced by Benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) (Rigano et al., 2025).

Chironomus riparius, a non-biting freshwater midge, has emerged as a key model in evolutionary ecotoxicology due to its short generation time, comprehensive genomic resources, and ecological relevance as a sentinel species in freshwater ecosystems (Foucault et al., 2019). Its sensitivity to environmental pollutants, combined with its ability to adapt to chemical stressors, makes it an ideal system for studying evolutionary responses to complex contaminant mixtures. Moreover, unlike many bioassays that use only sediment extracts or water-based samples, the *Chironomus* assay can be carried out with whole sediments, making it possible to assess toxicity in conditions that more closely reflect natural environments (OECD, 2023). As these previous studies have shown, *C. riparius,* as a model organism for freshwater biodiversity, may experience strong selective pressure from such complex environmental stressors, yet the long-term evolutionary consequences of such anthropogenic exposure remain largely unexplored.

To address this gap, experimental evolution combined with whole-genome sequencing offers a robust framework to investigate how populations adapt to environmental stressors across generations. In particular, the Evolve and Resequence (E&R) approach combines experimental evolution with high-throughput sequencing of pooled individuals (Pool-Seq) and has proven to be a reliable method for identifying genomic signatures of adaptation. In E&R studies, replicate populations are exposed to controlled selection pressures over multiple generations, and allele frequency changes are monitored over time to identify candidate loci under selection (Long et al., 2015; Pfenninger and Foucault, 2020; Schlötterer et al., 2015).

In this study, we used a multi-generational E&R approach with *C. riparius* exposed to urban runoff sediment to investigate: 1) How chronic exposure to this complex mixture impacts key fitness traits over multiple generations; 2) Whether genomic signatures of selection emerge in response to these conditions, and what is the nature of the adaptive changes at the genomic level; and 3) How phenotypic and genomic responses co-evolve over time under this ecologically relevant stressor.

2. Materials and Methods

2.1 Sediment collection, preparation and composition

In February 2023, sediment samples were collected from an urban runoff sedimentation basin located in Aachen, Germany (50°48'03.6"N 6°06'29.7"E). This basin receives runoff via a separate sewer system from a large area in the district of Aachen Soers, including a sports park and a heavily trafficked road, Bundesstraße B57 Krefelder Straße. The total drained area covers approximately 68 hectares. The B57 serves around 26,000 vehicles per day, based on a 2021 traffic count by the Federal Highway Research Institute (BASt). Sampling was conducted during routine cleaning of the basin. Sediment was collected from multiple locations within the basin using a stainless-steel bucket, pooled to form a composite sample, and manually homogenized. The homogenized sample was stored in a cold room at 4 °C until further processing, which was completed in the following weeks. For subsequent

analysis, aliquots were freeze-dried and sieved using a stainless steel mesh (1 mm) to remove large particles (Shuliakevich et al., 2022).

The sediment used in this study corresponds to the same matrix previously characterized in detail by Rigano et al., (2025). The urban runoff sediment contained a broad spectrum of urban and traffic-related contaminants, including tyre rubber particles (up to 5.2 g/kg), heavy metals (e.g., Fe: 19.5 g/kg, Zn: 1.74 g/kg, Cu: 144 mg/kg), road salts (46.12 g/kg), and mineral oils (983 µg/kg). The total concentration of PAHs was 4.0 mg/kg, with high molecular weight compounds such as Benzo[*b*]fluoranthene and Benzo[*g,h,i*]perylene accounting for approximately 80% of the total. LC-HRMS/MS screening further detected 124 synthetic organic compounds, including tyre additives (e.g., phosphate esters, phthalates, benzothiazoles, bisphenols), as well as personal care products, pesticides, biocides, and PFAS. A comprehensive chemical profile is available in Rigano et al., (2025).

2.2 Source population and establishment of laboratory population

The *C. riparius* culture used in this study originated from a local population collected from a small river in Hasselbach, Hessen, Germany (50.167562N, 9.083542E). The culture was maintained as an in-house laboratory standard and regularly supplemented with individuals from the field to preserve genetic diversity. Stock culture conditions followed a modified protocol based on OECD guideline No. 219 (Foucault et al., 2019).

2.3 Evolutionary life-cycle test

An uncontaminated control and two treatment groups exposed to urban runoff sediment (0.5% and 10% by total weight) were tested. The control group consisted of 500 g of quartz playground sand. The 0.5% group contained 497.5 g of quartz sand mixed with 2.5 g of urban runoff sediment, while the 10% group consisted of 450 g of sand and 50 g of sediment.

To initiate the life-cycle test in each generation, 10 freshly laid egg ropes were collected at the beginning of each generation from the same E&R replicates (see below) and transferred to 6-well plates containing 3 mL of medium per well. Nine glass bowls (Ø 20 × 10 cm) were prepared one day prior to the start of the experiment: three were assigned to the control group, three to the 0.5% group, and three to the 10% group.

Each bowl was filled with 1.25 L of medium and aerated continuously, except for the first 6 hours to allow larvae to settle in the sediment. Sixty first-instar larvae were introduced into each bowl at the beginning of each generation. The test was conducted over a period of 28 days, approximately corresponding to the duration of one generation, in accordance with OECD Guideline No. 233. (OECD, 2010). The organisms were fed daily with finely ground Tetramin® Flakes, with feeding amounts adjusted according to developmental stage (Foucault et al., 2019).

Adult emergence was recorded daily to calculate mortality by subtracting the number of emerged adults from the initial number of exposed larvae. The sex of emerged adults was recorded daily to calculate the mean emergence time (EmT50), defined as the time when 50% of the females had emerged. The number of fertile egg ropes was recorded, and egg counts were determined (Foucault et al., 2019). Finally, all measured parameters were summarized into PGR (Nemec et al., 2013). Data for the sixth generation of the control group could not be collected due to technical limitations.

Raw data were entered, and fitness parameters were calculated using Microsoft Excel® (MS Excel®). Statistical and graphical analyses were performed in R, applying linear, quadratic (second-order polynomial), and constant models to assess patterns in fitness parameters across contaminant exposure levels over multiple generations.

2.4 Establishment of the test replicates for E&R

Twenty-five egg ropes from the laboratory population were collected and hatched, and the ~ 15,000 larvae pooled to establish nine experimental replicates. From these pools, at least 1,000 first-instar larvae were transferred to glass bowls (Ø20 × 14.5 cm) to initiate each replicate. Three replicates were assigned to each treatment group (0, 0.5%, 10%).

All replicates were maintained at 23°C, 60% humidity, and a 16:8 light:dark photoperiod, following OECD guideline No. 233 (OECD, 2010). The quartz playground sand was pH-neutral and washed before use. Urban runoff sediment and sand were mixed thoroughly using a laboratory spatula to homogenize the sediment column. The bowls were filled with 1.5 L of medium consisting of deionized water adjusted to a conductivity of 550–650 µS/cm with aquarium sea salt (TropicMarin®) and a pH of ~8. Water loss due to evaporation was compensated by adding deionized water. The organisms were kept in swarming cages (40 × 40 × 60 cm) for the entire E&R experiment and fed weekly with finely ground Tetramin® fish flakes. Replicates were maintained under these consistent conditions for seven generations (Foucault et al., 2019).

2.5 Pool-seq-based detection of selection and haplotype analysis

Every 28 days, corresponding to the generation length at the experimental temperature, 120 adults were collected and pooled from each replicate over seven generations. In the F0 group, pooling was performed only at the end of the first generation, resulting in a total of 64 pools, including the F0 group and three replicates for each treatment: control (0), 0.5%, and 10%.

DNA was extracted from pooled samples of 120 individuals from the F0 and each replicate using the Qiagen blood and tissue extraction kit. Sequencing was performed on the Illumina NovaSeq platform, with an effective mean coverage ranging between 30× and 85×. Adapters and low-quality reads were trimmed using Trimmomatic (Bolger et al., 2014). Clean reads were then mapped to the latest *C. riparius* reference genome (v.4, unpublished) using the BWA mem algorithm (unpublished data) (Li and Durbin, 2009). Low-quality reads were filtered, and single nucleotide polymorphisms (SNPs) were initially called using samtools (Li et al., 2009).

The softwares Popoolation and Popoolation2 (Kofler et al., 2011a, 2011b) were used to call SNPs, remove indels, and estimate genetic diversity as Watterson's theta in the first and last generations of each replicate. A synchronized file was generated using PoPoolation2 and processed with a custom Python script to estimate allele frequencies. The sync file was filtered using the following parameters: minimum SNP coverage (≥ 15), minimum read count per population (≥ 15), minor allele frequency (≥ 0.10), and variance among allele frequencies (≥ 0.10).

Potentially selected SNPs were identified by detecting AFC larger than expected under genetic drift. AFC across generations were assessed using two complementary statistical approaches: linear regression, to detect SNPs exhibiting consistent frequency shifts over time, and Fisher's exact test, to identify SNPs showing significant differentiation between the initial and final generations. The top 1% of outliers from both tests were cross-referenced to identify selection signals.

To account for the expected effect of genetic drift, replicate-specific effective population sizes (N_e) were first estimated from the observed AFC between F0 and the final generation. Expected allele frequency trajectories under drift were then simulated using the Wright-Fisher model with the estimated N_e values over seven generations. Significance of the observed allele frequency changes was tested using a chi-square test, followed by the Benjamini-Hochberg (BH), comparing observed and expected values under drift. A 99.9th percentile threshold was

used to define highly significant SNPs according to the chi-square test and to compare real observations with expected drift. Only SNPs with -log10(p-value) exceeding the 99.9th percentile of the simulated distribution were retained for further analysis. All calculations and simulations were performed with the R package *PoolSeq* (Taus et al., 2017).

The number of candidate SNPs was likely inflated by the hitchhiking of neutral variants linked to selected SNPs, driven by the low number of recombination events expected during the experiment, which increased the frequency of large haplotype blocks (Franssen et al., 2017). To identify haplotypes, potential marker SNPs were defined as those falling within the top 20th percentile of AFC values. Pearson correlation coefficients were calculated between each marker SNP and neighbouring SNPs to define haplotypes boundaries. Haplotype blocks were extended from each marker SNP until the correlation coefficient with the focal marker fell below that with the next neighbouring SNP. If the correlation with the next marker exceeded that with the focal marker, the block was discontinued, and a new haplotype was initiated. Haplotypes were considered to overlap within and between treatment groups if they extended at least 25% in both directions from the marker SNP. After haplotype assignment, a Monte Carlo intersection analysis was performed to assess whether their presence within and between treatments aligned with random expectations.

The selection coefficient (s) was estimated from the change in allele frequency over the course of the experiment for each selected SNP (Crow, 2000). For each haplotype, the SNP with the highest selection coefficient was identified as the most likely selection target. Although a single primary selection target may exist within each haplotype, the entire haplotype with all its phenotype-relevant variants is likely under selection.

The gene nearest to the SNP with the highest selection coefficient within each haplotype was tentatively considered a potential selection target across all replicates. The resulting list of candidate genes was analysed for overrepresentation of gene ontology (GO) terms related to "biological function" using Fisher's exact test implemented in the R package TopGO (Alexa, Adrian, and Jörg Rahnenführer, 2009).

3 Results

3.1 Evolutionary life-cycle test

Mortality, mean emergence time (EmT50), fertility, and PGR exhibited distinct patterns across the control, 0.5%, and 10% groups over seven generations. In the control, mortality increased steadily over generations, following a linear model ($R^2 = 0.277$, $p = 0.0248$), whereas in the 0.5% group, mortality increased in a polynomial fashion ($R^2 = 0.251$, $p = 0.0464$). In contrast, mortality in the 10% group followed a quadratic pattern ($R^2 = 0.444$, $p = 0.2177$), with an initial increase followed by partial stabilization in later generations (Figure 1a).

EmT50 exhibited contrasting patterns across treatments (Figure 1b). In the control, EmT50 showed a slight, non-significant decrease over generations ($R^2 = 0.647$), following a loess model. In the 0.5% group, EmT50 declined significantly ($R^2 = 0.25$, $p = 0.021$), following a logarithmic model, while the most pronounced decline was observed in the 10% group ($R^2 = 0.405$, $p = 0.0019$), following a power model.

Fertility decreased over generations in the control, following a segmented linear pattern, but showed recovery after the third generation ($R^2 = 0.828$, $p = 0.00001$). In the 0.5% group, fertility declined with no significant trend ($R^2 = 0.186$). In contrast, fertility in the 10% group decreased significantly over generations ($R^2 = 0.605$, $p = 0.00005$) (Figure 1d).

PGR in the control exhibited a significant downward trend, following a segmented linear model ($R^2 = 0.655$, $p = 0.0006$), with a decline up to the third generation, followed by stabilization in subsequent generations. In the 0.5% group, no significant change in PGR was

observed (R^2 = 0.201). In the 10% group, PGR showed a similar trend to the control, declining significantly (R^2 = 0.613, p = 0.0485), but the decrease was less pronounced after the third generation, followed by partial recovery (Figure 2a).

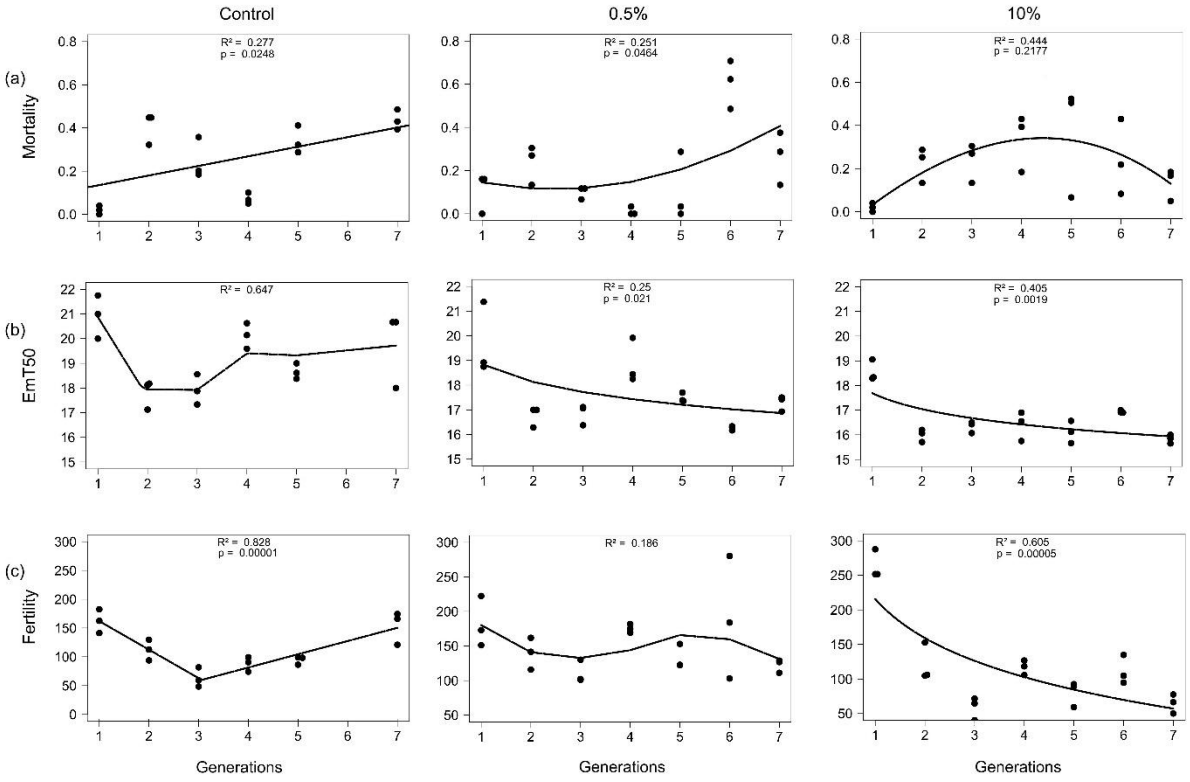

Figure 1. *C. riparius* life-cycle endpoints measured over seven generations: (a) Mortality, (b) EmT50, and (c) Fertility. Each data point represents the value from an independent replicate population, based on multiple individuals per generation and treatment. Trend lines represent the best-fitting models for each treatment and endpoint, selected based on visual inspection and model fit (R^2), and include linear, polynomial, quadratic, logarithmic, segmented linear, and loess models. Loess models were used for descriptive smoothing and do not yield standard p-values.

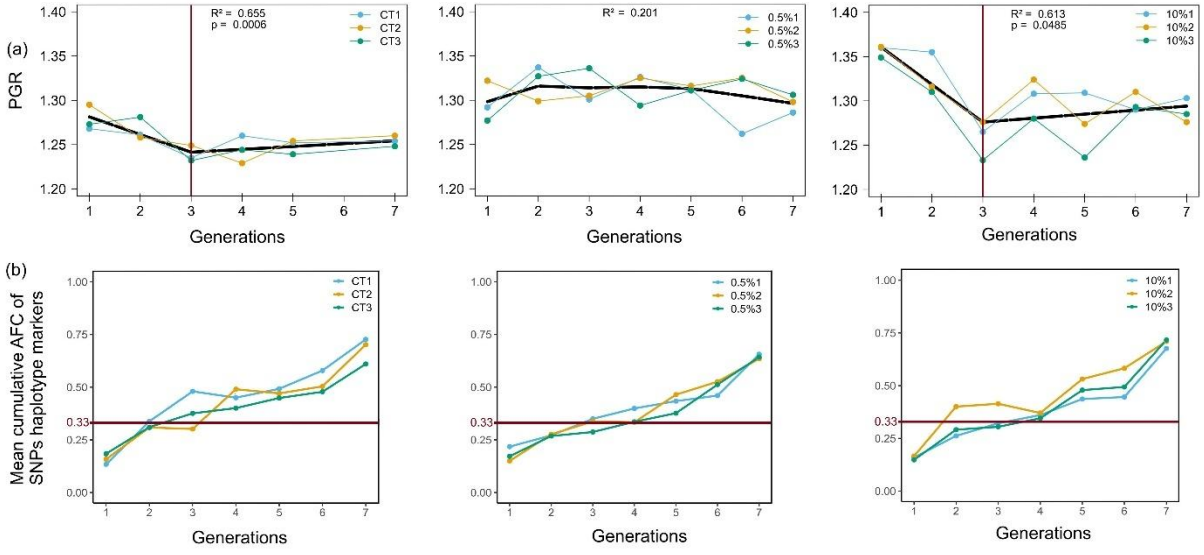

Figure 2. (a) PGR measured across seven generations for each replicate. Red vertical lines indicate the generation at which the inflection point in the PGR trajectory was observed. (b)

Mean cumulative AFC of haplotype-defining SNPs per replicate. The horizontal red line marks the upper 95% confidence limit expected under genetic drift.

3.2 Pool-seq-based detection of selection and haplotype analysis

The raw sequencing data and pool mapping files are available at the European Nucleotide Archive (ENA), under accession number PRJEB89250.

The replicate-specific effective population size (Ne) during the experiment varied between 29 and 115 (mean ± SD: 63.75 ± 27.18) (Table 1). The F0 population had a mean genome-wide population mutation parameter theta of 0.012. Based on the species' point mutation rate of 2.1×10^{-9} per site per generation (Oppold and Pfenninger, 2017), this corresponds to a long-term N_e of approximately 1.43 million. In the evolved replicates, theta ranged between 0.005 and 0.012. Selection and drift jointly reduced genetic variation by up to 53.1%, although in some replicates the reduction was negligible compared to the F0 population (Table 1).

The average AFC per replicate, calculated between the F0 and F7 generations for the selected SNPs, ranged between 0.61 and 0.72 (Table 1). A total of 1,171 SNPs in the control, 538 in the 0.5% urban runoff sediment treatment, and 674 in the 10% urban runoff sediment treatment exceeded the expected threshold and were retained for haplotype analysis. These SNPs allowed the identification of 318 haplotypes: 127 in the control replicates, 98 in the 0.5% urban runoff sediment replicates, and 93 in the 10% urban runoff sediment replicates (Table 1). An ANOVA followed by a Tukey post-hoc test showed significant differences in haplotype numbers only for two replicates of the 0.5% urban runoff sediment treatment ($p = 0.025$). A Kruskal-Wallis test followed by a Dunn post-hoc test with Bonferroni correction also showed significant differences in haplotype length between the same two replicates ($p = 0.014$). Haplotypes were considered overlapping if their lengths, extending in both directions from the marker SNP, overlapped by at least 25%. Based on this criterion, 9 haplotypes were assigned to the control, 1 to the 0.5% treatment, and 2 to the 10% treatment. Additionally, 12 haplotypes were shared between the control and 0.5%, 17 between the control and 10%, 15 between 0.5% and 10%, and 8 were shared among all three treatments (Figure 3b).

Table 1: Demographic and genetic parameters of each evolved replicate population. Reported are the estimated effective population size (Ne), the percentage loss of nucleotide diversity relative to the ancestral (F0) population (Genetic reduction %), the average AFC of selected SNPs, and the number of haplotypes detected.

Replicate	Ne	Genetic reduction (%)	AFC of sel. SNPs	N. haplotypes
Control1	28.75	6.81	0.72	39
Control2	40.42	8.60	0.70	44
Control3	71	17.02	0.61	44
05%1	89.72	10.27	0.65	25
05%2	77.79	52.61	0.63	56
05%3	115.24	31.55	0.64	17
10%1	53.66	8.35	0.67	34
10%2	47.78	8.74	0.71	27
10%3	49.35	2.61	0.71	32

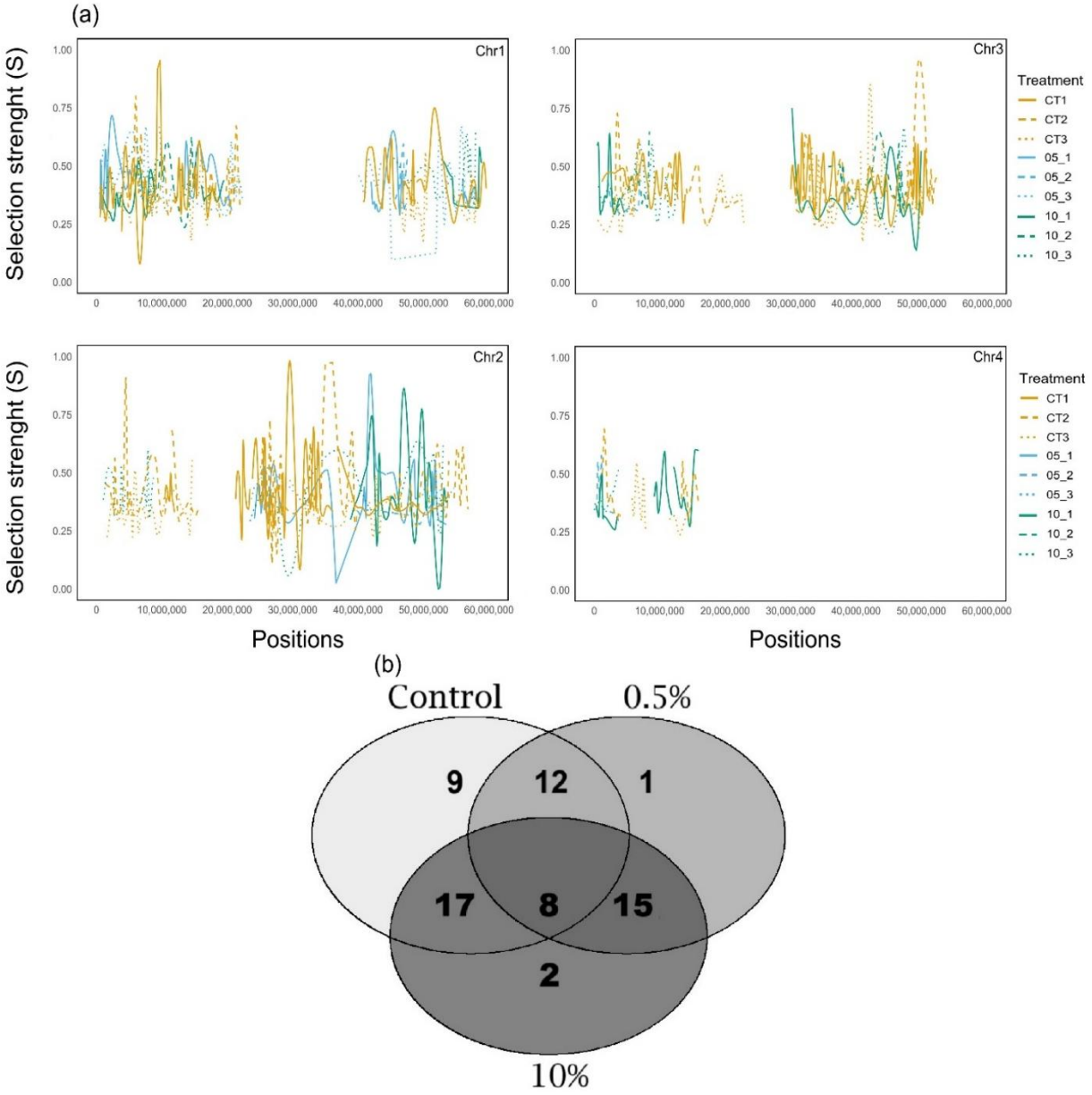

Figure 3. (a) Distribution of haplotypes across chromosomes in the different treatments. (b) Venn diagram showing the number of exclusive and shared haplotypes among treatments.

The Monte Carlo intersection analysis revealed that the number of observed haplotypes largely aligned with the expected random distribution, with some deviations depending on the treatment (Table 2). In the control group, the number of observed haplotypes (9) was very close to the expected value (8.63) and showed no significant deviation ($p = 0.89$).

The degree of haplotype sharing between treatments showed more pronounced differences. The number of haplotypes shared between the control and 0.5% groups (12) was higher than expected (7.22) but did not reach statistical significance ($p = 0.058$). In contrast, haplotype sharing between the control and 10% groups (17) and between the 0.5% and 10% groups (15) was significantly higher than expected ($p = 0.003$ and $p = 0.0003$, respectively). The overlap between all three treatments (8) was not significantly different from the expected value (6.54) ($p = 0.548$). In contrast, the number of haplotypes in the 0.5% and 10% treatments (1 and 2, respectively) was lower than expected (7.01 and 9.83), and this reduction was statistically significant in both groups ($p = 0.016$ and $p = 0.006$).

Estimated selection coefficients for significant SNPs ranged between 0.35 and 0.45. The GO term enrichment analysis identified several biological processes significantly overrepresented among the genes closest to SNPs within shared haplotypes across treatments. In haplotypes shared between the control and 0.5% groups, the most enriched terms were linked to dipeptide import across the plasma membrane (GO:0140206; p = 0.00000018), unsaturated fatty acid biosynthetic process (GO:0006636; p = 0.00003454), and synapse organization (GO:0050808; p = 0.00018).

Haplotypes shared between the control and 10% groups showed enrichment for processes such as anion transport (GO:0006820; p = 0.00000016), sulfation (GO:0051923; p = 0.00000898), and proteolysis (GO:0006508; p = 0.00005742).

For haplotypes shared between the 0.5% and 10% groups, the most enriched terms included NAD-cap decapping (GO:0110155; p = 0.00000004), O-glycan processing (GO:0016267; p = 0.000000065), and proteolysis (GO:0006508; p = 0.000007469), suggesting selective pressure on post-transcriptional and protein turnover pathways. Strong enrichment was also observed for nucleic acid phosphodiester bond hydrolysis (GO:0090305; p = 0.000016749) and regulation of transcription by RNA polymerase II (GO:0006357; p = 0.000086369), indicating targeted selection on gene expression and nucleic acid metabolism.

4 Discussion

Environmental contaminants are well known to impair fitness and alter the genomic architecture of exposed organisms. However, it remains poorly understood whether these ubiquitous stressorscan act as selective agents and drive adaptive evolution, especially when occurring in complex mixtures. To address this, we employed a multigenerational evolution approach that integrates life-history data with the E&R framework. This combined design allowed us to simultaneously assess phenotypic responses and genome-wide AFC in *C. riparius* populations exposed over seven generations to different concentrations of urban runoff sediments.

Our study provides one of the first integrative assessments of both adaptive and mutational responses to a realistic contaminant mixture over multiple generations, offering novel insight into the evolutionary dynamics triggered by chronic pollutant exposure.

4.1 Phenotypic and genomic responses to multigenerational exposure

To assess the effects of chronic exposure on fitness, we monitored mortality, EmT50, fertility and PGR. Phenotypic responses revealed nonlinear trajectories and potential trade-offs, suggesting that adaptive changes do not scale linearly with contaminant concentration. These patterns were accompanied by detectable changes in allele frequency, discussed below, indicating that the observed phenotypic dynamics were underpinned by genomic responses. We also detected evidence of selection in the control group, indicating that laboratory conditions imposed selective pressures, highlighting the importance of distinguishing these effects from those caused by contaminant exposure.

In the control group, mortality progressively increased over successive generations, likely due to nutritional limitations or rearing conditions differing from those of the ancestral stock culture. The temporal consistency of this pattern supports the hypothesis of a sustained physiological burden, rather than random demographic fluctuations. Conversely, the initially low mortality in the 0.5% group may reflect a nutritional benefit from organic content in the sediment, temporarily offsetting toxic effects (Rigano et al., 2024). However, this benefit did not prevent long-term physiological compromise, as mortality increased over time, highlighting that even low-level exposure can lead to significant fitness costs. In the 10% group, mortality

exhibited a biphasic trajectory, with an initial peak, presumably caused by acute toxicity, followed by partial stabilization in subsequent generations. Therefore, in contrast to the lower concentration, the high contaminant load in the 10% group likely neutralized any potential nutritional advantage, with toxicity emerging as the dominant driver of phenotypic responses.

EmT50 was also influenced by treatment, reflecting stress-induced developmental shifts. In the control group, EmT50 initially decreased and later recovered. In contrast, urban runoff sediment-exposed individuals, particularly at 10%, exhibited accelerated development across generations. This may be linked to increased nutrient availability in the urban runoff sediment, consistent with findings by Rigano et al. (2024), who reported accelerated development and increased larval size after a single generation of exposure to a similar matrix. However, faster development may also represent a stress-induced life-history adjustment. Kolbenschlag et al. (2024) showed that exposure to *Bacillus thuringiensis israelensis* (Bti), a biological larvicide, induced earlier emergence in *C. riparius*, together with morphological alterations and reduced adult fitness. These findings suggest that accelerated development may arise from distinct environmental pressures and involve physiological trade-offs, such as reduced energy reserves or compromised reproductive capacity (Zera and Harshman, 2001).

In the control group, fertility declined during generations F2 and F3 and then recovered from F4 onwards, reflecting an initial response to stress imposed by laboratory conditions, followed by recovery in subsequent generations. The 0.5% group maintained a relatively stable fertility across generations, suggesting that this exposure level did not significantly impair reproductive function, at least within the timeframe of the experiment. In contrast, fertility steadily declined in the 10% group, consistent with a likely cumulative reproductive cost associated with sustained contaminant pressure.

The observed fitness dynamics became particularly evident when considering PGR, a composite measure that integrates all the fitness parameters. In both the control and 10% groups, PGR declined during generations F2–F3, followed by a recovery in subsequent generations. In contrast, the 0.5% group showed no consistent deviation in PGR, suggesting that this lower exposure level remained below the threshold required to elicit long-term effects.

The similarity in PGR dynamics between the control and 10% groups suggests a shared underlying mechanism. This segmented pattern contrasts with theoretical expectations, which predict that fitness responses to environmental stress occur rapidly, usually within one generation, as selection quickly favours traits improving survival and reproduction (Fisk et al., 2007; Goitom et al., 2018; Sibly and Calow, 1989). We hypothesise that stress experienced by females in the early F1 generation compromised resource allocation into reproduction, thereby diminishing egg rope quality. This might have delayed visible fitness restoration in subsequent generations, despite the immediate onset of molecular selection (see below). These types of transgenerational effects, although often transient, have been documented in both invertebrate and vertebrate models. For instance, Salesa et al. (2022) reported that *Daphnia magna* exposed to prochloraz exhibited no reproductive effects in the F0 generation but showed significant phenotypic changes in F1. Similarly, exposure of pregnant rats to endocrine disruptors impaired the fertility of male offspring up to the F4 generation (Anway et al., 2005).

Although allele frequency changes were detectable from the earliest generations, phenotypic recovery did not occur immediately, suggesting that beneficial alleles required time to rise sufficiently in frequency to produce a measurable effect on population fitness. Suggestively, from generation F3 onward, allele frequency changes at haplotype-defining SNPs exceeded expectations under genetic drift across all treatments. Thus, the initial decline and subsequent recovery of PGR from generation F4 onward in the control and 10% groups

may reflect the interaction between transgenerational physiological constraints and evolutionary adaptation.

Genetic drift can strongly affect small experimental populations (Lynch et al., 2016). Additionally, limited recombination and a small number of generations constrain the breakdown of linkage disequilibrium (LD), causing selected alleles to remain physically linked to nearby neutral variants and hindering the identification of loci directly under selection (Kofler and Schlötterer, 2014). Although the E&R approach is limited in short-term experiments, due to low recombination and small population sizes that can reduce resolution and hinder causal loci (Franssen et al., 2017; Otte and Schlötterer, 2021), the consistent deviations from drift expectations across independent replicates, along with the functional enrichment of selected regions, support the conclusion that selection drove the observed genomic changes. Moreover, the temporal correspondence between molecular shifts and phenotypic recovery aligns with models of polygenic adaptation, in which selection acts on multiple small-effect loci distributed throughout the genome (Barghi et al., 2020; Berg and Coop, 2014).

These selective responses, despite differences in phenotypic outcomes across treatments, revealed shared genomic patterns. Functional annotation of candidate regions indicated that selected haplotypes were significantly enriched for biological processes involved in membrane transport, lipid metabolism, proteolysis, and gene expression regulation. This suggests that adaptation predominantly targets general stress-response pathways aimed at maintaining cellular homeostasis under chronic exposure, rather than pathways specific to contaminant concentration. These observations align with models of polygenic adaptation, in which selection acts on standing genetic variation across broad regulatory networks (Barghi et al., 2020, 2019).

4.2 Evolutionary implications and ecological relevance

Our study revealed coordinated phenotypic and genomic responses in both the control and 10% treatments. While the changes observed in the control group likely reflect adaptation to laboratory conditions, the common response in the sediment treatments suggested that chronic exposure to urban runoff sediment imposed selective pressures that contributed to evolutionary change. Similar patterns have been reported in previous multigenerational experiments involving single contaminants, such as heavy metals and microplastics, which are also major constituents of the complex mixture investigated in this study. For example, *C. riparius* exposed to increasing cadmium concentrations over eight generations exhibited genome-wide allele frequency changes indicative of strong selection, despite only modest phenotypic changes (Doria et al., 2022). Likewise, exposure to polyamide microplastics initially caused high larval mortality, followed by a rapid compensatory response and substantial allele frequency changes, consistent with polygenic adaptation (Khosrovyan et al., 2022). In another organism, *Daphnia magna* exposed to perfluorooctane sulfonate (PFOS) experienced reduced individual fitness during chronic exposure, while population growth recovered over successive generations, suggesting a potential for adaptive compensation despite sustained physiological stress (Tae-Yong Jeong et al., 2016).

Beyond selection, prior work using the same sediment matrix showed a significant increase in germline mutation rate in *C. riparius*, by approximately 50%, regardless of concentration (Rigano et al., 2025). This highlights a dual role of urban runoff sediment in shaping evolutionary trajectories, acting both as a selective pressure that promotes adaptive change and as a source of mutational input that increases genetic variation. This combination may accelerate evolutionary dynamics but also raises concerns about long-term genomic stability and fitness in natural populations.

By linking phenotypic trajectories with genome-wide allele frequency dynamics across generations, our study provides an integrated perspective on how aquatic organisms respond to sustained environmental stress. Although conducted under controlled laboratory conditions with relatively small populations, we detected clear adaptive responses at both the phenotypic and genomic levels. This suggests that natural populations, typically characterized by greater effective population size and thus genetic diversity and subjected to continuous exposure, may also evolve in response to similarly complex pollutant mixtures. However, the increased ecological complexity and co-occurrence of multiple stressors in natural environments are likely to influence both the direction and pace of such adaptation (Rullens et al., 2022).

In summary, our findings underscore the urgent need to integrate evolutionary perspectives into environmental risk assessment and regulatory frameworks. As pollution continues to reshape the genetic and ecological fabric of natural populations, ecotoxicology must move beyond short-term toxicity endpoints to consider the long-term evolutionary consequences of chronic exposure. Understanding how contaminants alter not only survival but also the heritable basis of adaptation is essential to safeguard biodiversity and ecosystem stability. In an era of accelerating environmental change, failing to do so risks underestimating the true cost of pollution, for nature and for society.

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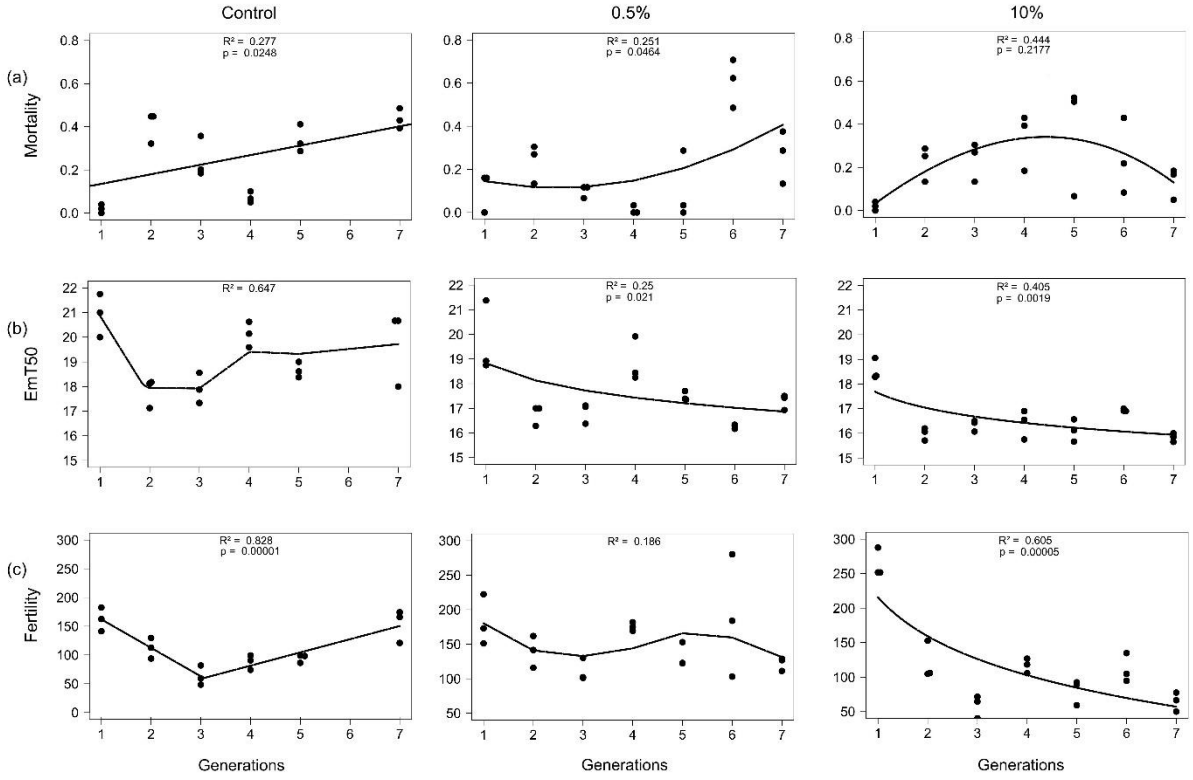

Figure 1. *C. riparius* life-cycle endpoints measured over seven generations: (a) Mortality, (b) EmT50, and (c) Fertility. Each data point represents the value from an independent replicate population, based on multiple individuals per generation and treatment. Trend lines represent the best-fitting models for each treatment and endpoint, selected based on visual inspection and model fit (R^2), and include linear, polynomial, quadratic, logarithmic, segmented linear, and loess models. Loess models were used for descriptive smoothing and do not yield standard p-values.

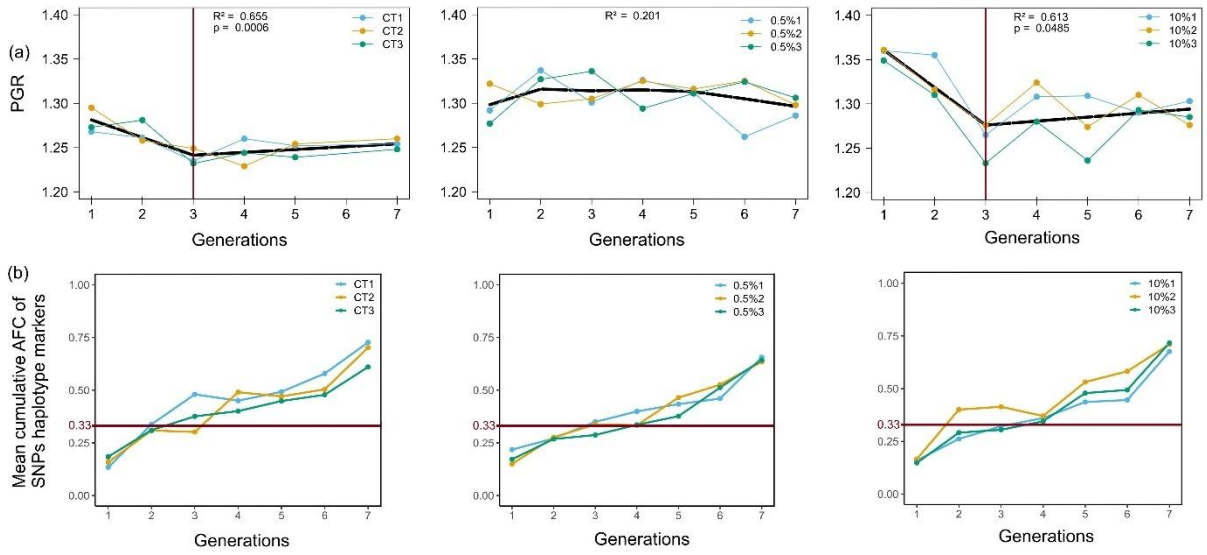

Figure 2. (a) PGR measured across seven generations for each replicate. Red vertical lines indicate the generation at which the inflection point in the PGR trajectory was observed. (b) Mean cumulative AFC of haplotype-defining SNPs per replicate. The horizontal red line marks the upper 95% confidence limit expected under genetic drift.

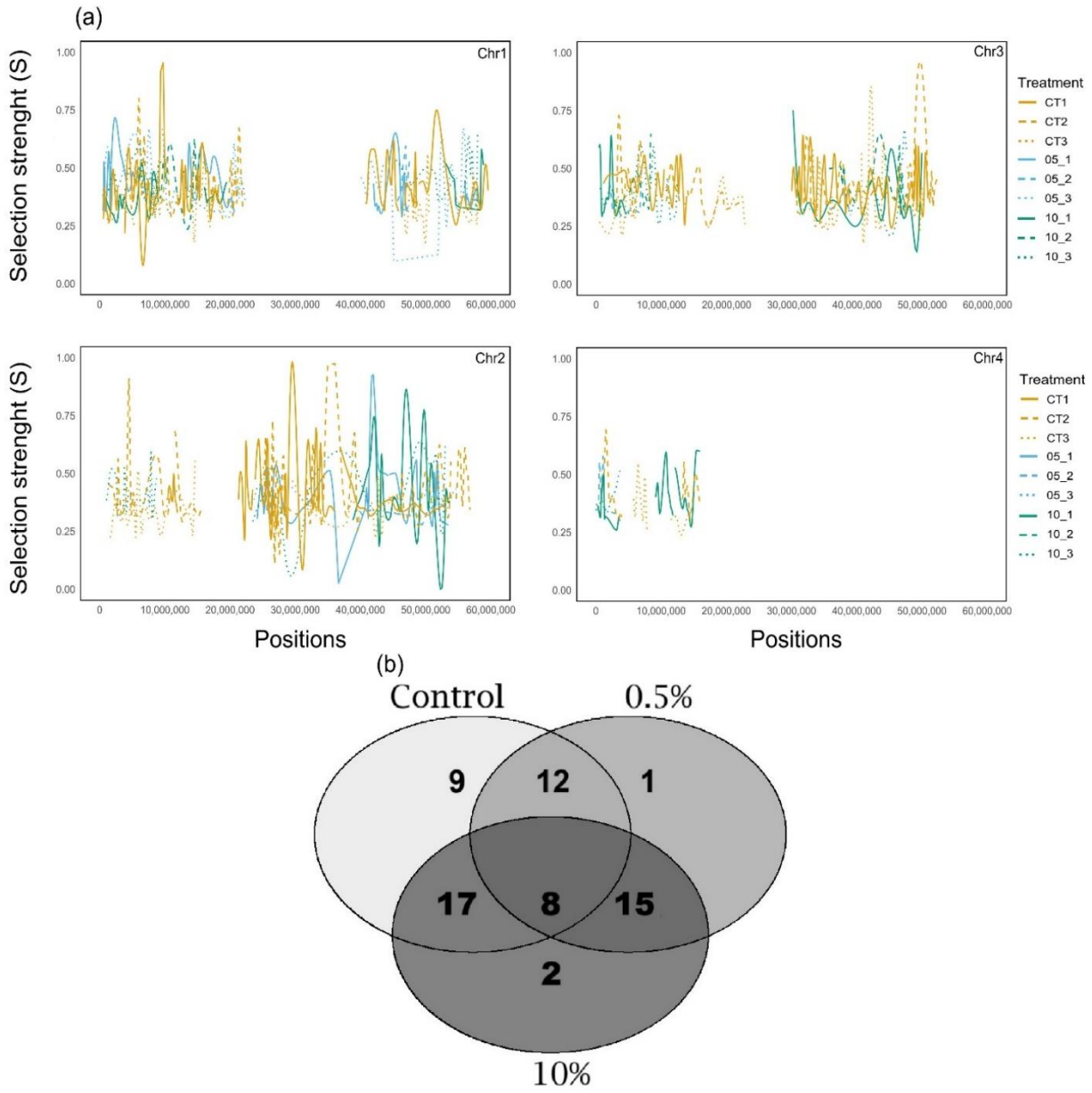

Figure 3. (a) Distribution of haplotypes across chromosomes in the different treatments. (b) Venn diagram showing the number of exclusive and shared haplotypes among treatments.

DISCUSSION

1. Toward More Realistic Environmental Scenarios in Evolutionary Ecotoxicology

In the Anthropocene, the widespread release of chemical pollutants has reshaped the network of environmental stressors influencing species ecology and evolution. This transformation has introduced a novel dimension, characterised by long-lasting effects across all levels of biological organisation, from molecular and cellular processes to population dynamics and ecosystem functioning (Best, 2019; Boivin et al., 2016). In natural ecosystems, exposure to pollutants rarely occurs in isolation. Instead, organisms are exposed to chemically diverse mixtures whose composition and toxicity shift with anthropogenic inputs and environmental dynamics (Fischer et al., 2013; Samuel et al., 2023).

Despite this complexity, ecotoxicological research has long relied on single-substance approaches, primarily assessing acute or short-term effects. This reductionist paradigm has been crucial for establishing causal links between defined exposures and biological effects, as well as for quantifying dose–response relationships (McCarty, 2025; Ritz, 2010). Moreover, these insights are indispensable for hazard characterization and risk assessment, guiding the identification of effect thresholds, susceptible target organs, and reliable biomarkers (Coutellec and Barata, 2011; Rodea-Palomares et al., 2015).

Although single-compound tests alone cannot reliably predict the toxicity of complex mixtures, they also remain fundamental for identifying primary toxicity drivers and for providing baseline data to interpret combined effects. For instance, a mixture's overall response may be largely driven by a single compound with high toxic potency or bioavailability, which could remain undetected without prior testing under isolated conditions (Sigurnjak Bureš et al., 2021). In this way, short-term or acute single-compound studies provide the necessary foundation for designing mixture experiments and parameterising predictive models of contaminant interactions (Liu and Sayes, 2024).

However, fully capturing pollutant impacts in natural systems requires incorporating the temporal and generational dimensions of ecological change, through a broader perspective that integrates ecological context with evolutionary dynamics. Over the past decades, progress in molecular, analytical and experimental methodologies, combined with a growing recognition of the evolutionary implications of chemical stress, has driven the integration of evolutionary perspectives into ecotoxicological research (Bickham, 2011). This has expanded

the field beyond immediate physiological responses to encompass heritable changes, long-term adaptations and shifts in population resilience under sustained chemical stress (Brady et al., 2017; Straub et al., 2020).

Despite this progress, evolutionary studies conducted under environmentally realistic conditions testing field-derived matrices remain exceedingly scarce. Most multigenerational investigations still follow the single-compound paradigm or involve exposures to synthetic mixtures of fixed and well-defined composition (Doria et al., 2021; Doria and Pfenninger, 2021; Khosrovyan et al., 2022). These approaches reflect only a limited representation of the contaminant complexity and variability that characterise real-world contamination scenarios. Consequently, significant gaps remain in understanding and predicting organisms' responses to multifactorial chemical pressures over ecologically relevant timescales.

This limitation is further compounded by the impossibility of reliably inferring mixture toxicity from single-substance effects. In real-world contexts, multiple contaminants frequently co-occur and exert mechanistically complex, non-linear effects. These may be synergistic, leading to greater toxicity than expected, or antagonistic, resulting in reduced effects (Martin et al., 2021). The overall toxicological impact depends on the chemical nature of each component, its speciation, and the biological pathways involved in uptake, metabolism, and detoxification (Petitjean et al., 2019; Rodea-Palomares et al., 2015). By affecting these processes, contaminant mixtures can alter bioavailability, interfere with detoxification mechanisms, and modulate key physiological responses. Collectively, these effects may disrupt homeostatic regulation and trigger oxidative stress or endocrine imbalance. In turn, this can lead to emergent toxicological outcomes not evident in single-compound exposure scenarios (Altenburger et al., 2013).

Furthermore, contaminant interactions may also involve mechanisms such as complexation and competitive binding, which influence their mobility and persistence in the environment (Cipullo et al., 2018). For instance, heavy metals may associate with dissolved organic matter or particulate surfaces, which reduces their bio-accessible fraction. Organic compounds, on the other hand, can undergo degradation, photolysis, or microbial transformation, resulting in metabolites that possess distinct and sometimes stronger toxicity profiles (Briffa et al., 2020; Zhou et al., 2023). Moreover, these dynamics are not static but depend on fluctuating environmental parameters such as pH, redox potential, temperature, and ionic strength. This variability influences the physicochemical behaviour of contaminants, as well as their capacity to interact and exert biological effects.

Within biological systems, complexity increases further. Co-occurring contaminants can interfere with absorption, distribution, metabolism, and excretion (ADME) processes by competing for cellular transporters, receptor sites, or metabolic enzymes (Casals-Casas et al., 2008; Tan et al., 2025). These interactions may alter intracellular concentrations and disrupt toxicokinetic and toxicodynamic pathways, ultimately modifying how contaminants are processed and how much accumulates in target tissues (Liu et al., 2024; Wu et al., 2024). Additionally, many pollutants can modulate the expression of genes involved in detoxification, oxidative stress response, and immune regulation. These include key enzymatic systems such as cytochrome P450 monooxygenases and glutathione S-transferases, which influence the organism's capacity to cope with chemical stress (Khoshmanesh et al., 2023; Suzuki et al., 2020; Vogel et al., 2020). These physiological adjustments can affect the net toxic burden experienced by the organism and reshape the nature, intensity, and direction of selective pressures acting on exposed populations.

From an evolutionary perspective, chronic exposure to complex contaminant mixtures that vary in concentration and composition over time represents a qualitatively distinct selective environment compared with isolated compounds. Organisms must contend with multiple, sometimes opposing physiological demands, where adaptation to one contaminant may compromise tolerance to others. Such trade-offs can constrain viable adaptive solutions, influence life-history strategies, and limit the evolutionary trajectories available to a population (Coutellec and Barata, 2011; Straub et al., 2020).

These broader evolutionary constraints often manifest through underlying physiological trade-offs. For instance, acute exposure of *Chironomus riparius* to urban runoff sediments, a chemically complex matrix collected from the field, revealed a pronounced physiological trade-off (Chapter 3). Larval size and mean emergence time displayed bell-shaped relationships with contaminated sediment concentration. At low concentrations (0.5%), the additional organic matter in the tested sediment likely enhanced food availability, enabling more efficient feeding and greater energy allocation to growth and accelerated development. At higher concentrations (10%), however, the elevated contaminant load appears to have imposed sufficient physiological stress to offset these benefits, reducing growth and delaying emergence. This interplay between nutritional gains and contaminant stress demonstrates how rapidly the balance of opposing pressures can shift with changes in exposure level, potentially constraining the scope for adaptive responses under chronic exposure.

The complexity of predicting performance across different exposure scenarios is further illustrated by Wijewardene et al. (2025), who tested whether tolerance to an isolated stressor

persists when multiple stressors act simultaneously. In their multifactorial experiment, six clonal lineages of the freshwater rotifer *Brachionus calyciflorus* were exposed to constant salinity and three temperature regimes (17 °C, 20 °C as control, and 23 °C). At both 17 °C and 23 °C, some lineages declined rapidly despite exhibiting the highest acute tolerance to salinity when tested individually. Conversely, lineages with relatively low acute tolerance under single-stressor conditions achieved greater long-term survival when stressors were combined. This reversal in relative performance demonstrates that acute tolerance to individual stressors offers little predictive value for long-term success in multifactorial environments, emphasizing the complexity of adaptive responses under realistic conditions.

Contamination patterns in natural systems vary across time and space, continuously reshaping the selective pressures acting on populations. This variability can redirect adaptive trajectories, particularly when stressors interact non-linearly. To capture these dynamics, experimental designs must combine ecological realism with multifactorial exposures, thereby providing a more reliable basis for predicting evolutionary outcomes under real-world contamination regimes.

2. Experimental Design Across a Gradient of Complexity

In this thesis, I investigated how contaminant complexity influences both acute and long-term biological responses, using the freshwater midge *C. riparius* as a model organism. The research was conducted in two complementary experimental contexts: the first addressed multigenerational exposure to a single model contaminant (Chapter 1), while the second extended the investigation to both acute and multigenerational exposure to a chemically complex, field-derived matrix (Chapters 2-4).

In Chapter 1, benzo[*a*]pyrene (BaP) was selected as the model contaminant. BaP is a well-characterized polycyclic aromatic hydrocarbon (PAH), known for its genotoxicity, environmental persistence, and occurrence in urban runoff sediments (Baptista Neto et al., 2016; Grung et al., 2016; Rigano et al., 2024). While heavy metals such as cadmium and microplastics have been widely investigated for their mutagenic potential, PAHs and BaP remain comparatively underexplored. The toxicological relevance of BaP, supported by extensive literature, provided a strong basis for assessing concentration-dependent effects and potential adaptive responses under controlled exposure conditions.

Populations of *C. riparius* were exposed across consecutive generations to two concentrations of BaP (10 and 100 µg/L). The lower concentration reflects levels reported in contaminated freshwater environments, thus representing a realistic exposure scenario

(Edwards, 1983). The higher concentration was selected to impose a strong selective pressure, maximising the likelihood of detecting evolutionary responses within the timeframe of the experiment. Findings from this first phase offered valuable insights into the evolutionary consequences of chronic BaP exposure, while also establishing a methodological baseline for interpreting responses to more complex contaminant mixtures.

In the second context, the experimental design was extended to urban runoff sediment, a chemically complex, field-derived matrix (Chapter 2-4). Based on acute test results (Chapter 3), two concentrations (w/w %) were selected for the multigenerational experiments. The lower dose (0.5%) represented environmentally realistic exposure to most contaminants present in the sediment, whereas the higher dose (10%) approached physiological stress thresholds. This gradient enabled the detection of subtle effects at low exposure while capturing pronounced biological responses under elevated stress.

The urban runoff sediment was collected from a retention basin serving the Soers district of Aachen, Germany. This basin receives stormwater runoff from a mixed catchment area that includes heavily trafficked roads and developed land. In such systems, stormwater mobilizes and deposits a wide range of anthropogenic contaminants from surrounding surfaces. The resulting sediment captures the chemical complexity typical of urban pollution, including polycyclic aromatic hydrocarbons (PAHs), tyre and road wear particles (TRWPs), heavy metals, petroleum derivatives, and variable amounts of organic matter (Baptista Neto et al., 2016; Rigano et al., 2024). Acting as a long-term sink for contaminants from multiple sources, urban runoff sediment displays a highly heterogeneous chemical profile. This heterogeneity arises from both spatial variation, related to differences in catchment characteristics and pollutant inputs, and temporal dynamics driven by fluctuating environmental conditions and human activity (Jartun et al., 2008; Rodriguez et al., 2003).

Urban runoff sediments are rarely incorporated into experimental evolution studies, as their chemical complexity and lack of standardisation pose considerable challenges. This complexity therefore also makes them valuable, as it reflects the heterogeneous and dynamic selective pressures organisms face in polluted environments. For this reason, I employed them as an ecologically grounded framework to capture how populations confront the shifting balance of pressures imposed by prolonged contaminant heterogeneity.

3. Evolutionary Responses to Single Compounds and Complex Mixtures

3.1 Influence of Chronic Contaminant Exposure on Mutation Rate and Spectrum

The mutagenic potential of environmental contaminants represents a fundamental dimension of evolutionary ecotoxicology. By altering germline mutation rates, these stressors influence the generation of new genetic variation, which serves as the raw material for natural selection (Eyre-Walker and Keightley, 2007; Loewe and Hill, 2010). Changes in the rate, spectrum, and distribution of mutations within the genome can shape both the pace and direction of evolutionary processes (Carlson et al., 2020). This dynamic has important implications for the adaptive capacity of exposed populations. On one hand, elevated mutation rates may increase the probability of beneficial variants arising, potentially enhancing adaptive responses to environmental challenges. On the other, they can also lead to the accumulation of deleterious mutations, which compromise genomic integrity and reduce overall fitness, especially under prolonged toxic stress (Mehlhoff and Ostermeier, 2023; Sprouffske et al., 2018). The balance between generating adaptive variation and avoiding mutational load is therefore critical, determining whether populations exposed to chronic contamination can evolve resilience or experience long-term decline (Bao et al., 2022; Langie et al., 2015).

The results from my thesis demonstrate that both chronic exposure to BaP and to urban runoff sediments led to significant increases in germline mutation rates. Specifically, exposure to BaP at the highest concentration (100 µg/L) resulted in a modest but statistically significant 20% increase in mutation rate compared to the control (Chapter 1). Similarly, multigenerational exposure to urban runoff sediments resulted in an approximately 50% increase in mutation rate compared to the control, observed at both tested concentrations (0.5% and 10%) (Chapter 2). Extending this comparison beyond chemical exposures, Waldvogel and Pfenninger (2021) showed that temperature can have a pronounced impact on the spontaneous mutation rate of *C. riparius*. In their multigenerational study, exposure to 12 °C and 26 °C increased mutation rates by approximately 180% and 350%, respectively, compared to the minimum observed at 17 °C. These values are substantially greater than those observed in my study for BaP (~20%) and urban runoff sediments (~50%), indicating that chemical stressors, under the tested conditions, exert a comparatively moderate influence on germline mutagenesis.

The modest effect of BaP on germline mutation rate likely reflects several, non-exclusive factors. This compound requires metabolic activation by cytochrome P450 enzymes to form DNA-reactive metabolites (Shiizaki et al., 2017). In *C. riparius*, this activation is assumed to involve insect-specific P450 families such as CYP6/9 and CYP4, which are known

to metabolise xenobiotics (Nair et al., 2013; Nauen et al., 2022, 2021). However, it remains unclear whether germline tissues express these P450 enzymes at levels sufficient to support substantial BaP activation. Limited bioactivation within germ cells, together with rapid detoxification of reactive intermediates and efficient repair of BaP-induced DNA lesions, could therefore restrict its mutagenic impact (Christmann et al., 2016; Wang et al., 2017). In addition, since the laboratory strain originates from a natural population, it is possible that previous environmental exposure to pollutants, including PAHs such as BaP, has contributed to a certain degree of inherent tolerance. This background adaptation could further limit susceptibility to BaP-induced changes in mutation rate (Arambourou et al., 2014; Armitage et al., 1995).

A similar pattern of limited germline response in *C. riparius* was documented in a multigenerational exposure study involving another individual stressor. Doria et al. (2021) reported that exposure to cadmium (32 µg/L) produced no statistically detectable change in mutation rate compared to the control. The authors attributed this outcome to cadmium's mode of action, which predominantly induces chromosomal rearrangements rather than single-nucleotide substitutions in the germline. As with BaP, they also suggested that pre-existing tolerance in *C. riparius*, together with the low effective bioavailability of cadmium under the tested conditions, may further constrain its mutagenic potential.

For urban runoff sediments, the higher but still moderate increase in mutation rate likely reflects the reduced bioavailability of many sediment-associated contaminants, which are often tightly bound to organic matter, limiting their uptake and mutagenic potential (Bräunig et al., 2016; Li et al., 2022). Antagonistic interactions among mixture components may further limit mutagenicity, for instance by reducing the metabolic activation of certain compounds or lowering their effective bioavailability (Martin et al., 2021). Consistently with the patterns observed for single stressors, a background level of tolerance in *C. riparius*, shaped by repeated exposure to polluted habitats, may further restrict the potential for additional mutagenic effects.

In contrast, temperature acts as a systemic driver of mutation rate, influencing all germline cells across the life cycle. Higher temperatures accelerate metabolism and shorten generation times (Clarke and Fraser, 2004; Gillooly et al., 2001), which can increase oxidative stress and overwhelm DNA repair systems (Berger et al., 2017). Conversely, lower temperatures are also associated with elevated oxidative stress and reduced efficiency of repair mechanisms (Lalouette et al., 2011; Park and Kwak, 2014). Thus, deviations in either direction from the thermal optimum increase the likelihood that replication errors persist (Chu et al., 2018; Waldvogel and Pfenninger, 2021), leading to substantially greater rises in mutation rate than those observed under chronic chemical exposure.

Taken together, my findings show that long-term pollutant exposure consistently induces mutagenesis, and that its magnitude can be reliably quantified through the multigenerational framework used in this study. However, this effect can be constrained by processes such as limited bioavailability, antagonistic interactions within mixtures, and pre-existing tolerance in exposed populations. Consequently, even in the presence of multiple contaminants, increases in mutation rate may plateau rather than scale proportionally with chemical complexity.

3.2 Divergent Evolutionary Trajectories under Different Contaminant Exposure Scenarios

Although both BaP and urban runoff sediments induced significant increases in germline mutation rates in *C. riparius*, their evolutionary trajectories diverged markedly. At the highest concentration of BaP, the mutagenic effect did not translate into detectable phenotypic adaptation within the three-generation timeframe (Chapter 1). These findings contrast with the theoretical expectation that a single, constant stressor in a simplified selective environment should facilitate adaptation by imposing consistent directional selection (Stephan, 2016; Stetter et al., 2018). In this regime, advantageous alleles from standing genetic variation or de novo mutation are expected to increase in frequency and produce measurable phenotypic change (Matuszewski et al., 2015; Stetter et al., 2018; Tellier et al., 2024).

Findings from other systems are consistent with this expectation. Indeed, Merlo et al. (2020) conducted a multigenerational evolution experiment to investigate how populations adapt under different selective regimes. *Saccharomyces cerevisiae* populations evolved for approximately 500 generations under a single limiting nutrient (glucose, histidine, or uracil) or under the simultaneous limitation of all three. Populations in single-stressor environments achieved clear and statistically significant fitness gains in their respective conditions. By contrast, populations evolved under all three simultaneous limitations did not show significant improvement in any individual nutrient-limited condition and displayed only slight fitness gains in the combined environment. A similar pattern emerges in natural environments, as shown by Shahid et al. (2024). In their population-level investigation, they found that the amphipod crustacean *Gammarus pulex* from pesticide-impacted streams exhibited markedly greater tolerance to the neonicotinoid clothianidin compared with those from uncontaminated reference sites. However, when additional stressors were introduced, specifically increased warming and the fungicide prochloraz, this adaptive advantage was reduced or even lost. Together, these findings show that while adaptation to isolated stressors is often feasible, adaptation to co-occurring stressors is considerably more challenging. Moreover, interactions

among stressors can severely limit or even erase the benefits gained under single-stressor conditions, thereby constraining evolutionary potential.

Even for single stressors, however, adaptation is not straightforward, as it depends on the supply of beneficial variants and is frequently constrained by demographic and physiological costs. At the highest BaP concentration (Chapter 1), the mutagenic effect observed was modest, likely producing only a small number of potentially advantageous alleles. This occurred in a context where the compound's intrinsic toxicity significantly reduced survival, fertility, and population growth, thereby lowering the effective population size (N_e) (Amorim et al., 2017; Loewe and Hill, 2010). Consequently, a smaller N_e makes populations more prone to genetic erosion and increases the influence of drift, potentially masking or even overriding selection (Lynch et al., 2016; Star and Spencer, 2013). This pattern is consistent with findings by Pedrosa et al. (2017). In their study, they showed that *C. riparius* populations with higher genetic diversity performed better in life-cycle assays under mercury and salinity stress. In contrast, genetically impoverished populations exhibited reduced performance, underscoring the key role of genetic diversity in mediating susceptibility to environmental stress.

Furthermore, the short, three-generation timeframe may have been insufficient for beneficial alleles to increase in frequency and produce detectable phenotypic change. Adaptive evolution often requires longer timescales, particularly when reliant on new mutations, because selection must act on variants initially present at low frequency (Perrier and Charmantier, 2019). Finally, metabolic costs of detoxification and DNA repair may have diverted resources from growth and reproduction, reducing the potential for detectable phenotypic adaptation over such a short timescale.

This lack of adaptation to BaP aligns with evidence from multigenerational Cd exposure in *C. riparius*. In a five-generation experiment, Doria and Pfenninger (2021) showed that increasing Cd levels reduced emergence, slowed development, and lowered fertility. At the highest concentration, the population growth rate (PGR) fell below the replacement threshold, and no adaptation was detected, consistent with the demographic constraints described above.

In contrast, exposure to urban runoff sediment led to a markedly different evolutionary outcome. Populations exposed to the higher sediment concentration showed clear signs of phenotypic recovery beginning as early as the third generation (Chapter 4). Interestingly, similar patterns also emerged in control populations, indicating that selection pressures inherent to the laboratory environment also shaped evolutionary trajectories. Genome-wide

analyses revealed consistent allele frequency shifts from the third generation onward, affecting loci involved in membrane transport, lipid metabolism, proteolysis, and regulation of gene expression. The broad genomic distribution and modest effect sizes are consistent with polygenic adaptation, potentially involving selection distributed across multiple traits that together may enhance fitness under chemically diverse and variable conditions.

Comparable genome-wide responses were reported by Khosrovyan et al. (2022). In their seven-generation evolve-and-resequencing experiment, *C. riparius* populations were chronically exposed to polyamide microplastics. The populations exhibited rapid, genome-wide allele-frequency shifts within only a few generations, consistent with polygenic adaptation, and overcame initial fitness losses.

These findings are not restricted to controlled laboratory conditions, as multigenerational recovery dynamics were also observed in more complex ecological setting. For instance, Zhang et al. (2022) investigated the multigenerational effects of long-term metal contamination on the collembolan *Folsomia candida*. Populations were exposed to nine naturally polluted agricultural soils differing in physicochemical properties (acidic, neutral, and calcareous) and in levels of combined metal pollution, mainly cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn). In the acidic soils, collembolans initially showed reduced reproduction, slower juvenile growth, and elevated body burdens of Cd and Pb. Over five generations, however, they gradually recovered reproductive output and juvenile length while simultaneously lowering Cd and Pb accumulation in their bodies.

Similar patterns have also been documented in natural populations exposed to multiple anthropogenic stressors. Briski et al. (2025) provided evidence that adaptation in urbanised populations can involve tolerance to multiple concurrent stressors. In 30-day factorial assays, *Mytilus mussels* and two gammarid species (*G. locusta* and *G. salinus*) from urban sites were exposed to combinations of elevated temperature, altered salinity, and high pCO_2. Compared with conspecifics from a nearby protected reserve, urban populations coped better, showing higher survival and delayed mortality. Furthermore, robustness scaled with disturbance intensity, with the most impacted sites producing the most tolerant populations, consistent with adaptation along anthropogenic stress gradients. This tolerance likely reflects a combination of rapid adaptation and phenotypic plasticity under chronic urban stress, potentially reinforced by cross-tolerance, whereby adjustment to one disturbance broadens resistance to others.

Overall, these results highlight that adaptive responses to environmental contamination arise from the interplay of standing genetic variation, physiological constraints, and ecological context. Mutation supply, selection strength and direction, demographic stability, and the

temporal scale of exposure jointly shape the course of adaptation. Under multiple concurrent stressors, broad resistance can evolve through cross-tolerance, whereby responses to one stressor enhance resistance to others. However, such outcomes are not universal, as interactions among stressors may constrain adaptation and even erase advantages observed under single-stressor regimes. While isolated toxicants often drive narrow, trait-specific responses, chemically complex mixtures are more likely to impose diffuse, multidimensional pressures that can favour polygenic responses, with subtle allele-frequency shifts across many loci contributing to long-term resilience.

4. *Chironomus riparius* as a Model of Rapid Adaptation

The findings presented here must be interpreted in the context of the biological characteristics of the model species employed throughout this thesis. *C. riparius*'s fast developmental cycle, high reproductive output, and considerable standing genetic variation provided favourable conditions for the emergence of both phenotypic and genomic changes within a limited number of generations (Armitage et al., 1995; Foucault et al., 2019; Pedrosa et al., 2017). These life-history traits make *C. riparius* particularly well-suited for multigenerational studies by increasing the likelihood of detecting early adaptive responses to environmental stress (Foucault et al., 2019).

However, this evolutionary potential is not equally shared across taxa. Many species possess slower life histories or reduced genetic diversity, and ecological and demographic factors such as N_e or dispersal can further constrain their capacity to respond adaptively to comparable contaminant pressures. In such cases, the probability of evolutionary rescue may be reduced, and in some cases effectively absent, increasing the risk of long-term decline under chronic pollution. These interspecific differences highlight the fundamental role of species-specific biology in shaping eco-evolutionary trajectories.

As such, while *C. riparius* provides a powerful model for investigating contaminant-driven evolution, its responses may not capture those of more vulnerable organisms. To strengthen evolutionary ecotoxicology, integrating a wider range of taxa spanning diverse niches and exposure pathways will be essential, thereby improving our understanding of species-specific vulnerabilities and enhancing the predictive scope of population-level risk assessments.

5. Reframing Ecological Risk Assessment (ERA): Accounting for Evolution and Contaminant Complexity

Ecological risk assessment (ERA) frameworks are still grounded in standardized short-term toxicity tests, such as OECD (2010) and OECD (2023) or USEPA (1998). In these protocols, model organisms are exposed to individual chemicals under controlled conditions, and endpoints like survival, growth, development and reproduction are measured. By ensuring reproducibility and comparability across laboratories, they have become the foundation of regulatory thresholds and hazard classifications and remain central to environmental decision-making.

However, the standardized design of these assays inevitably restricts the range of processes they capture. Their short duration and simplified conditions overlook key physiological, ecological, and evolutionary processes essential for long-term resilience, and fail to reflect the complexity of natural ecosystems where contaminant mixtures interact with fluctuating abiotic and biotic conditions (Brady et al., 2017; Panizzi et al., 2017; Rodríguez-Romero et al., 2021; Segner, 2007). Consequently, ERA frameworks risk underestimating not only the capacity of populations to adapt, but also the costs of adaptation, including genetic erosion, fitness trade-offs and altered ecological interactions. Overlooking these dynamics weakens the predictive value of risk assessments for long-term population persistence and threatens ecosystem stability under chronic contamination.

The results of this thesis provide empirical support for the need to incorporate evolutionary processes into the ERA framework, while accounting for adequate temporal depth. In BaP exposure experiments at the highest concentration, mutation rates increased significantly, but adaptive phenotypic changes remained undetectable within the first three generations (Chapter 1). By contrast, at the highest concentration of urban runoff sediments, genome-wide allele frequency shifts became clearly distinguishable from genetic drift after three generations, coinciding with coordinated life-history adjustments indicative of rapid polygenic adaptation (Chapter 4). These findings suggest that even multigenerational assays, when limited in duration, may not be sufficient to detect whether early evolutionary dynamics are taking shape.

Addressing these limitations requires both conceptual and methodological advances. Multigenerational testing should be incorporated as a standard element of risk assessment, particularly for chemicals known to be mutagenic, endocrine-disrupting, or bioaccumulative, ideally extending beyond five generations in fast-reproducing models to encompass key

evolutionary processes. In parallel, molecular and genomic tools such as whole-genome resequencing, transcriptomics and epigenetic profiling represent powerful approaches to enhance mechanistic resolution, allowing assessment of whether populations are adapting or declining, with direct implications for persistence and extinction risk.

Finally, risk assessment must better reflect the chemical complexity of real environments. Incorporating mixture-based experimental designs, as shown in this thesis, is crucial for producing more realistic evaluations. Recognising contaminants as agents of evolutionary change broadens the scope of ERA, as their effects extend beyond immediate toxicity to reshape genetic diversity, population dynamics, and ultimately ecosystem stability. Acknowledging these evolutionary consequences is essential not only for biodiversity conservation but also for the long-term maintenance of ecosystem services that support human societies.

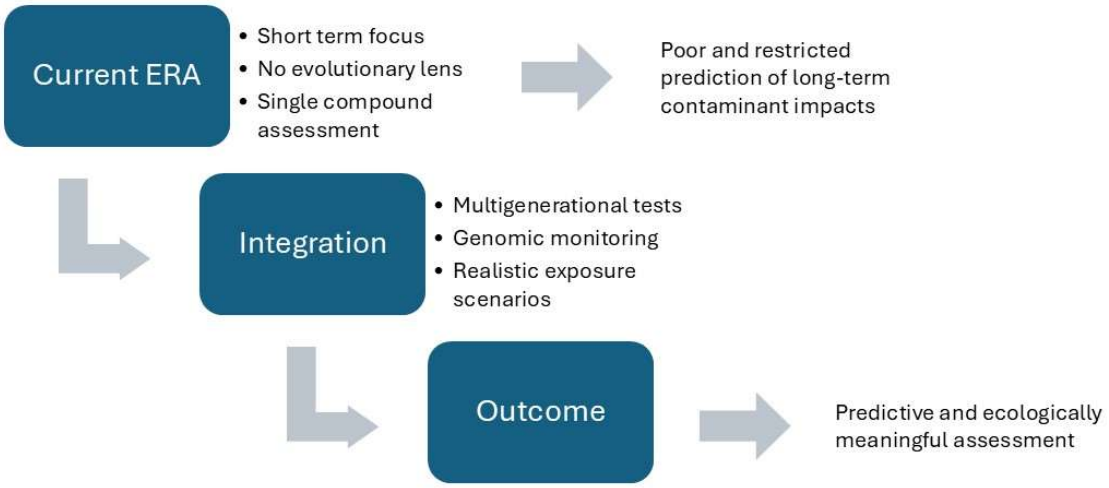

Figure 3. Schematic overview of the proposed transition.

From the current limitations of ERA to integrated approaches leading to predictive and ecologically meaningful risk assessment.

CONCLUSION

In conclusion, rethinking ecological risk assessment through an evolutionary lens is not a minor refinement but a scientific necessity. Populations are dynamic systems that adapt to persistent stressors, and, overlooking this, weakens our ability to anticipate long-term ecological outcomes. In this thesis I investigated how chronic contaminant exposure shapes evolutionary trajectories in *Chironomus riparius*, showing that benzo[a]pyrene increased mutation rates without evidence of adaptation, whereas urban runoff sediments induced both elevated mutagenesis and rapid polygenic adaptation. These results highlight that evolutionary responses to pollution depend on the nature of the stressor and the conditions under which populations are exposed. Exposure to urban runoff sediment over a single generation reinforced this perspective, highlighting physiological trade-offs and underscoring the inherent complexity of contaminant effects.

A more predictive ecotoxicological framework therefore requires multigenerational experiments, the integration of molecular and genomic tools to detect early signals of adaptation or decline, and exposure scenarios that better reflect chemical mixtures in nature. Embedding evolution and environmental realism into ecological risk assessment will provide a stronger basis for predicting whether species resist, persist, or decline under Anthropocene pressures, while safeguarding biodiversity and the ecosystem services on which human societies depend.

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