Universidade de Aveiro
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Transmissão geracional de alterações pro- e anti-genotóxicas induzidas por pesticidas

Generational transmission of pro- /anti-genotoxic alterations induced by pesticides

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Associado com agregação do Departamento de Biologia da Universidade de Aveiro e sob a coorientação científica da Doutora Sofia Isabel Antunes Gomes Guilherme, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro

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Aos meus pais e ao Carlos

o júri

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palavras-chave Pesticidas; Genotoxicidade; Metilação; Intergeracional

Resumo

O dano no ADN pode resultar em mutações, causando transformações malignas (em células somáticas) e/ou com potencial para originar alterações herdáveis (se ocorrer em gametas). Portanto, o impacto real dos pesticidas, através da genotoxicidade, só pode ser rigorosamente avaliado se for alcançado o entendimento da forma como uma exposição parental se traduz na prole, prevendo a repercussão na mesma. Nesse sentido, há a necessidade de perceber a capacidade de reversão do dano após a cessação da exposição, possibilitando uma melhor previsão das consequências, tanto na geração presente como nas subsequentes. Para além disso, é fundamental esclarecer em que medida o impacto dos pesticidas pode ser modulado pelo estado fisiológico do organismo, assim como, pelas condições ambientais.

Os níveis de pesticidas nos sistemas aquáticos aumentaram em todo o mundo devido ao seu uso intensivo e/ou indevido, nas práticas agrícolas. Muitos dos componentes das formulações comerciais de pesticidas são estáveis ao longo do tempo e podem ser transportados na água e no ar para áreas distantes de sua fonte de emissão. Consequentemente, o uso destes agentes tem afetado diversos ecossistemas, ameaçando organismos não-alvo. Em particular, o biota aquático pode ser exposto a agrotóxicos, gerando riscos a diversos níveis, com destaque para a instabilidade no ADN. Para além do impacto na integridade do ADN, vários estudos mostraram que os pesticidas podem ter um impacto a nível epigenético. Sabe-se que as alterações epigenéticas podem ser transmitidas por mitose e meiose, o que significa que essas modificações podem ser mantidas ao longo da vida do organismo e transpostas para a geração seguinte. Além disso, uma vez que uma dada geração pode ser afetada por múltiplas exposições a pesticidas, o conhecimento sobre as alterações induzidas, bem como a sua transmissão intergeracional, é crucial para entender os fenómenos de vulnerabilidade ou resistência adquiridos, como elementos-chave do impacto real na população.

O *Procambarus clarkii*, também conhecido como o lagostim vermelho do Louisiana, é uma espécie nativa do sul dos Estados Unidos e nordeste do México, embora, atualmente, possa ser encontrado em águas fluviais em todos os continentes, exceto na Austrália e na Antártida. O lagostim vermelho é visto como um paradigma de sucesso devido à sua plasticidade ecológica, apresentando alta resistência em condições adversas (por exemplo, cenários de contaminação por pesticidas). Assim, e considerando a sua ampla distribuição, este lagostim foi selecionado como “organismo-teste” para a realização do presente estudo ecogenotoxicológico.

Partindo dos anteriores pressupostos, este trabalho, dividido em 2 fases, teve como objetivo na 1ª fase (i) avaliar o potencial para induzir dano no ADN dos herbicidas glifosato e penoxsulame, dos inseticidas dimetoato e imidaclopride e dos fungicidas pirimetanil e imazalil, numa exposição de curto prazo. A 2ª fase teve como objetivos: (ii) avaliar a genotoxicidade do penoxsulame a longo prazo; (iii) aumentar o conhecimento sobre os efeitos das exposições parentais, considerando a integridade do metiloma e do ADN na prole; (iv) contribuir para a implementação do conceito de pesticidovigilância.

Na 1ª fase, começou-se por avaliar o potencial espermiotóxico dos pesticidas acima mencionados, no curto prazo, em concentrações ambientalmente relevantes (ou seja, glifosato 9 e 90 µg L^{-1}, penoxsulame 2,3 e 23 µg L^{-1}, dimetoato 2,4 e 24 µg L^{-1}, imidaclopride 13,1 e 131 µg L^{-1}, pirimetanil 2,2 e 22 µg L^{-1} e imazalil 16 e 160 µg L^{-1}), através de uma abordagem *ex vivo*. A integridade do ADN nos espermatozoides foi afetada pelas concentrações mais elevadas de glifosato, pirimetanil e imazalil, bem como por ambas as concentrações de penoxsulame e dimetoato. O imidaclopride foi o único pesticida (inseticida) que apresentou propriedades pró-oxidantes, apesar da ausência de danos inespecíficos no ADN. Esta (primeira) fase também demonstrou a adequabilidade da abordagem *ex vivo* para triagem de espermiotoxicidade, destacando o impacto de pesticidas em espécies não-alvo, como *P. clarkii*, devido ao papel crítico da integridade do ADN dos espermatozoides no sucesso da população. Como o penoxsulame mostrou ser o pesticida mais espermiotóxico, foi então o eleito para os testes subsequentes a realizar na 2ª fase.

Assumindo que os efeitos nocivos dos pesticidas no ambiente podem ser estendidos além da escala temporal da exposição, a 2ª fase desta investigação começou com a avaliação da progressão do dano genético após uma exposição (7 dias) ao penoxsulame (Px: 23 µg L^{-1}) e um após o período de pós-exposição (70 dias), considerando células somáticas e espermatozoides do lagostim. A mesma abordagem foi aplicada ao genotóxico-modelo metanosulfonato de etilo (EMS; 5 mg L^{-1}), como via complementar para melhorar o conhecimento sobre a dinâmica da genotoxicidade (indução de dano no ADN *vs.* recuperação). Os resultados desta etapa demonstraram a genotoxicidade do Px em todas as células/órgãos testados (*i.e.*, brânquias, hepatopâncreas e espermatozoides), mostrando também especificidades celulares e de género, com as células branquiais mostrando-se mais vulneráveis nas fêmeas, enquanto os machos demonstraram maior suscetibilidade no que concerne a células/órgãos internos (*i.e.*, hepatopâncreas e espermatozoides). Em relação à ação do genotóxico-modelo, os lagostins foram incapazes de recuperar do dano induzido no ADN pelo EMS nas brânquias e no hepatopâncreas (em ambos os géneros), bem como nos espermatozoides. Os gametas masculinos mostraram ser o tipo celular mais vulnerável, enquanto o dano no ADN do hepatopâncreas só foi percetível após o período pós-exposição. Assim, ficou claro que a caracterização do risco genotóxico de um determinado agente deve integrar um conjunto de informações, abordando diferentes tipos de danos no ADN, especificidades de células/órgãos e género, bem como uma avaliação de longo prazo da progressão temporal do dano.

A hipótese de que o dano a longo prazo causado pelo Px vai além da geração exposta foi testada através da observação de alterações na metilação e na integridade do ADN da prole, após uma exposição parental. Portanto, considerando um contexto intergeracional e o potencial genotóxico do Px, seguiram-se duas etapas (dentro da fase 2, supracitada). A primeira, consistiu na procura do entendimento da ação do Px sobre o metiloma de células musculares esqueléticas em duas gerações de lagostins.

Assim, os organismos de F_0 e da sua descendência (F_1) foram expostos a Px (23 µg L^{-1}) e a EMS (5 mg L^{-1}). Os indivíduos adultos (F_0) não apresentaram alterações no metiloma após a exposição ao herbicida. No entanto, a hipometilação que se observou nos juvenis da geração F_1 não expostos (com exposição parental a Px) demonstrou que o histórico de exposição, *per se*, pode modular o epigenoma, apontando um efeito epigenético intergeracional. Nos descendentes F_1 do grupo exposto a Px, a hipermetilação foi mais pronunciada nos machos do que nas fêmeas. Além disso, o EMS induziu hipometilação em fêmeas adultas (F_0), destacando uma especificidade de género. O papel modulador de exposições passadas (parentais) em relação aos efeitos do penoxsulame ou EMS mostrou também depender do estágio de desenvolvimento da prole. Estes resultados provaram que uma influência indireta (ou seja, eventos de exposição ocorridos na geração anterior) pode ter um impacto maior na dinâmica epigenética do que exposições diretas. A segunda etapa incluiu exposições indiretas, adotando uma abordagem *ex vivo*, onde a integridade do ADN de espermatozoides dos indivíduos da geração F_1 foi avaliada após uma exposição a Px ou EMS, com e sem a influência de uma exposição parental (F_0), aos mesmos compostos. A exposição parental, isoladamente, não afetou a integridade do ADN dos espermatozoides da geração F_1 (não expostos). No entanto, o histórico de exposição a Px aumentou a vulnerabilidade a lesões oxidativas de ADN na prole exposta ao mesmo agente. Os descendentes da geração exposta a EMS pareceram desenvolver mecanismos de proteção do ADN expressos quando também foram expostos a esse desafio genotóxico específico, revelando traços protetores advindos da história parental, revelando-a como um escudo toxicológico. O potencial espermiotóxico do Px só foi observado com um histórico de exposição parental a EMS, revelando, desta vez, a história de vida (exposição a Px) como uma "sombra" toxicológica (características negativas) para a descendência.

Globalmente, os presentes resultados demonstraram que as recomendações de procedimentos regulatórios para proteção do ambiente aquático devem ser aprimoradas. Portanto, a presente investigação reforça a importância de uma avaliação (epi)genotóxica (combinando dois biomarcadores, a integridade do ADN e a metilação do ADN) abrangendo várias gerações. Além disso, no quadro da autorização de comercialização de pesticidas, deve-se considerar uma avaliação contínua desses agentes para determinar seu real impacto ambiental, o que pode ser considerado como um apelo à criação do conceito pesticidovigilância.

keywords Pesticides; Genotoxicity; DNA methylation; Intergenerational

abstract

The DNA damage may result in mutations, leading to malignant transformation (in somatic cells) and/or having the potential to cause altered heritable traits (if it occurs in gametes). Therefore, the real impact of agrochemicals through genotoxicity can be thoroughly assessed only if the understanding on how a parental exposure is translated into the offspring is achieved, predicting the repercussion on the prole. In the same direction, there is a need to unveil the ability of damage reversion after the exposure cessation, enabling a better forecast of the consequences both at the present and subsequent generations. In addition, it is critical to clarify in what extent this potential impact of agrochemicals can be modulated by the organism physiological state and surrounding environmental conditions.

The levels of pesticides in water have increased worldwide due to their intensive use and/or misuse in the modern agricultural domain. Many components of pesticide formulations are stable over time and can be transported in water and air far from their point source. Consequently, the use of these agents has affected several ecosystems, threatening non-target organisms. In particular, aquatic biota can be exposed to waterborne pesticides, generating health risks at several levels, with emphasis on genomic instability. In addition to the impact on DNA integrity, several studies have shown that pesticides can have an epigenotoxic impact. It is known that epigenetic changes can be transmitted by mitosis and meiosis, which means that these modifications can be maintained throughout the organism's life and transported to the next generation. Moreover, since each generation might be affected by multiple exposures to pesticides, the knowledge about the induced alterations, as well as their intergenerational transmission, is crucial to understand the phenomena of acquired vulnerability or resistance as key determinants to the real impact on the population.

Procambarus clarkii, the red swamp crayfish, is a native species to the southern United States and north-eastern Mexico, though, nowadays, can be found in inland waters on all continents except Australia and Antarctica. This crayfish is seen as a paradigm of success due to its ecological plasticity, showing high resistance in adverse conditions (*e.g.,* pesticide contamination scenarios). Thus, and considering its wide distribution, this crayfish was selected as a “tool organism” to perform the current ecogenotoxicological study. Bearing all this in mind, this biphasic work aimed (i) to assess the DNA damaging potential of the herbicides glyphosate and penoxsulam, the insecticides dimethoate and imidacloprid, and the fungicides pyrimethanil and imazalil (Phase 1).

In a 2nd phase, it was aimed: (ii) to evaluate the penoxsulam long-term genotoxicity; (iii) to increase the knowledge concerning the effects of parental exposures on offspring, considering methylome and DNA integrity; (iv) to contribute to the implementation of the pesticidovigilance concept. Within phase 1, it was first assessed the short-term (7 days) spermiotoxic potential of the above-mentioned pesticides, at environmentally relevant concentrations (*i.e.*, glyphosate at 9 and 90 µg L^{-1}, penoxsulam at 2.3 and 23 µg L^{-1}, dimethoate at 2.4 and 24 µg L^{-1}, imidacloprid at 13.1 and 131 µg L^{-1}, pyrimethanil at 2.2 and 22 µg L^{-1}, and imazalil at 16 and 160 µg L^{-1}), through an *ex vivo* approach. Sperm DNA integrity was affected by the higher concentrations of glyphosate, pyrimethanil and imazalil, as well as by both concentrations of penoxsulam and dimethoate. Imidacloprid was the only pesticide (insecticide) displaying pro-oxidant properties, despite the absence of non-specific DNA damage. This (first) phase also demonstrated the suitability of the *ex vivo* approach to spermiotoxicity screening, highlighting the impact of pesticides on non-target species, such as *P. clarkii*, due to the critical role of sperm DNA integrity on the population's success. Since penoxsulam showed to be the most spermiotoxic pesticide, it was then elected to perform phase 2.

Assuming that harmful effects of environmental noxious compounds can be extended beyond the exposure time scale, phase 2 began with the evaluation of the genetic damage progression following an exposure (7 days) to penoxsulam (Px: 23 µg L^{-1}) and a post-exposure period (70 days), considering crayfish somatic and germ cells. The same approach was applied to the model genotoxicant ethyl methanesulfonate (EMS; 5 mg L^{-1}), as a complementary path to improve the knowledge concerning the genotoxicity dynamics (DNA damage induction *vs.* recovery). The outcomes of this stage of work pointed out Px genotoxicity in all cells/organs tested (*i.e.*, gills, hepatopancreas and spermatozoa), also disclosing cells- and gender-specificities, with gill cells showing to be more vulnerable in females, while males demonstrated higher susceptibility when internal cells/organs (*i.e.*, hepatopancreas and spermatozoa) were considered. Regarding the model genotoxicant, crayfish were unable to recover from the DNA damage induced by EMS in gills and hepatopancreas (both genders), as well as in spermatozoa. Male gametes proved to be the most vulnerable cell type, while DNA damage in hepatopancreas was only perceptible after the post-exposure period. Thus, it became clear that the characterization of the genotoxic hazard of a given agent must integrate a complete set of information, addressing different types of DNA damage, cell/organ- and gender-specificities, as well as a long-term appraisal of the temporal progression of damage.

The supposition that the long-term damage caused by Px went beyond the exposed generation was confirmed by the effects on methylome and sperm DNA currently reported in the prole, following a parental exposure. Therefore, considering an intergenerational context and the demonstration of the Px genotoxic potential, two steps followed (within the above-mentioned phase 2). The first one consisted in the understanding of the action of Px on methylome of skeletal muscle cells in two crayfish generations.

Thus, F_0 and its progeny (F_1) were exposed to Px (23 µg L^{-1}) and to EMS (5 mg L^{-1}). Adult crayfish (F_0) didn't present alterations in the methylome following the herbicide exposure. However, the hypomethylation occurring in unexposed F_1 juveniles (with parental exposure to Px) demonstrated that the history of exposure, *per se*, can modulate the epigenome, pointing out an intergenerational epigenetic effect. In F_1 descendants of the Px-exposed group, hypermethylation was more pronounced in males than females. Moreover, EMS induced hypomethylation in adult females (F_0), highlighting a gender-specificity.

The modulatory role of past (parental) exposures to penoxsulam or EMS showed also to depend on the offspring developmental stage. These outcomes proved that an indirect influence (*i.e.*, exposure events occurring in the preceding generation) can have a higher impact on epigenetic dynamics than direct exposures. The second step included indirect exposures, adopting an *ex vivo* approach, where the DNA integrity of F_1 spermatozoa was evaluated following a Px or EMS exposure, with and without the influence of a parental exposure (F_0) to the same compounds. The parental exposure, alone, did not affect the DNA integrity of F_1 spermatozoa (unexposed). However, the historical of a Px exposure increased the vulnerability to oxidative DNA lesions in the Px-exposed offspring. The descendants from the generation exposed to EMS seemed to develop DNA protection mechanisms expressed when they were also exposed to this specific genotoxic challenge, unveiling protective traits arising from the parental history, disclosing it as a toxicological shield. The spermiotoxic potential of Px was only observed with a parental exposure background to EMS, disclosing, this time, life history (Px-exposure) as a toxicological “shadow” (negative traits) to progeny.

Overall, the present findings demonstrated that the recommendations of regulatory procedures for aquatic environment protection must be improved. Therefore, the current research pointed out the importance of performing an (epi)genotoxic evaluation (combining two useful biomarkers, namely DNA integrity and DNA methylation) encompassing several generations. Moreover, during pesticide marketing authorization, it should be considered a continuous evaluation of these agents in order to determine their real environmental impact, which can be regarded as a call for pesticidovigilance.

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Chapter I

General Introduction

1. General introduction

1.1 Genome and epigenome analysis in ecogenotoxicology

In ecotoxicology it is important to comprehend the molecular mechanisms that underlying the response of organisms to different chemical and physical environmental stressors (Šrut, 2021). It has been shown that the exposure to environmental pollutants can induce DNA damage (Barranger et al., 2016; Guilherme et al., 2015; Marques et al., 2014) and alter the epigenome, namely the DNA methylation (Rondon et al., 2017; Yauk et al., 2008). Some authors pointed out to the complex interaction between DNA damage and DNA methylation, since it has been shown that the presence of DNA lesions could interfere with DNMT activity (Akcha et al., 2021; Yauk et al., 2008). Even after the initial stressor has been removed, environmental-induced epigenetic alterations can last for a long time and even be passed on to following generations (Mirbahai and Chipman, 2014). Thus, if chemical exposure to one generation can have effects on multiple subsequent non-exposed generations, the risk assessment of these chemicals should incorporate this time interval between effects and exposure in previous generations (Vandegehuchte and Janssen, 2014). Considering this, DNA damage and DNA methylation parameters present highly promising tools as sensitive and predictive biomarkers of environmental exposure in the field of ecotoxicology.

DNA damage

The genome is the central base, where the organism cell's information is encoded by the genes. The genome's integrity is vital for every living being and interferes with the organism' biological levels, *i.e.,* from gene to protein, cell to tissue, and then to organism, population, and, at the top, ecosystem. A single change in the DNA molecule can induce major biological effects, such as interrupting normal cell functions, and even resulting in cell death. For instance, mutations in some genes are associated with later stages of progression of some types of tumors, and the formation of DNA adducts (products of the

covalent reaction of electrophilic molecules with DNA) may be a beginning of the process of carcinogenesis (Phillips and Arlt, 2009). At the level of the germline cell, severe implications not only for the individual but also for the entire population since it may cause reproductive impairment, teratogenesis and, very importantly, tumorigenesis (Martins and Costa, 2017).

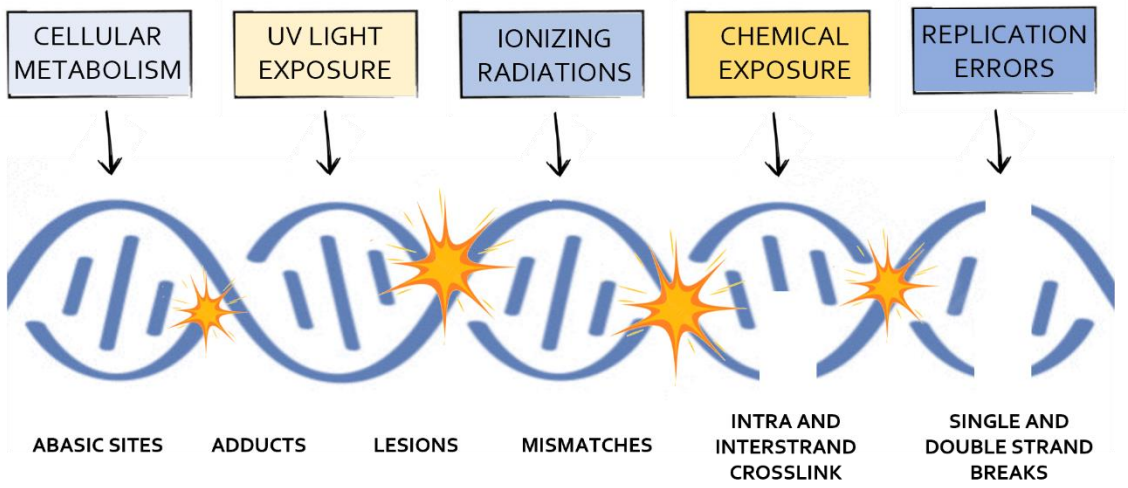

Fig. 1. Schematic representation of genotoxic sources and DNA damage (based on Chatterjee and Walker, 2017).

Genotoxicants may induce on the genome point mutations, deletions, insertions, gene amplifications, chromosomal rearrangements, or numerical chromosomal changes (aneuploidy) (Phillips and Arlt, 2009). In the field of ecotoxicology, the evaluation of the genotoxicants potential presents in the environment has become an essential tool, aiming to assess the actual risk to organisms, as well as to help in the development of protection and mitigation regulatory strategies against these compounds. According to the EU, risk assessment of carcinogens includes hazard identification and characterization (dose-response), and exposure assessment (*e.g.,* intensity, frequency, pathway and duration of the exposure). These pillars are then merged into the assessment of potential risks for cancer induction in exposed populations (European Commission, 2009).

DNA damage caused by genotoxic promoters can take numerous forms (Fig. 1), and it can result in:

(i) breaks in the sugar-phosphate backbone of the molecule, either in one strand (single strand break; SSB) or in both strands of the double helix (double-strand breaks; DSB);

(ii) covalent binding of the genotoxicant;

(iii) the most abundant oxidation lesion in DNA, the 8-oxo-dGuo; it can be formed by free radical attack on DNA or through normal aerobic metabolism (Phillips and Arlt, 2009).

To assess genotoxicity, the occurrence of DNA-adducts, chromosomal aberrations, sister chromatid exchanges, and micronuclei frequency could be used as biomarkers (EC, 2009b). Most genotoxicants do not react directly to DNA, but may be metabolically activated, inside the cells, to produce reactive intermediates and, some of them, can also induce oxidative DNA damage (Phillips and Arlt, 2009). In reaction to the DNA damage, vertebrate organisms have developed DNA repair mechanisms such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ), which are active throughout different stages of the cell cycle, allowing the cells to repair the DNA injuries (Chatterjee and Walker, 2017). DNA repair mechanisms (*e.g.*, BER) were also reported in invertebrates (Braga et al., 2021; Pruski and Dixon, 2002). Some genotoxic agents may interfere with the DNA repair ability, and persistent genotoxic damage depends upon the balance between repair and replacement of damaged cells (El-Bibany et al., 2014). In fact, failures in antioxidant defense and in DNA repair may increase base oxidation and DNA strand breaks (Azqueta et al., 2009). As mentioned above, studies on aquatic invertebrates have shown that DNA damage can lead to genotoxicity, mutation, cell death, and carcinogenesis, with long-term consequences of which may include embryonal aberrations (Barranger et al., 2014) reduced hatching rates, gamete development, and reduced fitness (Lewis and Galloway, 2009).

Alkaline comet assay as a methodology to assess DNA damage

The DNA damage assessment provide an early warning signal of genotoxic exposure (Rybakovas et al., 2009). As shown in Fig. 1, there are several forms of damage and for each type of harm there is one or more techniques to detect and evaluate them (*e.g.*, Figueroa-González and Pérez-Plasencia, 2017). One of the most common techniques to measure DNA strand breaks (single or double) in eukaryotic cells is the alkaline comet assay (Collins, 2004). This method was developed in 1984 by Ostling and Johanson (1984), when they demonstrated the migration of DNA fragments from nuclei under a neutral condition (used to detect double-strand DNA breaks). Later, Singh et al. (1988) showed that an alkaline condition substantially increased the specificity and reproducibility of the assay. The alkaline comet assay (Fig. 2) is considered sensitive, inexpensive, and fast, having been used in genotoxicity testing for *in vitro* screening of innovative cosmetics, medications, and chemicals, for many years (Azqueta and Collins, 2014). Also, is one of the most commonly used methods in ecotoxicology since it can easily quantify small amounts of DNA damage (Azqueta and Collins, 2014; Collins, 2004; de Lapuente et al., 2015, Tice et al., 2000). It is also used to detect UV-induced pyrimidine dimers, oxidized bases and alkylation damage following the introduction of lesion-specific endonucleases (Collins and Azqueta, 2012). Moreover, this assay can be applied to a single-cell suspension of material from any animal tissue (Phillips and Arlt, 2009).

The comet assay require preparations with dissociated cells, either cells already in suspension (*e.g.* spermatozoon) or cells previously separated from the tissues (*e.g.* gills and hepatopancreas cells) (Sahlmann et al., 2017). Cells with high DNA damage display increased migration of the genetic material from the nucleus resembling the shape of comet-like structures (Fig. 3). The level of damage is measured by the stained DNA tail intensity (Azqueta et al., 2009; Phillips and Arlt, 2009). As mentioned, a simple alteration to the original protocol allows the comet assay to detect specific DNA damage, *e.g.,* oxidative DNA damage, for instance the lesion-specific bacterial repair enzymes Endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) which

recognize oxidized pyrimidines and purines, respectively (Azqueta et al., 2009; Collins, 2014).

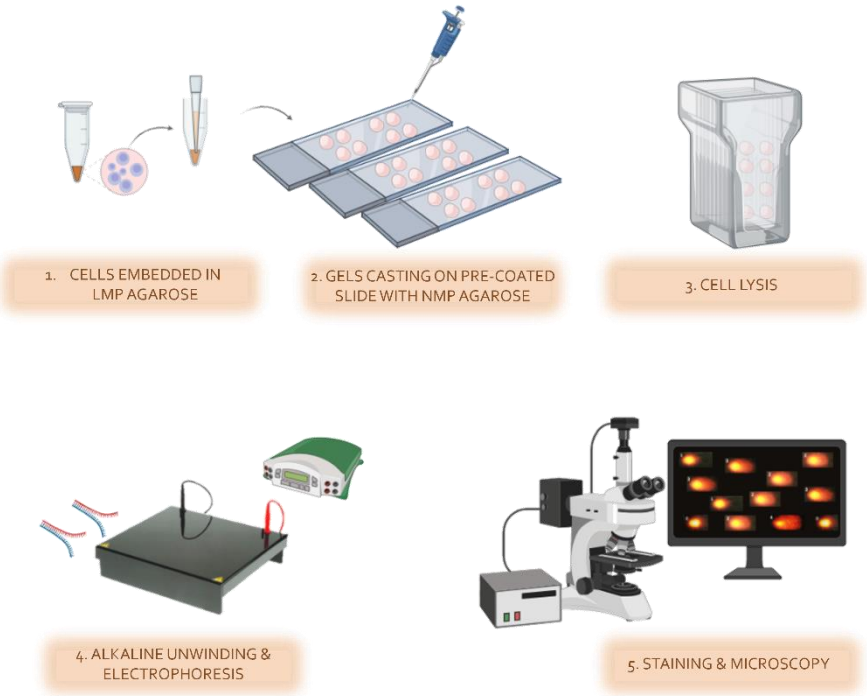

Fig. 2. Procedure of the comet assay. (1) microtube with cell suspension; (2) cell suspension embedded in agarose; (3) lysis (pH 10); (4) alkaline unwinding and electrophoresis (pH 13); (5) representation of stained comet-shaped nucleoids.

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Besides, comet assay application gained great relevance in the field of ecotoxicology, where mammals, amphibians, fish, and invertebrate species are used as

bioindicators for environmental genotoxicants (de Lapuente et al., 2015; Frenzilli et al., 2009; Jha, 2008; Lee and Steinert, 2003).

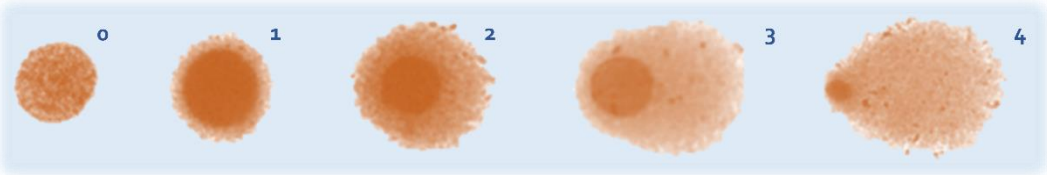

Fig. 3. Schematic representation of visual comet classification into five categories (0: no tail; to 4: almost all DNA in tail) proposed by Collins et al., 1997 (Figure adapted from Valencia et al., 2011).

In ecotoxicology context, this technique is used in many studies to assess DNA damage and repair (Braga et al., 2021; Marques et al., 2014) and has a widespread application considering genotoxicity testing of *in vitro* (Bajpayee et al., 2013), *ex vivo* (Marçal et al., 2020) and *in vivo* (Braga et al., 2021) approaches. Though the knowledge regarding DNA damage and repair in invertebrates species is still scarce, crustaceans (Costa et al., 2018; Hook and Lee, 2004; Lacaze et al., 2010), bivalves (Akcha et al., 2012; Barranger et al., 2014; Sahlmann et al., 2017), and other invertebrates (Gallo et al., 2018; Zang et al., 2000) have served as useful indicator species in ecogenotoxicological studies. Among invertebrates, different cell types were used in the comet assay, being the hemocytes the most examined (Barranger et al., 2016; da Silva Rocha et al., 2012; Klobučar et al., 2012; Lacaze et al., 2010; Lewis et al., 2010; Lewis and Galloway, 2009; Malev et al., 2010; Noventa et al., 2011; Patetsini et al., 2013), followed by gills (Costa et al., 2018; El-shorbagy and Hamdi, 2017; Martins et al., 2013; Raimundo et al., 2010; Wilson et al., 1998), hepatopancreas (Choi et al., 2018; Li et al., 2016; Pavlaki et al., 2016; Valant and Drobne, 2012; Wang et al., 2009) and spermatozoa (Caldwell et al., 2011; Erraud et al., 2018; Lacaze et al., 2010, 2011; Nagarajappa et al., 2006).

DNA methylation in epigenotoxicity

"Epigenetic modifications can be considered as the punctuation marks in the genome."

Professor Marnie Blewitt

It could be said that if the DNA code was an history written in a book, the epigenetic markers will be those that will allow to understand it.

Epigenetics refers to an annotation in the form of chemical marks on top of the DNA code (which is present in every cell), but the pattern of gene expression (*i.e.*, which genes are expressed or non-expressed) determines the cell type and function (Head et al., 2012). The term epigenetics was first introduced in 1942 by Conrad Waddington and was used to describe the class of internal and external interactions between the environment and the genes leading to the development of phenotype (Tronick and Hunter, 2016; Waddington, 1942). Nowadays, it is clear that epigenetic processes can also be passed down from generation to generation in organisms (Tollefsbol, 2011). Moreover, another important aspect of epigenetic markers is that they are susceptible to chemical and nonchemical environmental factors (Gavery and Roberts, 2017, Head et al., 2012, Vandegehuchte and Janssen, 2014). The epigenome serves as a link between the changing environment and the genome (stable and resistant to environmental changes) (Fig. 4).

Effects of early life experiences, such as toxic exposure or malnutrition, can show up later in life and even be passed to future generations (Head et al., 2012; Kucharski et al., 2008; Vandegehuchte and Janssen, 2011). Epigenetic mechanisms have been increasingly used to explain the rapid adaptation of species, since this cannot be justified only by the action of natural selection on genetic variants (Carneiro and Lyko, 2020). Ardura et al. (2017) considered epigenetics as a DNA signature that affects gene expression and enables a rapid reaction of organisms to environmental changes. Indeed, epigenetic mechanisms could promote phenotypic plasticity and adaptation to different environments, therefore, the extent to which environmental effects can trigger epigenetic responses is particularly

interesting for understanding the role of epigenetics in animal adaptation (Verhoeven et al., 2016).

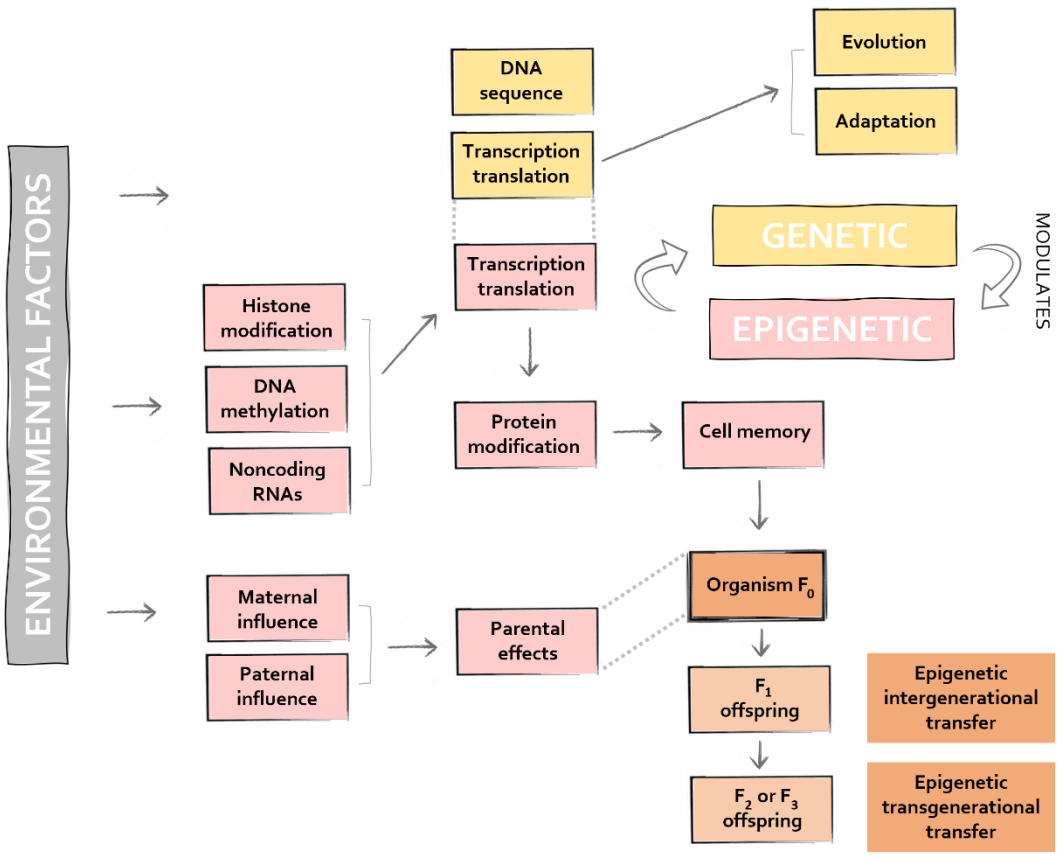

Fig. 4. Schematic diagram relating the genome, epigenome and environment with respect to transgenerational phenotypic characters (adapted from Ho and Burggren, 2010).

Epigenetic biomarkers have been identified as a potential risk-assessment tool in the future. Epigenetics-related alterations can change in response to a variety of environmental circumstances, influencing the gene expression and, as a result, the phenotype of the organism (Vandegehuchte and Janssen, 2014). Once the epigenetic changes to gene expression are it can potentially affect not only the organism but the future generations' evolutionary paths (Anway et al., 2008; Head et al., 2012; Oppold et al., 2015). Over time clear pathways or biomarkers of heritable epigenetic alteration may become

apparent, which may enable risk assessors to predict the likelihood of heritable contamination-induced epigenetic changes (Shaw et al., 2017).

In laboratory experiments, several authors have investigated the epigenetic mechanisms underlying environmentally induced phenotypes under controlled conditions (Gavery and Roberts, 2010; Kucharski et al., 2008; Nilsen et al., 2016; Vandegehuchte et al., 2010b). An important characteristic regarding epigenetic marks is that they can be easily influenced by environmental signals, but, unlike mutations, they are potentially reversible and can be affected by competing signals, as it was showed by the example of the study with the Agouti mouse (Dolinoy et al., 2010), where deleterious changes on the fetal epigenome were reversed with maternal dietary supplementation.

Epigenetics markers include DNA methylation, histone modifications and non-coding RNA activity, influencing gene expression primarily through the local modification of chromatin (Hu and Barrett, 2017). DNA methylation is the most studied epigenetic marker, mainly in mammalians (Carneiro and Lyko, 2020; Vandegehuchte and Janssen, 2011; Yagound et al., 2020), which consist in the addition of a methyl group, from the methyl donor S-adenosylmethionine (SAM) to the fifth carbon atom of cytosine (or at the sixth nitrogen atom of adenine in bacteria) (Šrut, 2021) in certain CpG dinucleotides (Bird, 2002). CpG islands (regions of the genome that contain a large number of CpG dinucleotide repeats) are often located around gene promotor regions, playing a role in regulating gene transcription (Šrut, 2021). Methylation can also occur within gene bodies, and it is a prevalent type of CpG methylation in invertebrates. This important transfer reaction is catalyzed by the DNA methyltransferases (DNMTs). The position of DNA methylation (*i.e.*, within the gene, at the transcription start site or in the promotor) will determine the effects on gene expression (Norouzitallab et al., 2016). For instance, the hypermethylation of a gene promoter is associated with a decreased expression of that gene, while the hypomethylation of non-coding region has been linked to chromosome instability (Collotta et al., 2013).

DNA methylation marks, in mammals, are erased and re-established twice during embryogenesis, which constitute a significant barrier to transfer of epigenetic information via DNA methylation between generations (Yagound et al., 2020). However, in non-mammalian species, such as in fish, it seems that there is an absence of global DNA methylation remodeling during embryogenesis and so the transfer of DNA methylation patterns across generations can occur (Yagound et al., 2020). Likewise, invertebrates seemed to lack epigenetic reprogramming, which could allow the transfer of DNA methylation through generations (Yagound et al., 2020).

The first studies concerning invertebrate organisms stated that DNA methylation was linked with the cell memory system, the silence and repress of genomic repeated DNA sequences, the mode of development and amount of cell (Regev et al., 1998). Some invertebrates as *Caenorhabditis elegans* lack this epigenetic marker (Simpson et al., 1986) and others, such as the *Drosophila melanogaster*, has only 0.034% of methylated cytosines (Capuano et al., 2014). Other insects, crustaceans and mollusk has their DNA methylated from 1-15% (Fallet et al., 2020; Šrut, 2021; Vogt et al., 2015). The DNA methylation has been comprehensively studied in the marble crayfish, *Procambarus fallax* (Martin et al., 2010; Vogt, 2008). The methylome of this crayfish showed that 41% of genes are substantially methylated, while 26% are unmethylated (Falckenhayn, 2016 *apud* Vogt, 2018). CpG-specific DNA methylation is seen in genes and repetitions, while most repeats are hypomethylated, except for repeats within genes, which have greater methylation levels (Vogt, 2018). Similar to many other invertebrates, the DNA methylation modification in the crayfish is directed to the gene bodies of housekeeping genes. When compared to mammals, the methylation and demethylation machinery of marbled crayfish, although effective, is simple (Vogt, 2018). The marble crayfish shares crucial features of DNA methylation such as CpG methylation, gene body methylation and repeated methylation with many other animals (Vogt, 2018). DNA methylation seems to be associated with *P. fallax* successful adaptation to different environments, where it seems that its genome can adapt to specific environmental conditions (Carneiro and Lyko, 2020).

In an ecotoxicological context, an epigenetic approach could be beneficial since epigenetic marks on specific genes, or even global DNA methylation, could be used as biomarkers for stressor exposure or harmful effects, since exposure to non-genotoxic compounds has been shown to induce DNA methylation changes (Vandegehuchte and Janssen, 2011). For instance, global DNA methylation could serve as broad indications of accumulated stress during the lifespan of an organism (Head et al., 2012). Considering inter- and transgenerational studies, epigenetic inheritance could explain why a population is: (i) slow to recover after, for instance, remediation of contaminated sediments; (ii) rapidly adapted to local contamination; (iii) able to thrive in very contaminated areas (Head et al., 2012). The presence of environmental chemicals (*e.g.*, metals, pesticides, endocrine-disruptors) and alterations in environmental conditions (*e.g.,* temperature and salinity changes, nutritional deficiencies) are significantly more likely to affect gene expression through epigenetic processes than by mutations (Ardura et al., 2017; Cribiu et al., 2018; Oppold et al., 2015; Rondon et al., 2016; Šrut, 2021; Vandegehuchte et al., 2010; Zhou et al., 2001). Most of the studies, regarding DNA methylation changes induced by contaminants, are performed in vertebrates (Anway et al., 2005; Head, 2014; Nilsen et al., 2016; Pilsner et al., 2010; Yauk et al., 2008), being only a few addressed in invertebrate species (Gavery and Roberts, 2010; Vandegehuchte, et al., 2010; Vogt et al., 2015). In invertebrates, some authors observed changes in the DNA methylation concerning specific genes (*e.g.* Jeremias et al., 2018; Vandegehuchte et al., 2010), while others studied the global DNA methylation (Akcha et al., 2021; Lian et al., 2015; Oppold et al., 2015; Vandegehuchte et al., 2009a). Among the numerous intergenerational effects that can be selected to assess the effect of environmental toxics at population-level (*e.g.* hatching rate, embryo development, embryo abnormalities, among others), the epigenetic inheritance (Head, 2014), such as the changes in global genome methylation, can be a starting point once, and as mentioned above, modifications in this epigenetic marker may affect severely the whole individual. For instance, global DNA methylation changes in germline, induced by environmentally factors can be inter- and transgenerational transmitted (Guerrero-Bosagna et al., 2014). These germline epigenetic modifications also induce epigenetic

alterations in somatic tissues which correlate with transgenerational transcriptome changes and phenotypic abnormalities (Nilsson and Skinner, 2015).

It can be considered that environmental genotoxicants can also have epigenotoxics potential. Yauk et al. (2008) observed DNA strands breaks and hypermethylation in mice spermatozoa (after being exposed to environmental contaminants). These authors suggest that the DNA damage caused an increase in DNMT activity (DNMTs are known to be upregulated during DNA damage and bind with high affinity to many DNA lesions), which resulted in the observed hypermethylation (Yauk et al., 2008).

So far, it is known that:

(i) changes in DNA methylation can affect species' phenotype negatively (*e.g.* cancer occurrence) or positively (*e.g.* increase adaptation) (Sargsyan et al., 2019);

(ii) stressing environmental conditions may induce changes in the global methylation levels (Aniagu et al., 2008; Cleary et al., 2019), that could be involved in phenotypic plasticity which can be important for adaptation to changing environmental conditions (Bossdorf et al., 2008; Suarez-Ulloa et al., 2015);

(iii) the direction of the change in global methylation may depend on particular stressors, *i.e.*, some chemicals present in environment may reduce methylation while others tend to increase it (Akcha et al., 2012; Rondon et al., 2017; Wang et al., 2009; Zhou et al., 2001).

DNA methylation assessment

There are three basic approaches to assess the methylation state of DNA: (i) the methylation sensitive amplification polymorphism (MSAP) method (Pérez-Figueroa, 2013); (ii) the full genome sequencing via bisulphite sequencing that profiles the genomic-scale methylation patterns; and (iii) the kit based quantitative tools such as ELISAs that capture

global DNA methylation patterns (Kurdyukov and Bullock, 2016). ELISA-like methods are spectrophotometric assays that can detect the levels of methylated cytosines in a sample of genomic DNA. These methods are examples of the tools that have become commercially available as the field of epigenetics expands in biomedical science and have already been used in animals' studies. The limitation of this approach is that the data reflect only global levels with no information about changes in specific regions of the genome; however, this is an affordable and approachable method to gain insight into changes in global patterns (*e.g.*, tissue-wide, or as a function of developmental stage) of DNA methylation between discrete samples (Hofmann, 2017).

Intergenerational effects

The transfer of epigenetic marks can contribute positively to the offspring equipment, being a gift to the success of the population (*e.g.*, stress adaptation); however, there may be situations in which the altered epigenetic marks lead to the production of hazardous phenotypes (*e.g.*, tumor development). In this later situation, the inter- or transgenerational inheritance could be a burden for the population (Skinner et al., 2011). The term transgenerational inheritance is only used to describe epigenetic modifications that are able to persist from F_1 (or F_2) generation to their respective offspring F_2 (or F_3), or later generations, in the absence of direct exposure to the factor that initiated the change (Xavier et al., 2019). While, intergenerational epigenetic inheritance is used to define epigenetic modifications in response to direct exposure to environmental factors that are transmitted from one generation to the next, *i.e.*, from parent's generation to the offspring's generation (F_1) (Fig. 4) (Heard and Martienssen, 2014).

Intergenerational and multigenerational studies are crucial to ecotoxicology field, since the cause (*e.g.* a chemical exposure) and effect (*e.g.* DNA damage) don´t need to occur at the same time, life stage, or even generation (Head et al., 2012). Some studies have reported that the parental exposure can have an impact on the prole' DNA integrity (Lewis and Galloway, 2009) and on DNA methylation pattern (Ding et al., 2020, Rondon et al., 2017). Changes in DNA methylation, even with the environmental stressor remotion, the

alteration can last for generations (Anway et al., 2006; Yauk et al., 2008). One of the most cited study concerning the impact of parental exposure, observed that pesticide-treated pregnant rats produced male offspring with lower sperm number and viability, and the effect persisted up to the F_4 generation and was correlated with altered patterns of DNA methylation (Anway et al., 2005).

The potential consequences of environmental chemical exposures for the health of non-exposed future generations should be considered in the ecological risk assessment process (Vandegehuchte and Janssen, 2011), taking into account the cause-effect relationships between specific environmental factors and the subsequent epigenetic modifications (Rondon et al., 2017). Epigenetic inheritance (*e.g.,* intergenerational) points out the long-term effects of pollutants, which affect how organisms cope with current and historical pollution burdens. The understanding of contaminant-related risks is critical for reducing environmental and health impacts, developing appropriate regulations, and implementing appropriate risk management strategies, all while taking into account the biological and functional effects caused by various exposure levels and pathways (Shaw et al., 2017). Furthermore, to really comprehend the influence of epigenetic alterations on organisms, as well as their adaptation to changing environmental conditions, it is critical to link epigenetic modifications to gene expression patterns and, as a result, with phenotypes. Some authors stated that species adaptation would benefit from relaxed gene expression control, *i.e.*, low levels of DNA methylation would increase transcriptional opportunities and enhance activity of transposable elements (Roberts and Gavery, 2012).

1.2 Aquatic contamination by pesticides

Environmental fate and risk

Freshwater contamination in surface and groundwater is a great worldwide concern, being its protection part of the European Water Framework Directive (WFD) milestones (European Commission, 2008), and integrated in the United Nations

Sustainable Developmental Goals. In the last century, approximately 80 000 new chemicals (*e.g.* fertilizers, plasticizers, surfactants, PCBs, PAHs, metals and pesticides) have been release into the environment (Selvaraju et al., 2021).

Pesticides are synthesized or natural substances that can prevent, destroy, or control harmful organisms (denominated as pests), protect plants or plant by-products during production, storage, and transport. These compounds can be classified based on their chemical structure (*e.g.*, organophosphates, neonicotinoids, azoles), target (*e.g.*, fungicides, herbicides, insecticides) or mode of action (*e.g.*, acetylcholinesterase inhibitors, calcium channels inhibitors) (Collotta et al., 2013).

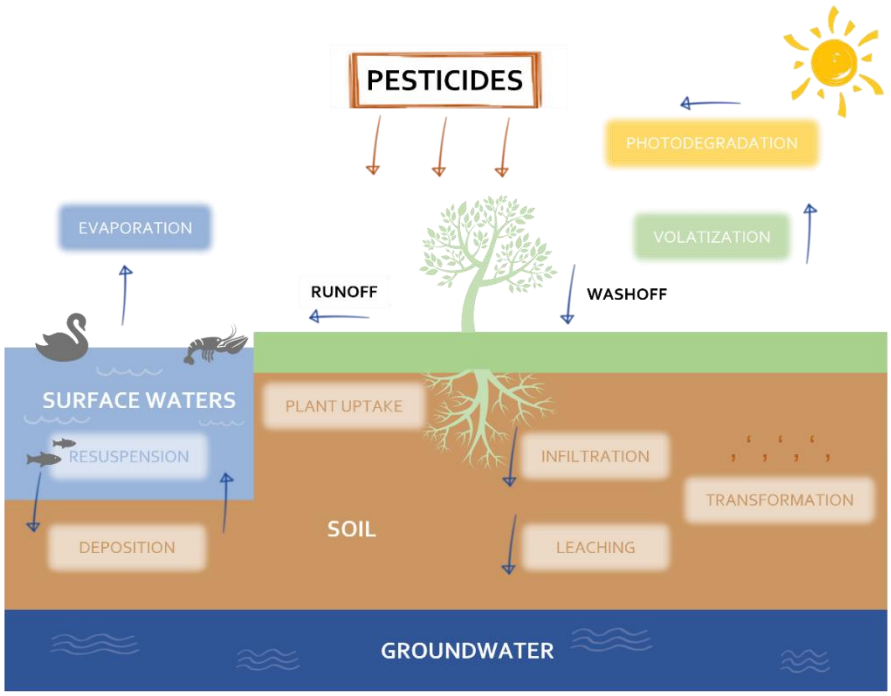

Fig. 5. Pesticide fate (adapted from Zacharia, 2011).

Pesticides can be found almost everywhere worldwide (Fig. 5), since due to their physical and chemicals properties, can enter water bodies through surface runoff, leaching, and/or erosion (Khan and Law, 2005). Furthermore, pesticide residues can reach the atmosphere (through drift, evaporation, and wind erosion processes) and contaminate surface waters, soils and biota via precipitation (Dubus et al., 2000). These compounds are one of the main contributors to the decrease of groundwater quality (Skevas, 2020) and,

therefore the control of pesticides misuse and their permanence in the environment is still a major challenge (Hofstetter et al., 2010). The intensive use of these kind of compounds in agriculture is related to the need of increasing production, considering the high global demand for food. Bearing in mind the pesticides ubiquity in the environmental scenario, ecotoxicological studies have grown significantly in recent decades from the standpoint of environmental protection (Lushchak et al., 2018).

The magnitude of damage caused by pesticides on aquatic environment is very difficult to estimate, since even when employed in accordance with acceptable agricultural practices, they have the potential to disrupt the ecosystem's natural equilibrium, and create significant ecological changes (Lushchak et al., 2018; Negro et al., 2011). Moreover, since pesticides have different modes of action, and the information of their effects stills scarce, their impact on non-target species is of great importance (Negro et al., 2011). Considering this, the biochemical responses assessment concerning the pesticide-exposed organisms is an approach that can show the overall environmental quality (McCarty et al., 2002). It is well described that pesticides can enter into organisms in a variety of ways, depending on the species, metabolic characteristics, and toxin bioavailability (Hodgson, 2012). If a chemical has already entered an organism, it must be able to cope with it via biotransformation and excretion into the environment (or a combination of these mechanisms) to neutralize or limit the negative effects (Lushchak et al., 2018). Pesticides can be eliminated by the organism, in one of two ways: via excretion in their natural state or, after biotransformation with other substances (Van der Oost et al., 2003). However, it is important to consider that biotransformation can, occasionally, produce more harmful chemicals than the original compound.

Pesticides can alter the endocrine system, induce neurological problems, and have an impact on the immune system, reproduction, and development (Khan and Law, 2005). Pesticides can also increase the ROS (reactive oxygen species) levels (Slaninová et al., 2009). ROS can react with important cellular macromolecules, causing, for example, enzyme inactivation, lipid peroxidation, and DNA damage, which can eventually lead to cell death (via necrosis or apoptosis). More specifically, several studies have demonstrated that

certain pesticides are known to cause unintended damage on DNA of non-target aquatic organisms, namely strand breaks (Çavaş and Könen, 2007; Guilherme et al., 2015), mutations (Bhuvaneshwari et al., 2013) and epigenetic variations (Baccarelli and Bollati, 2009; Brevik et al., 2018; Sargsyan et al., 2019; Vandegehuchte and Janssen, 2011; Zhang et al., 2012).

In 2009, the EU adopted a framework 2009/128/EC Directive on the Sustainable Use of Pesticides (SUDP), which foresees that all farmers (professional users) should adopt practices and choose products with lowest risk to human health and the environment, and where non-chemical methods should be selected whenever possible (EU Directive, 2009). It was also stated that all EU member states should minimize or prohibit pesticide use in specific areas and establish safeguard zones for surface and groundwater used for the abstraction of drinking water, and also create appropriately-sized buffer zones to protect non-target aquatic organisms (EU Directive, 2009).

The concern with non-target species contamination goes further when it is considered, not only considering the individual but also the population's safety. For that reason, it should be considered in the evaluation of ecotoxicology risk the implementation of intergenerational studies. Several studies have been addressed on offspring effects due to parental exposure (Debier et al., 2003; Devaux et al., 2011; Jeremias et al., 2018; Lacaze et al., 2011; Lewis and Galloway, 2009; Santos et al., 2013; White et al., 1999), but only a few focused on pesticides (Barranger et al., 2014; Bouilly et al., 2007; Schlenk et al., 2001).

Top concerning pesticides

According to the latest EU data herbicides, insecticides and fungicides are the most used pesticides in Europe (EUROSTAT, 2019). Within these groups some of the most used active ingredients are: (a) herbicides - glyphosate (organophosphorus) and penoxsulam (triazolopyrimidine); (b) insecticides - dimethoate (organophosphorus) and imidacloprid (neonicotinoid) and, (c) fungicides – pyrimethanil (anilinopyrimidine) and imazalil (azole)

(DGAV, 2016). Therefore, these compounds are of great concern, from the user and from the scientific community that assesses the risk.

Glyphosate [2-(phosphonomethylamino)acetic acid] (Fig. 6) is an active ingredient of commercial formulations, as for instance the well-known Roundup®. This compound is the most widely used herbicide worldwide, resulting in contamination of surface waters and raising great concern regarding potential environmental and human health impacts (Van Bruggen et al., 2018). Glyphosate is water-soluble and its half-life in aquatic environment is approximately 30 days.

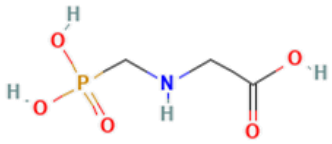

Fig. 6. Chemical structure of the herbicide glyphosate [2-(phosphonomethylamino)acetic acid] (Pubmed, 2022a).

This compound acts on the shikimate pathway in plants, through the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is involved in the metabolism of aromatic amino acids (Mesnage et al., 2015). Inhibition of EPSPS by glyphosate causes protein shortage, and, consequently, plant death. This compound is approved by the EU until December 15 of 2022, and, according to the press release, it is expected that its market sales growth of 5.1% worldwide until 2027 (Wood, 2021). Despite this information, several studies have pointed the genotoxic potential of this herbicide on aquatic animal species, namely fish (Guilherme et al., 2010; Leveroni et al., 2016; Marques et al., 2014) and shrimp (Hong et al., 2018).

The market sales of the herbicide penoxsulam (Fig. 7) are predicted to grow around 4.1% (worldwide), through 2026 (A. More, 2021). This recent post-emergent herbicide [2-

(2,2-difluoroethoxy) - N - (5, 8 – dimethoxy - [1, 2, 4] triazolo [1, 5-c]pyrimidin- 2 -yl) -6-(trifluoromethyl)benzenesulfonamide], developed by Dow Agrosciences, received registration in 2005 in southern U.S. (Jabusch and Tjeerdema, 2008), and is approved in the EU, until July 31st of 2023. This compound has an excellent herbicidal activity against a broad-spectrum of annual and perennial weeds, especially to broad-leaved weeds and sedges (Walton et al., 2005). After being absorbed by plant species, penoxsulam will inhibit the acetolactate synthase enzyme (ALS) activities, which leads to the hindrance in the biosynthesis of branched amino acids valine, leucine, and isoleucine (Walton et al., 2005). It is water-soluble, mobile, hydrolytically stable, and non-volatile, with degradation half-life (DT_{50}) in the aquatic system ranging between 1.28 and 1.96 days (Kogan et al., 2011). As glyphosate, penoxsulam genotoxic potential was observed on aquatic species, both vertebrates (Cattaneo et al., 2011) and invertebrates (Patetsini et al., 2013).

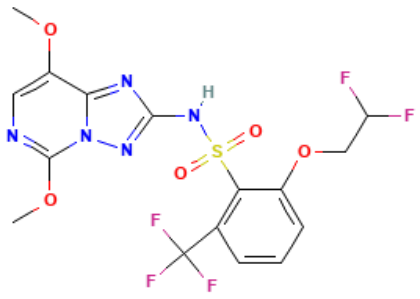

Fig. 7. Chemical structure of the herbicide penoxsulam [2-(2,2-difluoroethoxy)-N-(5,8-dimethoxy-[1,2,4]triazolo[1,5-c]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide] (Pubmed, 2022).

Dimethoate [2-dimethoxyphosphinothioylsulfanyl-N-methylacetamide] (Fig. 8), is an organophosphate insecticide that has no longer approvement to be used in the EU (the approval period ended on June 30 of 2019). Despite this, dimethoate continues to be used by several countries, and its market sales growth rate of 5% is predicted (IndustryARC, 2021). This active principle is used as a systemic insecticide for control of a wide variety of insect pests of fruits, vegetables and crop plants, as well as for non-agricultural purposes,

such as landscape maintenance and pest control (Van Scoy et al., 2016). Like other organophosphates, dimethoate is also an acetylcholinesterase inhibitor, therefore affecting impulse conduction through synapses and neuromuscular junctions, which is reflected in uncoordinated abnormal behavior of exposed animals (Singh, 2017). The genotoxic potential of dimethoate was already reported in the invertebrate *Procambarus clarkii* (Oskoei, 2018).

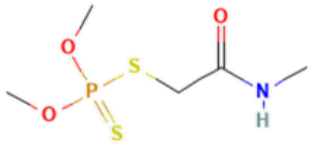

Fig. 8. Chemical structure of the insecticide dimethoate [2-dimethoxyphosphinothioylsulfanyl-N-methylacetamide] (Pubmed, 2022b).

Although it has also been banned in Europe (since December 2020), the application of the insecticide imidacloprid (Fig. 9) continues approved by the US-EPA (Reaves, 2020). Imidacloprid [1-(6-chloro-3- pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine] is used as a seed dressing to manage arthropod crop pests and in topical flea and tick control treatments for pets, where it will disrupt synaptic transmissions by acting as a nicotinic acetylcholine receptor agonist (Sohn et al., 2018). Its genotoxic potential was already reported in the aquatic (non-target) species *Rana nigromaculata* (Feng et al., 2004).

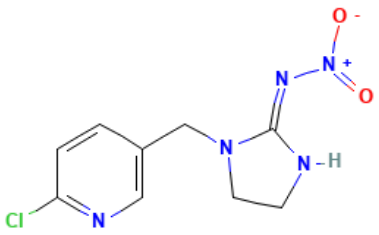

Fig. 9. Chemical structure of the insecticide imidacloprid [(NE)-N-[1-[(6-chloropyridin-3-yl)methyl]imidazolidin-2-ylidene]nitramide] (Pubmed, 2022b).

Among the major groups of pesticides, fungicides recorded the highest sales volumes in 2011 and 2019. Pyrimethanil (Fig. 10) is one of the most used in EU and US (Araújo et al., 2015). The pyrimethanil (4,6-dimethyl-N-phenylpyrimidin-2-amine) is a type of broad-spectrum fungicide used to prevent and control pathogenic fungus and often used in a variety of fruits and vegetables crops, ornamental trees, and lawns (Meng et al., 2020). It was designed to inhibit the secretion of hydrolase from pathogenic fungus, and thus, preventing infection and killing the pests, and its use was approved in the EU until April 30 of 2022. Pyrimethanil is characterized by its high chemical stability, low biodegradability, and long durability in water, and stable to hydrolytic degradation in water (Meng et al., 2020). To the author's best knowledge, no genotoxic effects on aquatic (non-target) species were reported so far, and the only study demonstrating pyrimethanil genotoxic potential was described in human mononuclear leukocytes (Lebailly et al., 1998).

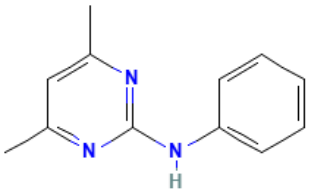

Fig. 10. Chemical structure of the fungicide pyrimethanil [4,6-dimethyl-N-phenylpyrimidin-2-amine] Pubmed, 2022c).

Along with pyrimethanil, the fungicide imazalil [1-[2-(2,4-dichlorophenyl)-2-prop-2-enoxyethyl]imidazole] (Fig. 11) is also used extensively to protect vegetable and fruit plantations, or post-harvest crops from rot, and to increase the lifetime of products on the market (Jin et al., 2016) This compound belongs to the group of imidazole and triazole derivatives which inhibit fungal cell wall synthesis by interfering with a specific cytochrome P450 enzyme (only found in animals, plants, fungi, and prokaryotes) (Lushchak et al., 2018). As pyrimethanil, no genotoxic potential to (non-target) aquatic species is known, although imazalil genotoxic properties were described on mammals (Đikić et al., 2012).

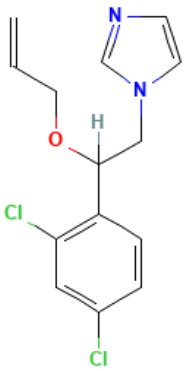

Fig. 11. Chemical structure of the fungicide imazalil [1-[2-(2,4-dichlorophenyl)-2-prop-2-enoxyethyl]imidazole] (Pubmed, 2022b) .

1.3 *Procambarus clarkii* as a tool in (epi)genotoxicity assessment

Invertebrates are often used as models in ecotoxicology research, both in the field and laboratory studies, due to their wide distribution, accessibility, easy maintenance, and handling. Moreover, these organisms are considered of high interest in what concerns to their limited ethical issues, when compared to vertebrate organisms.

Freshwater decapod crustaceans (*e.g.* shrimps, lobsters, crayfish, hermit crab and crabs) share a number of special features: all stages are 'homotopic', living within the same aquatic or semi-aquatic environment (although there may be habitat partitioning among life stages); most adult decapods are often much larger, than other freshwater invertebrates; many are longer-lived, and most are omnivorous or polytrophic (Reynolds et al., 2013). Freshwater crustaceans were considered as potential sentinel species for genotoxicological studies, due to environmental exposure to possible genotoxicants present in their habitat (Donato et al., 2016; Mistri et al., 2020). Specifically, among freshwater decapod organisms, crayfish were considered as an important component of aquatic ecosystems and are seen as sensitive bioindicators of water pollution (Kuklina et al., 2013). Moreover, crayfish possess key pre-requisites for a laboratory model, such as (i) suitable size (crayfish intern organ/tissues are easily identified), (ii) resistance to handling stress, (iii) high fertility, and (iv) a relatively short generation time (Vogt, 2019).

The red swamp crayfish (Fig. 12), *Procambarus clarkii* (Girard, 1852), native to north-eastern Mexico and south central USA (Lindqvist and Huner, 1999), was introduced for commercial purposes in Europe in the early '70s (Anastácio, 1993). *P. clarkii* can occupy a wide variety of habitats, including subterranean locations, seasonally flooded swamps and marshes, permanent lakes and streams, rivers and rice fields (Reynolds et al., 2013).

Fig. 12. *Procambarus clarkii* male adult dorsal view.

Red swamp crayfish is well adapted to life in seasonally flooded wetlands, feeding on plant and animal food and detritus, and retreating into burrows during periods of low water. *P. clarkii* have short-lifespan, rarely exceeding 12 – 18 months, and may mature between 45- and 125-mm total length. The breeding is in summer and early autumn, although in Kenya there are reports that introduced populations breed throughout the year (Anastácio, 1993), allowing re-flooded habitats to be rapidly recolonized. The technical report of Huner and Barr (1991) presents some methods to artificially produce this species off-season. Based on their work, in this thesis it was developed conditions (*e.g.*, water temperature was approximately 24°C and was completely changed once a week) in laboratory to produce the species *P. clarkii*. Male crayfish (form I: enlarged claws and hooks on the bases of the walking legs) was placed in the mature female aquaria overnight. Females lay and incubate up to 600 eggs in their burrows; they hatch in two to three weeks at 22°C (Reynolds et al., 2013). When most of the juveniles had already left the female's abdomen, the mother had to be removed to avoid cannibalism.

Figures 13 and 14 show the artificial spawning of the red swamp crayfish in laboratory conditions based on the methods described in Hunter and Barr (1984). The reproductive behavior of this species includes a phase in which the male courts the female through a sequence of movements, where the terminal phase of mating consists of the turning of the female and the deposition of the spermatophore by the male (Anastácio, 1993).

Fig. 13. Crayfish mating. (A) Examples of aquariums adapted for reproduction. (B) Mating.

Fig. 14. Female crayfish and juvenile development of *P. clarkii*. (A) Ventral view of an adult female. (B) Female with eggs in early development. (C and D) Female with hatchlings attached to swimmerets beneath the abdomen. (E) Crayfish juveniles.

This crayfish can be daily in contact with chemical and physical challenges/stressors (*e.g*. in rice fields) while, at the same time, being well succeeded, making them potential good organisms to perform ecotoxicological studies (Costa et al., 2018; Esposti et al., 2019). Specifically, *P. clarkii* has been used as a bioindicator for metals (Alcorlo et al., 2006; Mistri et al., 2020; Wei and Yang, 2015; Zhang et al., 2019) and nanoparticles (El-Atti et al., 2019), to study reprotoxic effects (Zhao et al., 2019) and pesticide genotoxicity (Costa et al., 2018). Moreover, crayfish provides tissue amount for analyses comparable to that obtained from fish, such as gills, gonads, hepatopancreas (as observed in Fig. 15), which can make them an alternative to fish (vertebrates) in biological assays, which may be seen as more ethical.

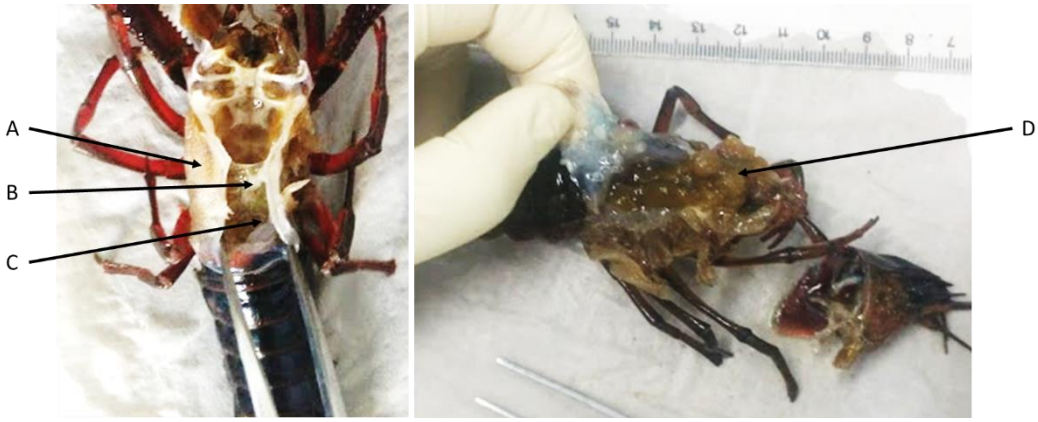

Fig. 15. Dissected crayfish: (A) gills; (B) testes; (C) vas deferent; (D) hepatopancreas.

1.4 Aims and thesis structure

Under the umbrella of the genotoxic risk of waterborne pesticides to aquatic (non-target) species, this thesis aimed: (i) to assess the DNA damaging potential of the herbicides (glyphosate, penoxsulam), insecticides (dimethoate, imidacloprid), and fungicides (pyrimethanil, imazalil); (ii) to evaluate penoxsulam genotoxic long-term effects; (iii) to increase the knowledge concerning parental exposure effects on offspring,

considering their methylome and their DNA integrity, and also (iv) to contribute to the implementation of the pesticidovigilance concept.

To achieve these main goals, the following specific objectives were established:

- the evaluation of the spermiotoxic potential of each pesticide (mentioned above), considering environmental realistic concentrations;
- the assessment of somatic and germinative tissue-specificity response (adult crayfish; somatic and germinative), concerning the genotoxic action of penoxsulam (non-specific and oxidative DNA damage);
- the genotoxic potential of penoxsulam assessment, upon a period of post-exposure;
- the evaluation of the parental exposure influence on the offspring (F_1) DNA and methylome integrity, in three different scenarios:
    - after being exposed to the same genotoxicant as their parents (F_0) (juvenile stage – F_1);
    - after being exposed to a different genotoxicant as their parents (F_0) (juvenile stage – F_1);
    - after they have grown up in a free-genotoxicant environment (juvenile and adult state – F_1).

The present thesis is composed of six chapters. The first chapter corresponds to the general introduction, in which diverse pertinent topics were considered to fully understand the global perspective of the present research. Following, the middle chapters (2 to 5) described the scientific work performed to achieve the thesis' objectives (described above). The thesis ends with a general discussion chapter, in which the results gathered and described in the previous chapters are discussed according to a comprehensive and integrated perspective.

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Chapter II

DNA of crayfish spermatozoa as a target of waterborne pesticides – an *ex vivo* approach as a tool to short-term spermiotoxicity screening

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2. DNA of crayfish spermatozoa as a target of waterborne pesticides – an *ex vivo* approach as a tool to short-term spermiotoxicity screening

Keywords

Ecogenotoxicity; DNA damage; Reprotoxicity; *Procambarus clarkii*

Abstract

The spermiotoxic properties of aquatic contaminants might be the cause of low fertilization rate and decreased prolificacy, affecting the success of the impacted populations. The genotoxic potential of pesticides in spermatozoa as an undesirable effect on non-target organisms, namely aquatic invertebrates with external fertilization, emerges as a key question in ecogenotoxicological research. Thus, this study aimed to clarify if DNA integrity of red swamp crayfish (*Procambarus clarkii*) spermatozoa is affected by waterborne pesticides at environmentally relevant concentrations. By adopting an *ex vivo* approach, six pesticides were addressed in a short-term assay: herbicides glyphosate (9 and 90 μg L^{-1}) and penoxsulam (2.3 and 23 μg L^{-1}); insecticides dimethoate (2.4 and 24 μg L^{-1}) and imidacloprid (13.1 and 131 μg L^{-1}); fungicides pyrimethanil (2.2 and 22 μg L^{-1}) and imazalil (16 and 160 μg L^{-1}). Genotoxicity was observed in higher concentrations of glyphosate, penoxsulam, dimethoate, pyrimethanil, and imazalil. Imidacloprid was the only pesticide that did not cause non-specific DNA damage, although displaying pro-oxidant properties. Overall, the present study demonstrated the suitability of the *ex vivo* approach on spermiotoxicity screening, highlighting the potential ecological impact of pesticides on non-target species, such as *P. clarkii*, compromising sperm DNA integrity and, subsequently, the population success.

2.1 Introduction

Spermatozoa are highly specialized cells, crucial in the definition of genetic and functional traits, as well as in determining the continuous success of the species. The physiological and structural features of this type of cells have been challenging research topics in environmental toxicology, namely concerning aquatic animals with external fertilization, firstly because of its adaptability to the reproductive strategy (Lewis and Ford, 2012), and secondly, for its ability to cope with the environmental pressures immediately following the release into the external milieu. After mating or during fertilization, gametes could be exposed to several aquatic contaminants, which may adversely affect the reproduction processes (Arizza et al., 2009; Lahnsteiner et al., 2005; Lee and Steinert, 2003), leading to the noxious effects on short- and long-term population survival (Jha, 2008). So, there is an environmental concern related to the spermiotoxicity on aquatic populations. In the aquatic invertebrates context, most studies addressed the impact of parental exposures on gametes and offspring (*e.g.* Barranger et al., 2014; Bouilly et al., 2007; Debier et al., 2003; Devaux et al., 2011; Lewis and Galloway, 2009), while only a few assessed the effects on spermatozoa from the direct exposure to contaminants, namely petroleum aromatic hydrocarbons (Beirão et al., 2018), CuO (Gallo et al., 2018) and ZnO nanoparticles (Oliviero et al., 2019). Hence, more research on spermiotoxic properties of water contaminants is needed as a path to protect those populations, mitigating reproductive impairments and the induction of transmissible disruptions to offspring.

The spermiotoxicity in aquatic invertebrates has been assessed through motility impairments (Esposito et al., 2020), metabolic disorders (*e.g.* acrosome-reaction, mitochondrial activity) (Favret and Lynn, 2010), and DNA damage (Lacaze et al., 2011). Sperm DNA integrity has crucial importance since it will directly influence the quality of the male genome conveyed to the female one, and thus, to progeny. Concomitantly, several studies pointed out the lack of DNA repair machinery on spermatozoa from

distinct species, such as *Pomatoceros lamarckii* (Polychaeta) (Dixon et al., 2002), *Crassostrea gigas* (Bivalvia) (Mai et al., 2013) and *Gammarus fossarum* (Malacostraca) (Lacaze et al., 2011). Therefore, the inability to repair damaged DNA, combined with their lower antioxidant defenses, strongly contribute to making spermatozoa more susceptible to genotoxic agents than oocytes (Mai et al., 2013). Previous studies on aquatic invertebrates' spermatozoa also found a link between the DNA damage and the abnormal development and growth of progeny (Barranger et al., 2014; Caldwell et al., 2011; Lacaze et al., 2011; Oliviero et al., 2019). These facts highlight the interest of spermatozoa as a peculiar cell population, namely in the framework of ecogenotoxicological research. Moreover, the suitability of DNA integrity as a tool for spermiotoxicity assessment gains prominence in species with immotile spermatozoa (due to the lack of a true flagellum), like crayfish and other decapod crustaceans (Kouba et al., 2015; Yazicioglu et al., 2016), where motility cannot be evaluated.

Pesticides runoff from agricultural land and its misuse are a major concern (Hofstetter et al., 2010). Few studies evaluated the pesticide spermiotoxicity on non-target aquatic invertebrates, relying the available reports on the appraisal of fertilization success (and failure) (Díaz et al., 2015; Mai et al., 2013; Yang et al., 2008), while the assessment of genotoxic effects in spermatozoa remains unexplored. Herbicides, insecticides and fungicides are the most currently used pesticides (FAO, 2019; INE, 2018). Within the first group, glyphosate is the best-selling, but studies regarding its genotoxicity in aquatic invertebrates' spermatozoa are still scarce (Akcha et al., 2012). Akcha et al. (2012) reported that no genotoxic effect was found on oyster spermatozoa after glyphosate exposure. Notwithstanding, glyphosate genotoxicity has been demonstrated in fish blood cells (Guilherme et al., 2010, 2012). Within herbicides, there is a new pre-emergent agent, penoxsulam, for which some studies have already warned for its genotoxic potential on aquatic invertebrates (Patetsini et al., 2013, Costa et al., 2018), although there is no information on sperm cells. Concerning insecticides, dimethoate and imidacloprid are among the most used for agriculture purposes (DGAV,

2015), but no data are available on their genotoxic effects in male gametes of aquatic invertebrates. However, genotoxicity was demonstrated for dimethoate in other cell types (Zang et al., 2000, Oskoei, 2018). In respect to fungicides, pyrimethanil and imazalil are among the most employed on crop production (DGAV, 2015). Although, their genotoxicity was described for human leukocytes (Lebailly et al., 1998) and mice hepatocytes (Đikić et al., 2012), the impact on aquatic invertebrates′ spermatozoa remains unknown.

The lack of studies on the risk to spermatozoa of aquatic invertebrates resulting from the exposure to waterborne pesticides is disquieting. Concerning non-target species, the crayfish *Procambarus clarkii*, due to the broad range of habitats occupied, is likely to be impacted. Despite the crayfish spermatozoa being transferred to the female within the spermatophores (Niksirat et al., 2014; Niksirat and Kouba, 2016), *P. clarkii* appears as a suitable experimental model, regardless the absence of studies on pesticide spermiotoxicity. Moreover, since its reproduction is external, the vulnerability to water-quality status cannot be discarded.

Organism-based (*in vivo*) approaches including field surveys and/or laboratory experimental exposures represent the methodological frontline of ecotoxicology. *In vivo* assays are designed to achieve the widest range of biological responses, considering a statistical data validation. In the end, the number of organisms used may reach ethically questionable levels. Since the implementation of Directive 2010/63/EU on animal protection scientific purposes, legal requirements based on 3R's principles (replacement, reduction and refinement) were settled out, imposing new approaches for toxicological tests. So, here it is proposed an *ex vivo* approach as an alternative cell-based methodology to screen for potential spermiotoxic effects of pesticides. Spermatozoa of aquatic invertebrates with external fertilization are highly suited for *ex vivo* studies since their natural physical environment at the time of fertilization is easy to simulate on the incubation cell medium. Moreover, it allows a high-throughput toxicological screening,

simplifying complex-toxicology trials into low-cost and low-time consuming assays, providing a larger volume of relevant information with a reduced number of organisms (in the limit, using spermatozoa from a single animal) (Cazarin et al., 2004). The proposed approach was successfully adopted by Santos et al. (2013) to assess toxicologically induced DNA damage in fish spermatozoa.

This work aimed (i) the evaluation of the genotoxic impact of waterborne pesticides on spermatozoa of *P. clarkii,* after a direct exposure through an *ex vivo* approach, (ii) the identification of eventual oxidative DNA damage, and (iii) the contribution to the knowledge of the long-lasting effects. It was tested the herbicides glyphosate and penoxsulam, the insecticides dimethoate and imidacloprid, as well as the fungicides pyrimethanil and imazalil. Accordingly, sperm DNA damage was assessed through the alkaline comet assay, with an extra step including DNA lesion-specific repair enzymes to assess the oxidative DNA damage mechanisms.

2.2 Materials and methods

2.2.1 Chemicals

All pesticides used, *viz*. glyphosate (CAS 1071-83-6), penoxsulam (CAS 219714-96-2), dimethoate (CAS 60-51-5), imidacloprid (CAS 138261-41-3), pyrimethanil (CAS 53112-28-0) and imazalil (CAS 35554-44-0), with purity above 98% (analytical standard), were obtained from Sigma-Aldrich Chemical Company (Spain). DNA lesion-specific repair enzymes, namely endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) were purchased from Professor Andrew Collins (University of Oslo, Norway). Trypan blue, proteinase K and all other chemicals were obtained from Sigma-Aldrich Chemical Company (Spain).

2.2.2 Animal maintenance and sperm extraction

Adult male red swamp crayfish specimens (*Procambarus clarkii*), with an average total length of 11.0 ± 0.9 cm, were collected at Rio Minho (Vila Nova de Cerveira, NW Portugal), a low impacted area concerning the presence of pesticides (Dagnac et al., 2012) as well as other inorganic and organic contaminants (Capela et al., 2016). In the laboratory, crayfish were kept in individual aquaria, during two weeks for acclimation, with the following water conditions: constant temperature (19 ± 1°C), freshwater (salinity 0) with aeration (dissolved oxygen 8.1 ± 0.5 mg L^{-1}), daily UV disinfection, weekly control of nitrites (0.06 ± 0.03 mg L^{-1}), nitrates (25 ± 6.0 mg L^{-1}) and ammonia (<0.1 mg L^{-1}) and pH (7.3 ± 0.2). Animals were daily fed *ad libitum* with crustacean feed, *Caridina Vita*, produced by Sparos® (Olhão, Portugal).

A total of 21 (7 x 3 assays/pesticide classes) crayfish were used to perform the assays. For sperm extraction, the vas deferent of each animal was excised and placed into 1.5 mL of cooled PBS, in a petri dish. Then, the distal section was gently pressed to release the sperm into the PBS medium, making a cell suspension that was immediately used (see point 2.3). Spermatozoa in crayfish consist of a huge acrosome, microtubular radial arms and nucleus, covered by an extracellular capsule, the spermatophore (Niksirat et al., 2013a, b).

2.2.3 *Ex vivo* assay set-up

An *ex vivo* exposure was performed to each pesticide class (*viz*. herbicides, insecticides, and fungicides), where two active principles of each were tested. Seven crayfish were used as sperm donors to perform each experiment (n=7).

Ex vivo incubations were carried out in 2 mL microtubes, for 2 hours, at 18 ± 1°C, with constant rotational movement (0.9 rpm) to avoid cells deposition. Briefly, 200 µL of

cell suspension in PBS (approx. 3 x 10^5 spermatozoa) were added to 1.8 mL of the proper medium, *i.e.*, PBS (control condition) or pesticide solution (prepared in PBS).

Two pesticide concentrations were selected based on environmentally relevant concentrations reported in the aquatic environment:

i) herbicides: glyphosate (organophosphate) 9 (G1) and 90 (G2) μg L^{-1} (Botta et al., 2009), and penoxsulam (triazolopyrimidine) 2.3 (P1) and 23 (P2) μg L^{-1} (Rodrigues and Almeida 2005 *apud* Murussi et al., 2014);

ii) insecticides: dimethoate (organophosphate) 2.4 (D1) and 24 (D2) μg L^{-1} (Van Scoy et al., 2016), and imidacloprid (neonicotinoid) 13.1 (I1) and 131 (I2) μg L^{-1} (Johnson and Pettis, 2014);

iii) fungicides: pyrimethanil (anilinopyrimidine) 2.2 (Py1) and 22 (Py2) μg L^{-1} (Verdisson et al., 2001), and imazalil (azole) 16 (Iz1) and 160 (Iz2) μg L^{-1} (Castillo et al., 2006).

At the end of the assay, cell viability was determined by the trypan blue exclusion method (Anderson et al., 2003; Strober, 2001). Sperm cell viability levels were above 90% in all experimental groups.

2.2.4 Genetic damage evaluation

At the end of *ex vivo* exposures, the 2.0 mL microtubes containing cell suspensions were centrifuged at 1,500 rpm, for 5 min, at 4°C. Then, the supernatant was removed, and the pellet was resuspended in 2.0 mL of glycerol (10%) to carry out the cryopreservation step (-20°C), for 48 h, to facilitate the cell lysis. This procedure is required due to the presence of the spermatozoon extracellular capsule. At the end of this step, samples were rapidly thawing at 37°C, the cryoprotectant solution was replaced by PBS, and the comet assay was initiated.

The comet assay alkaline version was performed based on the method presented by Collins (2004) with slight modifications according to Lacaze et al. (2010) and Shaposhnikov et al. (2010). Briefly, 60 µL of cell suspension was resuspended in 70 µL low melting point agarose (1%; dissolved in PBS). Twelve drops (gels) with 6 µL of cell suspension were placed on a pre-coated glass slide with 1% of normal melting-point agarose (1%; dissolved in distilled water), as two rows of 6 gels (6 groups of two replicates), without coverslips, containing approximately $2.2x10^3$ cells/gel. Gels were left for 5 min at 4°C to let agarose polymerase and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) overnight. To improve lysis step slides with gels were washed for 5 min in cooled PBS to remove lysis solution and then incubated with proteinase K (PK; 40 µg mL^{-1}) in the dark, at 37°C, for 60 min.

After PK incubation, three sets of slides were prepared and two of these were incubated with the endonucleases EndoIII or FPG, which convert oxidized pyrimidines and purines in DNA single-strand breaks, respectively (Azqueta et al. 2009), and a third was incubated only with endonuclease buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8). Slides were washed in endonuclease buffer for 3 times, 5 min each, at 4°C. Thirty µL of each endonuclease (diluted in endonuclease buffer) were placed in each gel. Gels were then immediately incubated in the dark at 37°C for 30 min. Gels without enzyme were incubated only with endonuclease buffer. After incubation, slides were gently placed in a horizontal electrophoresis tank, filled with freshly electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), for alkaline treatment. DNA was then allowed to unwind for 40 min. Electrophoresis was performed under 0.6 V cm^{-1} for 24 min. Lysis, DNA unwinding and electrophoresis were carried out in the dark, at 4°C. Once finished the electrophoresis, slides were washed in PBS (10 min), distilled water (10 min), and then gels were fixed for 15 min in absolute ethanol.

Slides were stained with ethidium bromide (20 g L^{-1}). Fifty nucleoids per gel were scored, using an Olympus BX 41 fluorescence microscope (400× magnification).

The DNA damage was quantified by visual scoring, where nucleoids were grouped into five discrete comet intensity and length classes (ranging from class 0, collecting nucleoids with no tail, to class 4 for nucleoids with almost all DNA in the tail; Collins 2004). A genetic damage indicator (GDI) was calculated as a major endpoint for each assay (GDI, $GDI_{EndoIII}$ and GDI_{FPG}) as follows:

$$\text{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where i is the number of each defined class (ranging within 0-4) and GDI values were inherently expressed as arbitrary units, in a scale of 0–400 per 100 scored nucleoids. The difference between $GDI_{EndoIII}$ and GDI (corresponding to $NSS_{EndoIII}$), as well as between GDI_{FPG} and GDI (corresponding to NSS_{FPG}), was calculated to indicate additional DNA breaks, which occur in net enzyme-sensitive sites solely (Azqueta et al. 2009).

2.2.5 Statistical analysis

Statistical analysis was performed with the software Statistica 7.0. Normality and homogeneity of variances were confirmed by Shapiro-Wilk's W and Brown-Forsythe (HOV) tests, respectively, in order to meet the required statistical demands, otherwise, a data transformation was applied. One-way ANOVA, followed by the post hoc Dunnett test, was used to compare treated groups with the corresponding control, by each experiment pesticide class. Additionally, the Student's t-test was used to compare treated groups (lower concentration *vs*. higher concentration) within the same pesticide. Differences between groups were considered as significant when $p < 0.05$ (Zar, 2010).

2.3 Results

The genotoxic assessment using the alkaline comet assay was expressed according to the genetic damage indicator (GDI) values, which translate non-specific DNA damage. With the incorporation of the lesion-specific repair enzymes EndoIII and FPG into the comet assay, it was possible to infer the specific occurrence of oxidative DNA damage, which was expressed by $GDI_{EndoIII}$ and GDI_{FPG}.

2.3.1 Herbicide assay

Considering glyphosate data, spermatozoa exposed to the higher concentration (G2) presented a significant increase (3-fold) of non-specific damage (GDI) in relation to the control group (Ct), as well as in relation to the lowest concentration (G1; 1.7-fold) (Fig. 16A). Oxidative damage measured as $GDI_{EndoIII}$ (Fig. 16B) and GDI_{FPG} (Fig. 16C) showed a pattern of results similar to GDI, with the exception that no differences between exposed groups were detected in the GDI_{FPG}. The net sensitive-sites (NSS) for each enzyme, $NSS_{EndoIII}$ (Fig. 16D) and NSS_{FPG} (Fig. 16E) showed a slight increase in both concentrations relatively to control, although without a statistical significance.

When exposed to penoxsulam, male gametes showed significantly higher GDI values for both concentrations (P1 and P2; 3.1 and 3.7-fold, respectively) in comparison to control (Fig. 1A). Oxidative damage assessment of P1 and P2 groups displayed as $GDI_{EndoIII}$ (Fig. 16B) and GDI_{FPG} (Fig. 16C), showed identical results to GDI, with increases in relation to control of 3.09 and 4.82-fold, respectively. In $GDI_{EndoIII}$, a significant difference was also found between P1 and P2, with the latter group displaying higher values (1.6-fold). The NSS values showed no statistically significant differences relative to control group or between treatments. However, P2 had a slight $NSS_{EndoIII}$ increase relative to the control group (Fig. 16D; P1 had no measurable net sensitive-sites) as well as NSS_{FPG} for P1 and P2 (Fig. 16E).

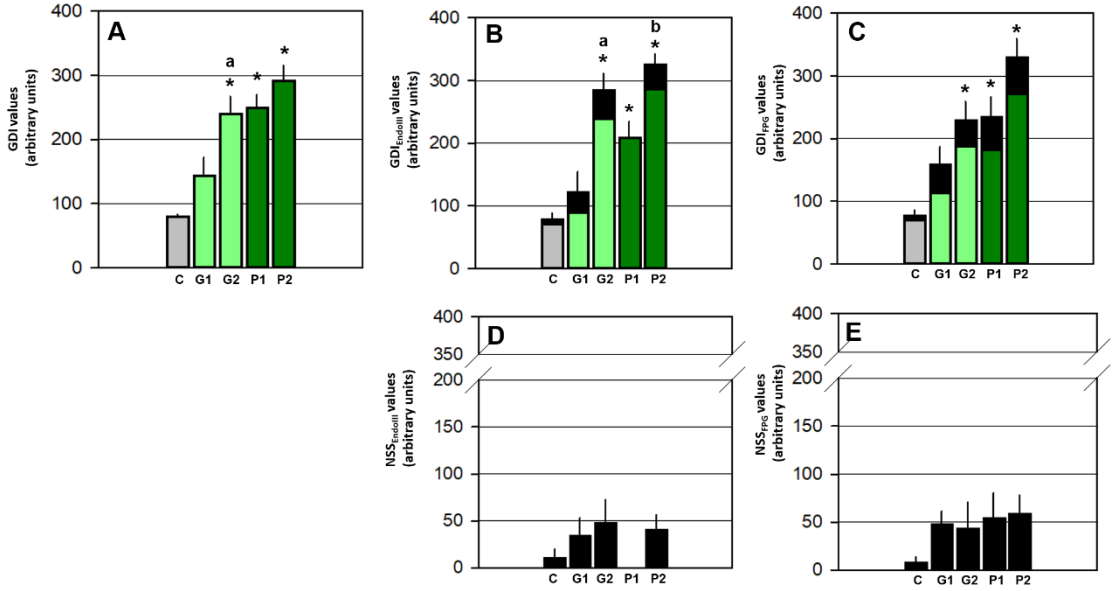

Fig. 16. Herbicides genotoxic assessment on crayfish spermatozoa. Mean values of spermatozoa DNA damage measured by alkaline comet assay on male gametes of *P. clarkii* exposed to 9 (G1) and 90 µg L^{-1} (G2) of glyphosate (light green), and to 2.3 (P1) and 23 µg L^{-1} (P2) of penoxsulam (dark green). Panels represent: (A) genetic damage indicator of non-specific DNA damage (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI (green) and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; black); (C) global and partial DNA damage (GDI_{FPG}), *i.e.*, GDI (green) and additional strand breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (D) $NSS_{EndoIII}$; (E) NSS_{FPG}. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) *vs.* control (Ct); (a) *vs.* G1; (b) *vs.* P1.

2.3.2 Insecticide assay

Spermatozoa exposed to dimethoate presented statistically significant increases in GDI levels for both D1 and D2 groups (2.2- and 2.1-fold-increment, respectively), in comparison with the control group (Fig. 17A). Similarly, oxidative damage measured as $GDI_{EndoIII}$ (Fig. 17B) displayed significant increases in both D1 and D2 groups (1.94- and 2.1-fold, respectively), comparing to the control group. Concerning GDI_{FPG} (Fig. 17C), no statistically significant differences were found. It is important to clarify that only D1 values were available as GDI_{FPG} since the sample corresponding to D2 had a mishap. Regarding $NSS_{EndoIII}$, D1 group had a slight decrease and D2 had a slight increase in relation to the control group (Fig. 17D). In NSS_{FPG}, like in $NSS_{EndoIII}$, D1 had a slight

decrease relative to the control group (Fig. 17E). The differences found in $NSS_{EndoIII}$ and NSS_{FPG} were not significantly different.

Considering imidacloprid results, no significant differences were found between exposed and control groups as GDI (Fig. 17A). On the other hand, regarding the oxidative damage presented as $GDI_{EndoIII}$ (Fig. 17B) and GDI_{FPG} (Fig. 17C), I1 group showed significantly higher values than control (2.2- and 1.6-fold, respectively). In $NSS_{EndoIII}$, I1 and I2 group had an identical increase in relation to the control group (Fig. 17D). In NSS_{FPG}, I1 and I2 groups had a slight decrease in relation to control (Fig. 17E). Even though, differences were not significant in both $NSS_{EndoIII}$ and NSS_{FPG} parameters.

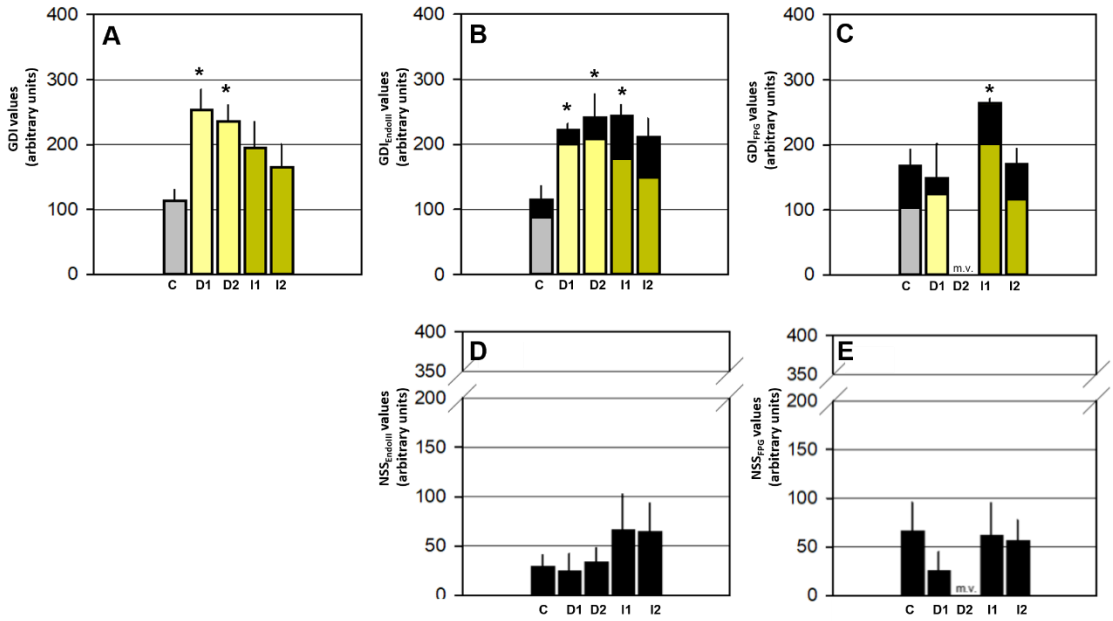

Fig. 17. Insecticides genotoxic assessment on crayfish spermatozoa. Mean values of spermatozoa DNA damage measured by alkaline comet assay on male gametes of *P. clarkii* exposed to 2.4 (D1) and 24 µg L^{-1} (D2) of dimethoate (light yellow), and to 13.1 (I1) and 131 µg L^{-1} (I2) of imidacloprid (dark yellow). (A) Genetic damage indicator of non-specific DNA damage, (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI (yellow) and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; black); (C) global and partial DNA damage (GDI_{FPG}), *i.e.*, GDI (yellow) and additional strand breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (D) $NSS_{EndoIII}$; (E) NSS_{FPG}. (m.v.) missing value. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) *vs.* control (Ct).

2.3.3 Fungicide assay

Following pyrimethanil exposure, only Py2 presented a significant DNA damage increment (1.37-fold), when compared with control, as GDI (Fig. 18A). In both $GDI_{EndoIII}$ (Fig. 18B) and GDI_{FPG} (Fig. 18C) parameters, no significant differences were displayed. Concerning $NSS_{EndoIII}$, Py1 and Py2 had a slight increase in relation to control group (Fig. 18D), but in NSS_{FPG}, both groups had a slight decrease also in relation to control (Fig. 18E). There were, also, no significant differences between treatments and the control group.

In what concerns imazalil, spermatozoa exposed to the higher concentration (Iz2) displayed significantly increased GDI values (1.55-fold) (Fig. 18A), compared to the control group. Moreover, Iz2 presented a significantly higher GDI value, 1.3-fold, comparing to Iz1. In relation to oxidative damage, Iz2 presented significantly higher $GDI_{EndoIII}$ values in relation to control (1.47-fold) and Iz1 (1.34-fold) groups (Fig. 18B). A different pattern was observed in GDI_{FPG}, where both exposed groups (Iz1 and Iz2) presented significantly elevated levels (1.2-fold) in relation to the control (Fig. 18C). Differently, to pyrimethanil groups, Iz1 and Iz2 had a slight decrease in $NSS_{EndoIII}$ (Fig. 18D) and a slight increase in NSS_{FPG} (Fig. 18E). Although here also the differences were not significant.

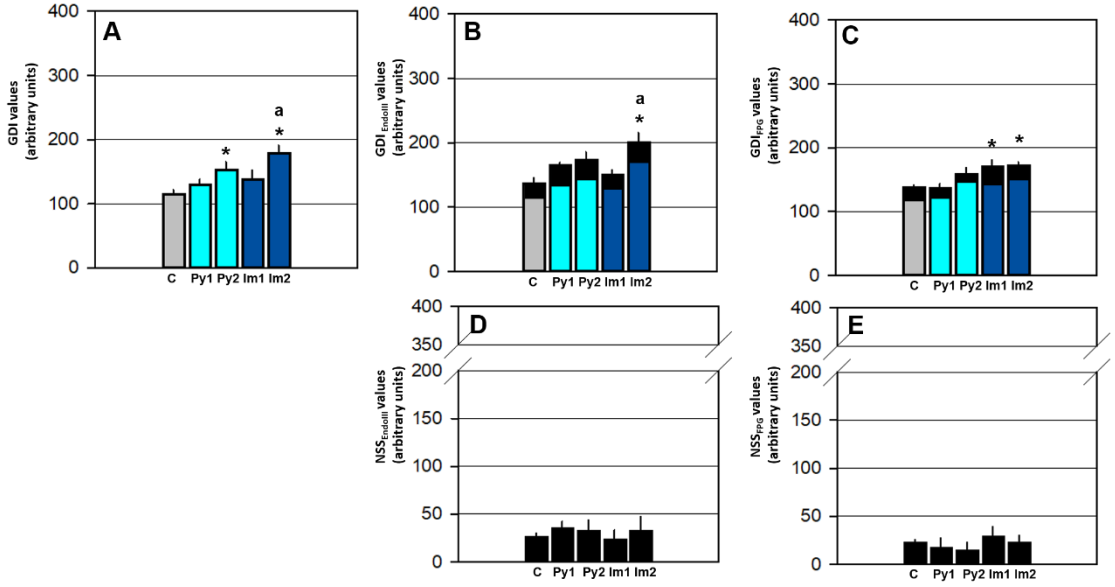

Fig. 18. Fungicides genotoxic assessment on crayfish spermatozoa. Mean values of spermatozoa DNA damage measured by alkaline comet assay on male gametes of *P. clarkii* exposed to 2.2 (Py1) and 22 µg L^{-1} (Py2) of pyrimethanil (light blue), and to 16 (Iz1) and 160 µg L^{-1} (Iz2) of imazalil (light blue). (A) Genetic damage indicator of non-specific DNA damage (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI (blue) and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; black); (C) global and partial DNA damage (GDI_{FPG}), *i.e.*, GDI (blue) and additional strand breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; black); (D) $NSS_{EndoIII}$; (E) NSS_{FPG}. Bars represent the standard error. Statistically significant differences ($p<0.05$) are: (*) *vs.* control (Ct); (a) *vs.* Iz1.

2.4 Discussion

Studies on pesticide toxicity based on LC50 (or LD50) values, despite their recognized usefulness, may lack some relevance and realism since it can be unrelated to the real concentrations found in the aquatic environment. Therefore, in this study, environmentally relevant waterborne concentrations were considered to assess the genotoxic potential of widely applied pesticides on the male germ cells of *Procambarus clarkii*.

During mating, at a given moment and even during a short period of time, the male gametes of *P. clarkii* are in direct contact to the surrounding water, exposing them

to the waterborne contaminants (McLay and van den Brink, 2016). Based on this information, and with the intuit of mimics what happens on the aquatic environment, the *ex vivo* approach was adopted as an alternative cell-based methodology. The results revealed that this approach allowed achieving a considerable amount of information, reducing costs and time consumed, where six pesticides (two concentrations of each) were tested, using a reduced number of individuals. In this direction and in line with the reduction goal of the 3R's principles, it should be highlighted that an 80% reduction on the number of animals sacrificed was achieved (21 crayfish instead of 105 that would be required for the equivalent experiment adopting the *in vivo* approach). Overall, the proposed *ex vivo* approach demonstrated its effectiveness, considering jointly the cell viability (trypan blue exclusion method) and the DNA integrity (comet assay) appraisals, as values > 90% were ensured for cell viability and, for instance, the capacity to detect effects at low levels of exposure and to distinguish pesticide concentrations differing by one order of magnitude (10x).

2.4.1 Spermatozoa DNA damage induced by herbicides

The existing research upon glyphosate reprotoxicity concerns mainly vertebrates (Anifandis et al., 2018; Lopes et al., 2014; Sánchez et al., 2017) while most of the studies in invertebrates addressed other parameters than genotoxicity, such as endocrine disruption and alterations on spermatogenesis (*e.g*. Gonçalves et al., 2018; Lopes et al., 2014; Nardi et al., 2017; Yousef et al., 1995). Specifically, Omran and Salama (2013) conducted a study with the freshwater mollusk *Biomphalaria alexandrina* where it was demonstrated that glyphosate interferes with testosterone, leading to azoospermia.

To the authors' knowledge, the only study regarding invertebrates' sperm cells in this framework was developed by Akcha et al. (2012), who tested glyphosate and diuron on the oyster. The glyphosate concentrations tested by Akcha et al. (2012) were below the maximum levels reported in the environment (Botta et al. 2009) and no

genotoxic effects were detected, which agrees with the current observations for the lower tested concentration. In the present study, the higher concentration of glyphosate induced DNA damage on crayfish sperm cells, pointing out a concentration-dependent profile of response.

Webster et al. (2014) detected an up-regulation of the mRNA of antioxidant enzymes in the gonads of zebrafish males exposed to glyphosate (10 000 μg L^{-1}), suggesting possible oxidative stress in the gonads. Inline, Lopes et al. (2014) hypothesized that a redox impairment and oxidative stress could be the mechanism responsible for glyphosate-induced damage to lipids and DNA in zebrafish *Danio rerio* spermatozoa. In addition, Guilherme et al. (2010) described the glyphosate potential to oxidatively damage DNA in blood cells of the teleost *Anguilla anguilla*. Given these previous findings, and in view of the knowledge about the low DNA repair capacity of male gametes, in the present study, it should be expected the occurrence of oxidative DNA damage. However, this hypothesis was not confirmed by the current $NSS_{EndoIII}$ and NSS_{FPG} data, which can be explained by the fact that the effects reported by Webster et al. (2014) and Lopes et al. (2014) concern a concentration around 100 times higher than that currently tested (and the maximum level measured in the environmental waters as well). Anyhow, it is possible to observe an increasing tendency of these parameters in the exposed groups, advising that the absence of a glyphosate pro-oxidant action on the crayfish sperm cells shouldn't be assumed as a closed question.

Penoxsulam is the most recent pesticide in the market among those tested in the present study. Consequently, its genotoxic potential has been scarcely addressed and no scientific studies focused on gametes were found, which reinforces the novelty of this research. Nevertheless, the genotoxicity of this compound to somatic cells, following *in vivo* exposures at environmentally realistic concentrations, was previously described. Patetsini and co-workers (2013) demonstrated the penoxsulam genotoxicity in *Mytilus galloprovincialis* hemocytes. Also, Costa et al. (2018) exposed two populations of

Procambarus clarkii (from a pesticide-free local *vs*. from a penoxsulam contaminated area) to a penoxsulam-based herbicide (Viper®), demonstrating that only that historically impacted presented DNA damage on gill cells. Hence, the penoxsulam genotoxic potential to sperm cells is described for the first time in the present study. Moreover, this compound could be considered highly genotoxic to crayfish's male gametes, since even the lower tested concentration (2.3 μg L^{-1}), which is 10 times below of the environmental found concentration, induced DNA damage.

Regarding the penoxsulam pro-oxidant potential, the present results tone down the assumption of those processes as key determinants of the DNA damage measured (despite the overall tendency towards NSS rise in treated groups and the $GDI_{EndoIII}$ increase of in P2 group when compared to P1). Moreover, no studies are available pointing out penoxsulam as an oxidative stress inducer.

2.4.2 Spermatozoa DNA damage induced by insecticides

To the authors' knowledge, there are no studies relatively to dimethoate genotoxicity on male gametes, concerning both invertebrates and vertebrates. Regarding dimethoate spermiotoxicity in vertebrates, a few studies reported pernicious effects, such as a decreased sperm cell concentration (Hassanin and El Asely, 2015), reduced motility, low viability, and increased abnormal morphology (Jallouli et al., 2015). Additionally, and regarding the eventual effect of dimethoate on the progeny fitness, a study with the freshwater crustacean *Daphnia magna*, involving a parental exposure, demonstrated that high concentrations could lead to a reduction in number and weight of offspring, strengthening the idea that this insecticide can affect germ cells (Andersen et al., 2006). Despite their intrinsic interest, these studies tested dimethoate concentrations highly above those found in the aquatic environment.

The present study, as the first linking dimethoate spermiotoxicity and DNA integrity, reinforced its biological risk since environmentally relevant concentrations

showed to be genotoxic to male gametes of *P. clarkii*, even at the lower concentration, regarding both GDI and $GDI_{EndoIII}$. Regarding the oxidative damage assessment, dimethoate did not come out as a pro-oxidant, since, unlike the previous pesticides, the NSS parameters did not show any increasing tendency.

Again, in what concerns imidacloprid genotoxic effects on male gametes, there is a lack of information. The few existing studies pointed this compound as reprotoxic to vertebrates (Lonare et al., 2016; Xia et al., 2016). Particularly in rats, imidacloprid caused spermatozoa malformations as well as a reduction in spermatozoa number and viability (Lonare et al., 2016). In aquatic animals, imidacloprid induced histopathological alterations on testis and a low sperm amount in fish (*Misgurnus anguillicaudatus*) (Xia et al., 2016). In the mentioned studies, the concentrations tested were found to be very high and environmentally non-relevant. So, the information provided should be taken cautiously to predict the real biological and environmental impact of imidacloprid.

Despite the absence of non-specific DNA damage, displayed by GDI parameter for both imidacloprid tested concentrations, the present findings bring new insights regarding the oxidative DNA damage occurrence on *P. clarkii*'s spermatozoa. NSS data did not unequivocally corroborate this pathway, but the observed increasing tendency for both tested concentrations presented by the $NSS_{EndoIII}$ parameter should not be neglected. Furthermore, the analysis of the $GDI_{EndoIII}$ and GDI_{FPG} parameters demonstrated that imidacloprid, at the lower tested concentration, might act as a pro-oxidant, affecting equally pyrimidines and purines. Moreover, an inverse concentration-effect relationship was apparent, mainly on the induction of purine lesions. This response profile has been frequently described in different somatic cells and explained on the basis of the modulation of the DNA damage repair. However, this explanation cannot be extended to spermatozoa due to the lack of DNA repair machinery in these cells (Dixon et al., 2002; Lewis and Ford, 2012; Mai et al., 2013). So, the inverse concentration-effect relationship observed for oxidatively damaged DNA may be justified by the action of the

antioxidant defense system of the sperm cell. In a recent scientific review concerning reactive oxygen species (ROS) and antioxidants in invertebrates and vertebrates (Len et al., 2019), it was described that, in the presence of oxidative events (*e.g.* cryopreservation) for sperm cells, the antioxidants catalase and GSH play a protective role, leading to a decrease in DNA damage. Therefore, in the present study, the high concentration of imidacloprid promoted the threshold conditions enabling the activation of the cellular antioxidant defenses, in contrast with the lower concentration where that threshold was not reached, and therefore, the oxidative damage on sperm cell DNA was barred.

2.4.3 Spermatozoa DNA damage induced by fungicides

Considering that pyrimethanil was described as causing reproduction and embryonic development impairments in a freshwater snail (*Physella acuta*) (Seeland et al., 2013) and that reprotoxicity could be related to DNA breaks on spermatozoa (Devaux et al., 2015), it would be conceivable to hypothesize its genotoxic potential to invertebrate male gametes.

As an important contribution, the current study demonstrated a mild spermiotoxic potential of pyrimethanil, relying on the ability of the higher concentration tested (22 µg L^{-1}) to exert genotoxic damage (measured only as GDI) to spermatozoa of *P. clarkii*. In what concerns the involvement of oxidative pressures, it can be regarded as a negligible DNA damage mechanism associated with this compound, within the tested conditions.

A few studies have been carried out concerning imazalil male reprotoxicity in invertebrate and vertebrate species, though it is noteworthy the absence of genotoxic studies. Pennati and co-workers (2006) pointed out imazalil as a spermiotoxic agent in *Phallusia mammillata* (ascidian), translated into a decrease in sperm viability, inhibition in fertilization and impairment of embryological development, though an environmentally unrealistic concentration was tested.

Here, imazalil showed to be genotoxic to crayfish male gametes. In addition, it was also shown that the spermiotoxic effect is dose-dependent (see GDI and $GDI_{EndoIII}$). DNA oxidation seems not to be a prominent damaging process for imazalil, though the observation that, for the lower concentration, DNA breaks occurred only in the presence of FPG recommends some caution on this assertion.

2.4.4 The potential impact of pesticides on the crayfish population success

The fact that male gametes have a high content in unsaturated fatty acids, including in crayfish (Subramoniam, 1993), makes them highly susceptible ROS (Luño et al., 2015). Additionally, the DNA repair machinery down-regulation during late spermatogenesis, namely on spermiogenesis phase (González-Marín et al., 2012), leads to a narrower range of possibilities to mitigate the genotoxic impact in spermatozoa. Nevertheless, some studies pointed that DNA repair machinery of the oocyte, and also of the early embryos, could have also the capacity to repair the male gametes' DNA, despite depending on the damage amount (the oocyte machinery only repair spermatozoa DNA if harmed less than 8%) (Baumgartner et al., 2007; González-Marín et al., 2012) and nature (*e.g.* Single-stranded DNA breaks and DNA adducts induced by oxidative stress are repaired but double-stranded DNA breaks may not) (García-Rodríguez et al., 2019; Kumar et al., 2017).

On the other hand, during spermiogenesis, the ability to complete apoptosis is also lost, meaning that gametes with severe DNA damage cannot be eliminated, which could lead, for instance, to unsuccessful fertilization (González-Marín et al., 2012; Lacaze et al., 2011).

As stated above, reprotoxicity was causally associated with DNA breaks on spermatozoa (Devaux et al., 2015). Moreover, DNA damage in these cells can have a severe impact on fertilization rate, embryonic development and offspring number, and, consequently, on population fitness (Baumgartner et al., 2007; González-Marín et al.,

2012; Kumar et al., 2017; Lewis and Ford, 2012; Lewis and Galloway, 2009; Lonare et al., 2015). Bearing in mind the limiting physiological features of spermatozoa above mentioned and the observed genotoxic effects caused by all the tested pesticides, it can be inferred the likelihood of ecological risk to the *P. clarkii* populations, even when short exposures are considered. The possibility of a risk overestimation cannot be neglected as the above-mentioned role of the DNA repair machinery lent by the oocyte was not into play in the current study. As mentioned above single-stranded DNA breaks (SSBs) are a direct consequence of oxidative attacks on sperm DNA (González-Marín et al., 2012) and low levels of SSBs are easily repaired by the oocyte (García-Rodríguez et al., 2019). Thus, if the DNA oxidized bases present in the groups I1 ($GDI_{EndoIII}$ and GDI_{FPG}) and Iz1 (GDI_{FPG}) become single-stranded breaks they could be repaired by the oocyte machinery.

Overall, it is possible to propose a pesticide hierarchy in terms of ecological risk as follows: penoxsulam = dimethoate > imazalil > glyphosate = pyrimethanil > imidacloprid.

It must be also kept in mind that pesticides utilization is not only based on the application of the active ingredients (Demetrio et al., 2014; Guilherme et al., 2015), but also on commercial formulations, which may be complex chemical mixtures. Since the commercial formulations were not tested herein, it was not possible to assess the eventual additive, synergistic or antagonistic effects. Furthermore, and considering the adopted experimental design, there is also a lacuna concerning the evaluation of the inherent effects of the corresponding metabolites generated by active compounds decomposition, which is also an approach ecologically relevant (Guilherme et al., 2014; Guzzella et al., 1997).

Future studies should look at the formulation of more specific pesticides, with less impact on non-target species and on the aquatic environment.

2.5 Conclusion

The genotoxicity evaluation of waterborne pesticides on spermatozoa of *Procambarus clarkii*, a non-target organism, was assessed after a direct exposure. In this spermiotoxic screening study, the *ex vivo* approach proved to be very effective since it allowed to point out a genotoxic impact of pesticides and to distinguished DNA damage magnitudes between different levels of exposure.

Environmental relevant concentrations of glyphosate, penoxsulam, dimethoate, pyrimethanil and imazalil showed spermiotoxic properties through a DNA damaging action. Moreover, imidacloprid revealed to oxidatively damage DNA.

Spermatozoa in crayfish during and after mating are protected against environmental stresses by the spermatophore layers. Hence, further investigation involving the exposure of spermatozoa with intact spermatophores is recommended to clarify their protective role against potential genotoxins in freshwater crayfish before fertilization.

Overall, the present study highlighted the potential ecological impact of pesticides on non-target species, such as *P. clarkii*, compromising sperm DNA integrity and, subsequently, the population success.

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Chapter III

Improving knowledge on genotoxicity dynamics in somatic and germ cells of crayfish (*Procambarus clarkii*)

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3. Improving knowledge on genotoxicity dynamics in somatic and germ cells of crayfish (*Procambarus clarkii*)

Keywords

Freshwater crustacea; Ecogenotoxicity; Long-term effects; Post-exposure period; Herbicide.

Abstract

The harmful effects of pesticides can be extended beyond the exposure time scale. Appraisals combining exposure and long-term post-exposure periods appear as an unavoidable approach in pesticide risk assessment, thus allowing a better understanding of the real impact of agrochemicals in non-target organisms. This study aimed to evaluate the progression of genetic damage in somatic and germ tissues of the crayfish *Procambarus clarkii*, also seeking for gender-specificities, following exposure (7 days) to penoxsulam (23 μg L^{-1}) and a post-exposure (70 days) period. The same approach was applied to the model genotoxicant ethyl methanesulfonate (EMS; 5 mg L^{-1}) as a complementary mean to improve knowledge on genotoxicity dynamics (induction *vs.* recovery). Penoxsulam induced DNA damage in all tested tissues, disclosing tissue- and gender-specificities, where females showed to be more vulnerable than males in the gills, while males demonstrated higher susceptibility in what concerns internal organs, *i.e.*, hepatopancreas and gonad. Crayfish were unable to recover from the DNA damage induced by EMS in gills and hepatopancreas (both genders) as well as in spermatozoa. The genotoxicity in the hepatopancreas was only perceptible in the post-exposure period. Oxidative DNA lesions were identified in hepatopancreas and spermatozoa of EMS-exposed crayfish. The spermatozoa proved to be the most vulnerable cell type. It became clear that the characterization of the genotoxic hazard of a given agent must integrate a complete set of information, addressing different types of DNA damage,

tissue- and gender-specificities, as well as a long-term appraisal of temporal progression of damage.

3.1 Introduction

Pesticide application is an important procedure in intensive agriculture, as an attempt to respond to global food demand. According to the report data of regulation No 1107/2009 (European Union; EU), there are almost 500 active substances (pesticides) approved in Europe, with a substantial number belonging to the class of herbicides (Amat et al., 2013). Though recognizing a trade-off between environmental impact and productivity benefits, a critical and elucidative analysis of the pesticide regulation was carried out by Milner and Boyd (2017), establishing a parallel with pharmaceuticals due to the similarities in terms of society dependence on their use, risk of inadequate/unproportionate applications, and the need to sustain high standards of human and environmental safety. Milner and Boyd (2017) concluded that pharmaceuticals and pesticides are differently regulated after approval, with the latter being monitored far less effectively. Surprisingly and worryingly, it stands out that post-approval or long-term monitoring of the effects is not clearly imposed to the pesticide authorization holders by the EU and United States regulations. Bearing this in mind, the role of environmental research emerges as a key element while the combination of exposure and long-term post-exposure appraisals, using non-target organisms, becomes a critical approach for pesticide risk assessment, allowing for a better understanding of the real impact of these agrochemicals on the impacted wild populations.

Due to their chemical and physical properties, herbicides can reach adjacent environmental compartments through surface runoff, leaching and erosion (Khan and Law, 2005). Consequently, their dispersion from land could lead to contamination of aquatic habitats. Thus, the toxicity of herbicides on aquatic organisms is a major concern

all around the world (Lushchak et al., 2018). Several studies in aquatic invertebrates have demonstrated that herbicides can have noxious effects on endocrine (Lagadic et al. 2007; Omran and Salama 2013) and nervous systems (Matozzo et al., 2018), also effecting antioxidant and ROS levels (Cochón et al., 2007), as well as DNA integrity (Costa et al., 2018; Patetsini et al., 2013). In fact, some herbicides have a pro-oxidant potential, which can induce oxidative DNA damage (Barranger et al., 2016; Marques et al., 2014).

The DNA damaging actions can lead to DNA adducts, single and/or double strand breaks, and in some cases base lesions can cause depurination or depyrimidination (Phillips and Arlt, 2009). In addition, the fate of lesions will depend upon repair and replacement of damaged cells (El-Bibany et al., 2014). Since DNA plays a pivotal role on organism's development, survival and reproduction, its integrity and stability are critical factors to constantly consider in the assessment of environmental hazard of herbicides.

Penoxsulam, a triazolopyrimidine herbicide, originally developed for weed control in rice cultivation, is also registered for use on turf, trees, vines and to control aquatic vegetation (Billington et al., 2010). It was classified as a reduced-risk herbicide by the US-EPA, showing no evidence of genotoxic potential measured by mutational and cytogenetics methodologies, such as Ames and micronucleus tests (Billington et al., 2010). However, another tool used to evaluate DNA integrity - the alkaline comet assay - demonstrated that exposures to environmentally relevant concentrations of penoxsulam generate DNA strand breaks in bivalves (alone, as active ingredient) (Patetsini et al. 2013) and crayfish (as a penoxsulam-based commercial formulation) (Costa et al., 2018).

Freshwater crustaceans have been suggested to be suitable sentinel species for genotoxicological studies due to their response to toxicants (Donato et al., 2016). Moreover, Stara and co-workers (2016) highlighted that crayfish are an interesting alternative to aquatic vertebrates, since they provide comparable tissues for analysis and similar responsivity to environmental pollution. The red swamp crayfish is currently

considered to be a major freshwater pest and is present in at least 15 European countries (Souty-Grosset, 2016). This species, which can inhabit highly impacted areas [contaminated, for instance, with herbicides (Anastácio, 1993; Costa et al., 2018)], became an appropriate non-model organism for ecogenotoxicological research. Gill's crayfish are directly exposed to waterborne herbicides, playing thus an important toxicokinetic role (Grosell et al., 2002; Henry et al., 2012; Peterson, 2015), while hepatopancreas plays similar functions to liver and pancreas of aquatic vertebrates. This tissue/organ, thus, represents the main structure of crayfish for metabolism and detoxification, leading to the elimination of xenobiotics that may enter the body. Regarding germ cells, the DNA integrity of spermatozoa appears as a crucial factor to the reproductive fitness and, ultimately, to the success of populations, but it remains poorly explored within the context of crayfish response to aquatic stressors, including pesticides.

Ecogenotoxicological studies concerning the effects of herbicides on non-target organisms are scarce. Only a few have gone beyond studies of the immediate effects of exposure to investigate long-lasting consequences, including following exposure cessation (*e.g.* Guilherme et al., 2014; Marques et al., 2014). To the authors' knowledge, there is no information regarding the progression of DNA damage in invertebrates during post-exposure periods. Nevertheless, in the fish *Anguilla anguilla*, following the exposure to environmentally realistic concentrations of a glyphosate-based herbicide, liver cells were able to recover from the genetic damage within one day (Marques et al., 2014), while in blood cells, the DNA integrity only returned to basal levels after 14 days, though oxidative DNA damage was still detectable (Guilherme et al., 2014). These outcomes showed that different tissues present distinct response patterns to the same genotoxicant. Therefore, within the context of pesticide genotoxicity evaluation, the integration of tissue-specific vulnerabilities must be considered.

Considering the gaps in scientific knowledge of penoxsulam toxicity, the focus of the present work is on progression of the genetic damage in different tissues (somatic

and germ cells) of the crayfish *P. clarkii*, upon a waterborne exposure and a subsequent post-exposure period. The aims of this study are to (i) assess tissue-specific DNA damage (including oxidative DNA damage) on somatic cells (gills and hepatopancreas) and male gametes; (ii) identify gender specificity; (iii) evaluate the progression of the DNA damage in crayfish upon a long-term post-exposure period; (iv) increase knowledge concerning the actual magnitude of risk posed by this herbicide to crayfish populations. For these purposes, DNA damage was assessed through the alkaline comet assay with the incorporation of the DNA lesion-specific bacterial repair enzyme endonuclease III (EndoIII) (Collins, 2004). The alkaline comet assay allows the detection of unspecific DNA damage (such as alkali-labile sites, single and double strand breaks), as well as the detection of specific DNA lesions, such as oxidized bases, through the addition of the EndoIII. Furthermore, the model genotoxicant ethyl methanesulfonate (EMS), already tested in invertebrates (Carmona et al., 2011; Kumar et al., 2014) including crayfish (Costa et al., 2018), was used as a complementary approach to improve knowledge on genotoxicity dynamics (induction *vs*. recovery).

3.2 Materials and methods

3.2.1 Chemicals

Penoxsulam (CAS: 219714-96-2) and ethyl methanesulfonate (EMS; CAS: 62-50-0) were obtained from Sigma-Aldrich Chemical Company (Spain). The DNA lesion-specific repair enzyme endonuclease III (EndoIII) was purchase from Professor Andrew Collins (University of Oslo, Norway). All other chemicals were obtained from Sigma-Aldrich Chemical Company (Spain).

3.2.2 Animal maintenance

Adult specimens of *Procambarus clarkii* (commonly known as red swamp crayfish of Louisiana), from both genders, with an average length of 11.00 ± 0.89 cm, were collected at Rio Minho (Vila Nova de Cerveira, NW Portugal), a low impacted area concerning the presence of pesticides, as well as inorganic and organic contaminants (Castro-Valencia et al., 2019; Rocha et al., 2021; Vera et al., 2021). In the laboratory, crayfish were kept in aquaria (60 L), separated by gender, during two weeks for acclimation, with the following water conditions: constant temperature (19 ± 1°C), freshwater (salinity 0), with aeration (dissolved oxygen 8.1 ± 0.5 mg L^{-1}), UV disinfection, weekly control of nitrites (0.06 ± 0.03 mg L^{-1}), nitrates (25 ± 6.0 mg L^{-1}) and ammonia (<0.1 mg L^{-1}) and pH (7.3 ± 0.2). During acclimation period, animals were daily fed *ad libitum* with the crustacean feed Caridina Vita, produced by Sparos® (Olhão, Portugal).

3.2.3 Experimental assay

To respond to the defined objectives, the experimental design encompassed two periods: exposure and post-exposure, with the duration of 7 and 70 days, respectively. An equal number of males and females were randomly separated into three groups: (i) exposed to 23 µg L^{-1} of penoxsulam (Px group), (ii) exposed to 5 mg L^{-1} of the model genotoxicant EMS (EMS group), and (iii) maintained in uncontaminated water (NC; negative control group). In the exposure period, animals were kept in individual aquaria (1 L) and experimental medium was renewed daily (100%). At the end of the exposure period, 7 animals from each condition and gender were sampled. Thereafter, to perform the post-exposure evaluation, 10 crayfish from each condition and gender were transferred to uncontaminated water and sampled after 70 days. The post-exposure period was intended to offer the animals in captivity conditions as close as possible to those experienced in the wild, mimicking a recovery in the field. For this reason, once the animals had reached sexual maturity, the temperature was set to 23°C and animals

were grouped in couples to allow reproduction. Individualized couples were kept in 60-L tanks (3-4 couples per tank). Every two days, the water conditions were checked to maintain the medium in similar conditions to the acclimation period, see section 2.2, and the water was completely renewed weekly. Following spawning (around 35 to 45 days after couples' formation), each crayfish was isolated in 1 L aquaria.

At each sampling time, crayfish were sacrificed, and somatic tissues (gills and hepatopancreas, in both genders) and vas deferent (in males) were excised. Briefly, after the carapace removal, gills and hepatopancreas were collected in 2.0 mL of chilled PBS in a petri dish (separately). Both somatic tissues were carefully cut and triturated to create a cell suspension. Finally, for sperm extraction, each excised vas deferent was placed into 2.0 mL of cooled PBS. Then, the distal section was gently pressed to release the mature sperm into the PBS medium, making a cell suspension. A volume of 1.5 mL of spermatozoa cell suspension was centrifuged at 200 g, for 5 min, at 4°C. Then, the supernatant was removed, and the pellet was resuspended in 2.0 mL of glycerol (10%) to carry out the cryopreservation step (-20°C), for 48 h, to facilitate the cell lysis. This procedure is required due to the presence of a spermatozoa extracellular capsule. At the end of this step, samples were rapidly thawed at 37°C, the cryoprotectant solution was replaced by PBS, and the comet assay procedure was initiated.

In the exposure period, there was no mortality in the negative control group. Groups exposed to penoxsulam and EMS had no mortality in males, although there was 10 and 20% mortality in females, respectively. In the post-exposure period, there was no mortality in females from the negative control group, although there was 10% mortality in males of the negative control group and in groups pre-exposed to penoxsulam and EMS (both genders).

3.2.4 DNA damage assessment

The alkaline version of the comet assay was performed based on the method presented by Collins (2004) with slight modifications according to Lacaze et al. (2010) and Costa et al. (2018). Briefly, 20 µL of cell suspension for gills and hepatopancreas, or 60 µL of spermatozoa suspension, were resuspended in 70 µL low melting point agarose (1%; dissolved in PBS). Eight drops with 6 µL of cell suspension were placed on a pre-coated glass slide with 1% of normal melting-point agarose (dissolved in distilled water), as two rows of 4 gels (4 groups of two replicates). Gels were left for 5 min at 4°C to let agarose polymerize and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) overnight. To improve spermatozoa lysis step, slides were washed for 5 min in cold PBS to remove lysis solution and then incubated with proteinase K (40 µg mL^{-1} dissolved in ultrapure water) at 37°C, for 60 min.

After lysis, two sets of slides were prepared and one of these was incubated with the endonuclease III (EndoIII), to convert oxidized pyrimidines in DNA single-strand breaks (Azqueta et al., 2009), and the other set was incubated only with endonuclease buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8). Slides were washed in endonuclease buffer 3 times, 5 min each, at 4°C. Thirty µL of each endonuclease (diluted in endonuclease buffer) were placed in each gel. Gels were then immediately incubated in the dark at 37°C for 30 min. Gels without enzyme were incubated only with endonuclease buffer. After incubation, slides were gently placed in a horizontal electrophoresis tank, filled with fresh electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), for alkaline treatment. Due to cell specificities, gills DNA and hepatopancreas DNA were then allowed to unwind for 20 min, and spermatozoa DNA for 40 min. Electrophoresis was performed under 1.04 V cm^{-1} for 15 min (for gills samples), 0.83 V cm^{-1} for 20 min (for hepatopancreas samples), and 0.6 V cm^{-1} for 24 min (for spermatozoa samples). Lysis, DNA unwinding, and electrophoresis were carried out in the dark at 4°C. Once electrophoresis was finished, slides were washed in PBS (10 min)

followed by distilled water (10 min), and then gels were fixed for 15 min in absolute ethanol.

Slides were stained with ethidium bromide (20 g L^{-1}). Fifty nucleoids per gel were scored, using a Leica DMLS fluorescence microscope (400× magnification). The DNA damage was quantified by visual scoring, where nucleoids were grouped into five discrete comet intensity and length classes (ranging from class 0, collecting nucleoids with no tail, to class 4 for nucleoids with almost all DNA in the tail; Collins 2004). A genetic damage indicator (GDI), was calculated as a major endpoint for each assay as follows:

$$\text{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where i is the number of each defined class (ranging within 0-4) and GDI values were inherently expressed as arbitrary units, in a scale of 0–400 per 100 scored nucleoids. The difference between $GDI_{EndoIII}$ (genetic damage indicator of slides treated with EndoIII) and GDI was calculated to indicate additional DNA breaks (corresponding to $NSS_{EndoIII}$), which occurs in net enzyme-sensitive sites solely (Azqueta et al., 2009).

3.2.5 Statistical analysis

Statistical analysis was performed with the software Statistica 7.0. Firstly, data were tested for normality (Shapiro-Wilk's W test) and homogeneity (Brown-Forsyth HOV test) to meet the required statistical demands. Data were transformed (log_{10} or sqrt) when it was not possible to meet these requirements. A one-way ANOVA was performed in somatic and germ cells data, followed by the post hoc Dunnett test. A t-test was used to compare the results between genders (male *vs.* female) in somatic cells (*i.e.,* gills and hepatopancreas). Differences between groups were considered as significant when $p < 0.05$ (Zar, 2010).

3.3 Results

3.3.1 Genotoxic assessment on crayfish gills

Non-specific DNA damage

In the exposure period, a significant increase of GDI values was observed in *Procambarus clarkii* exposed to Px for both males and females compared to unexposed controls (1.63- and 1.60-fold, respectively; $p < 0.001$) (Fig. 19A). Females exposed to Px had GDI values that were significantly increased when compared to males (1.22-fold; $p = 0.024$). The same pattern was observed in the negative control groups (NC), where females had a 1.24-fold higher GDI value than males ($p = 0.016$). Males exposed to EMS had their GDI values significantly higher (1.56-fold) than the control group ($p < 0.001$). Likewise, females exposed to EMS presented significantly higher DNA breaks when compared to NC (1.46-fold; $p = 0.003$).

For the post-exposure, a prolonged effect of Px was only observed in females, which displayed a significant increase in GDI in relation to the unexposed group (1.6-fold; $p = 0.017$) (Fig. 19A); moreover, when both genders of crayfish previously exposed to Px were compared, females presented a significantly higher level of damage (1.83-fold; $p < 0.001$). Concerning groups exposed to EMS, both genders kept GDI values significantly higher than the corresponding NC group (2.00- and 2.14-fold increase, respectively for males and females; both gender with $p < 0.001$).

Oxidative DNA damage

After the seven-day exposure, $GDI_{EndoIII}$ data (Fig. 19B) showed no significant differences between the Px-exposed crayfish (both genders) and the NC group. Likewise, groups exposed to the model genotoxicant EMS did not differ statistically from the NC group. On the other hand, $NSS_{EndoIII}$ data (Fig. 19C) showed that males and females exposed to penoxsulam presented lower values compared to the respective NC groups

(3.17- and 3.38-fold, respectively; both gender with $p < 0.001$). EMS-exposed groups presented $NSS_{EndoIII}$ results similar to those described for Px-exposed groups.

Regarding the post-exposure period, no significant differences between the Px and NC groups (both genders) were detected as $GDI_{EndoIII}$ and $NSS_{EndoIII}$, although Px females showed a higher $GDI_{EndoIII}$ value compared to males (1.85-fold) (Fig. 19B). In contrast, EMS groups displayed a significantly elevated $GDI_{EndoIII}$ value in comparison to the respective NC groups [(1.71-fold ($p = 0.002$) for males and 1.84-fold for females ($p < 0.001$)]. No differences between the treated groups and the respective negative control were found for $NSS_{EndoIII}$ results.

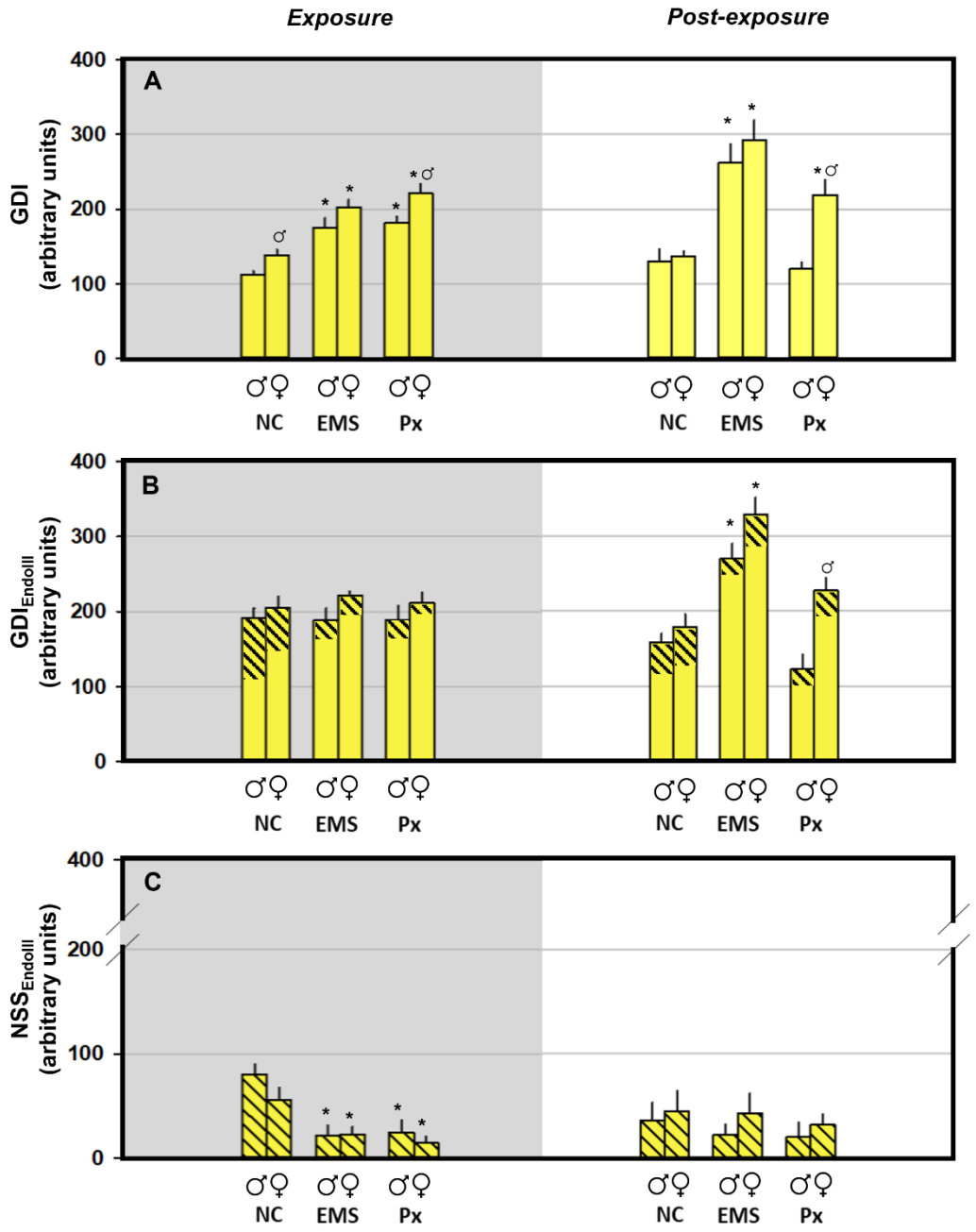

Fig. 19. Genotoxic assessment on crayfish gills. Mean values of DNA damage measured by the comet assay on gill cells of male (♂) and female (♀) *P. clarkii* exposed to 23 µg L^{-1} of penoxsulam (Px), 5 mg L^{-1} of ethyl metasulfosonate (EMS), or to uncontaminated water (negative control; NC). Crayfish were exposed for 7 days (grey background) and submitted to a period under xenobiotic-free water for 70 days (white background). (A) Genetic damage indicator of non-specific DNA damage (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; striped area); (C) $NSS_{EndoIII}$. Bars represent the standard error of the mean. Statistically significant differences ($p < 0.05$) are: (*) *vs.* respective negative control (NC); (♀) *vs.* respective male group.

3.3.2 Genotoxic assessment on crayfish hepatopancreas

Non-specific DNA damage

No significant differences were observed between the Px-exposed groups and their respective negative control (NC). Crayfish exposed to the model genotoxic and also did not show any difference with the corresponding NC (Fig. 20A).

After 70 days in uncontaminated medium, male crayfish exposed to penoxsulam changed their response pattern, showing higher GDI values when compared to NC (2.14-fold; $p < 0.001$) (Fig. 20A). Despite the absence of a significant alteration in relation to the respective control, females of Px group presented significantly lower DNA damage when compared to Px males (1.37-fold; $p = 0.03$) (Fig. 2A). The EMS groups (both genders) displayed GDI values significantly higher than their respective NC groups (males 2.08-fold, $p < 0.001$; females 2.01-fold, $p = 0.002$) (Fig. 20A).

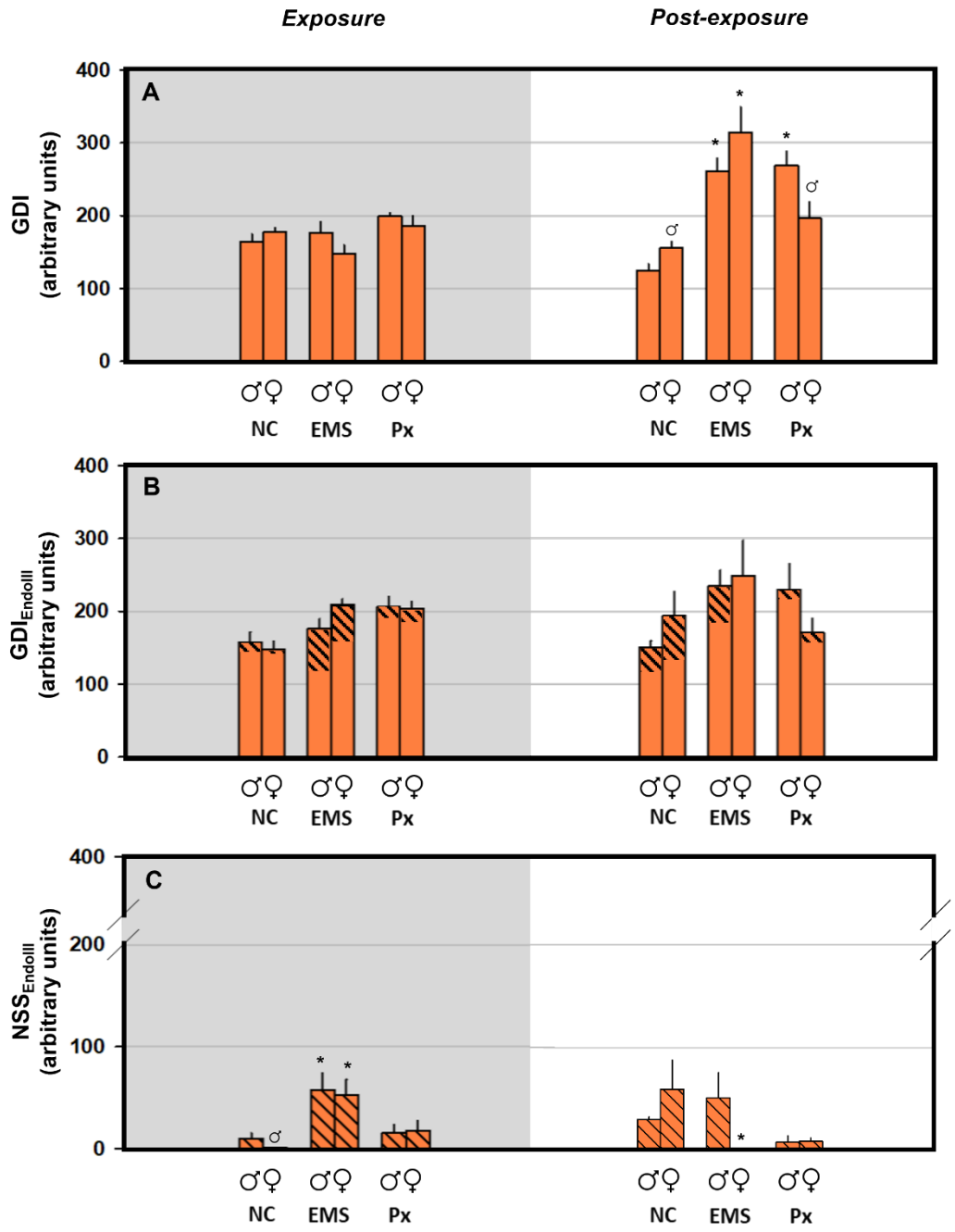

Fig. 20. Genotoxic assessment on crayfish hepatopancreas. Mean values of DNA damage measured by the comet assay on hepatopancreas cells of male (♂) and female (♀) *P. clarkii* exposed to 23 µg L^{-1} of Px, 5 mg L^{-1} of ethyl metasulfosonate (EMS), or to uncontaminated water (negative control; NC). Crayfish were exposed for 7 days (grey background) and submitted to a period under xenobiotic-free water for 70 days (white background). (A) Genetic damage indicator of non-specific DNA damage (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; striped area); (C) $NSS_{EndoIII}$. Bars represent the standard error of the mean. Statistically significant differences ($p < 0.05$) are: (*) *vs.* respective negative control (NC); (♀) *vs.* respective male group.

Oxidative DNA damage

After 7 days of exposure, there were no significant changes between the Px-exposed groups and their negative controls (NC). Similarly, crayfish exposed to EMS showed no significant differences to the control groups (Fig. 20B). No significance differences were observed in the $NSS_{EndoIII}$ parameter between Px-exposed crayfish and their respective NC group. In contrast, both genders of the EMS group showed $NSS_{EndoIII}$ values that were significantly higher than the control groups (males: 5.59-fold, $p = 0.01$; females: 73.84-fold, $p = 0.013$) (Fig. 20C).

Concerning the post-exposure period, no significant differences were observed in the $GDI_{EndoIII}$ parameter between the pre-exposed groups (Px and EMS) and the negative control group. Concerning the $NSS_{EndoIII}$, despite the lack of statistically significant differences, it is possible to observe a tendency to lower levels on Px groups of both genders when compared to their respective control groups (males: 3.67-fold; females: 6.73-fold) (Fig. 20C). Females from EMS group presented a significant decrease in the $NSS_{EndoIII}$ parameter in relation to those from NC group ($p = 0.015$).

3.3.3 Genotoxic assessment on crayfish spermatozoa

Non-specific DNA damage

Spermatozoa of crayfish exposed for 7 days to penoxsulam showed a significant increase in DNA damage measured as GDI in relation to NC group (2.19-fold; $p < 0.001$) (Fig. 3A). In the same direction, the EMS group presented a higher GDI value comparing to the NC group (1.70-fold; $p < 0.001$) (Fig. 21A).

After the post-exposure, the Px group had a GDI level that was significantly higher than spermatozoa of the control group (1.87-fold; $p < 0.001$) (Fig. 3A). Similarly, EMS group revealed a GDI value above that of the NC group (2.25-fold; $p < 0.001$) (Fig. 21A).

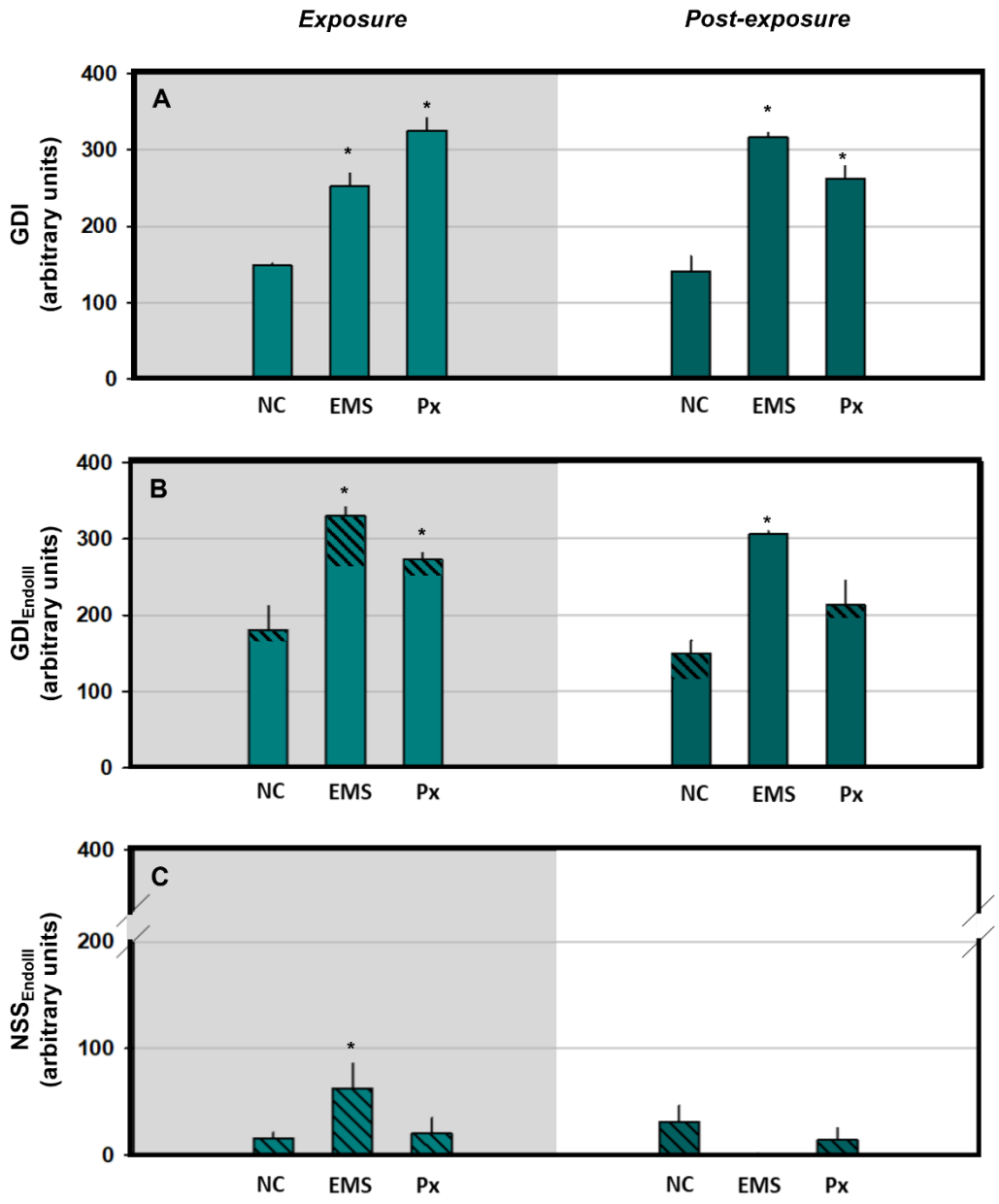

Fig. 21. Genotoxic assessment on crayfish spermatozoa. Mean values of DNA damage measured by the comet assay on male gametes of *P. clarkii*'s exposed to 23 µg L^{-1} of Px, 5 mg L^{-1} of ethyl metasulfosonate (genotoxic model; GM), or to uncontaminated water (negative control; NC). Crayfish were exposed for 7 days (grey background) and submitted to a period under xenobiotic-free water for 70 days (white background). (A) Genetic damage indicator of non-specific DNA damage (GDI); (B) global and partial DNA damage ($GDI_{EndoIII}$), *i.e.*, GDI and additional strand breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; striped area); (C) $NSS_{EndoIII}$. Bars represent the standard error of the mean. Statistically significant differences ($p < 0.05$) are: (*) *vs.* respective negative control group (NC).

Oxidative DNA damage

$GDI_{EndoIII}$ results demonstrated that spermatozoa from both Px-exposed and EMS-exposed crayfish presented significantly higher) DNA damage when compared to NC group (1.19- and 2.42-fold, respectively; both with $p < 0.001$) (Fig. 21B). Regarding the $NSS_{EndoIII}$ data, no significant difference was observed between Px-exposed and NC groups (Fig. 21C). On the other hand, spermatozoa from the EMS group presented a significant $NSS_{EndoIII}$ elevation in relation to the NC group (3.97-fold; $p < 0.04$).

With regard to the post-exposure condition, no significant differences were observed in $GDI_{EndoIII}$ results between Px and NC groups (Fig. 21B), while EMS group presented a significantly higher value comparing NC group (2.04-fold; $p = 0.002$) (Fig. 21B). The $NSS_{EndoIII}$ data did not show any significant difference (Fig. 21C).

3.4 Discussion

The assessment of DNA damage on crayfish cells/tissues, *viz*. gills, hepatopancreas and spermatozoa, following the removal of the genotoxicant source, appears to be a crucial step in improving the understanding of the dynamics of herbicide-induced toxicity. Therefore, and considering a long-term evaluation, the genotoxic assessment on post-exposure (*i.e.*, in organisms that stopped the contact with the pesticide) may help to improve risk assessment, allowing for more effective regulation of penoxsulam use. In fact, when the agrochemicals are used on a large scale [which can be extended up to 10 years (European Commission, 2009)], it is necessary to improve data collection, detection, assessment and monitoring so as to help with prevention of adverse effects such as on non-target plants and animals. What is needed is long-term monitoring, termed pesticidovigilance by Milner and Boyd (2017). The present study contributes to knowledge on the long-lasting effects of Px, having shown that its genotoxic potential in a non-target organism occurs 70 days after the exposure cessation.

However, to fully understand the magnitude of the risk posed by penoxsulam to aquatic populations, further studies regarding the absorption, distribution, metabolism, and excretion of the parental compound, and as well as its metabolites, are needed. A detailed discussion of tissue responses is presented below.

3.4.1 The tissue- and gender-specific progression of genetic damage

Response profiles of somatic cells/tissues

Crayfish gills provide a selective interface between the external environment and the internal milieu, raising the possibility that there may be a permanent threat of accumulating toxic substances from the aqueous phase (Henry et al., 2012). Some ecotoxicological studies with crustacean gills' epithelium showed that specific amino acids form complexes with waterborne contaminants, promoting its influx across the gills (Henry et al., 2012; Vogt, 2019).

The observation that *P. clarkii'* gills (of both genders) were vulnerable to the genotoxic action of penoxsulam, could mean that the herbicide compromised, at least partially, vital functions such as breathing, osmoregulation, and acid-base balance. The gill data showed that female crayfish were more susceptible to the genotoxic effects of Px compared to males. The differential genotoxic extent on males and females has been studied in some invertebrate species (Almeida et al., 2011, 2013; Gagné et al., 2008; Weber et al., 2013). Gagné et al. (2008) observed that males of the mussel *Mytilus edulis* (from areas contaminated with organotins and/or aromatic hydrocarbons) tended to exhibit higher DNA damage in gills than females. In a similar study, this gender-related response pattern was observed in *M. galloprovincialis*, where males' hemocytes presented higher DNA damage (Almeida et al., 2011). Nevertheless, in the clam *Ruditapes decussatus*, females' hemocytes were more sensitive to environmental genotoxicants than males (Almeida et al., 2013), in line with the current gill results for Px. A different perspective was expressed by Weber and co-workers (2013), who stated that, in the freshwater

amphipod *Quadrivisio aff. Lutzi*, highly variable responses in DNA damage were observed to be mostly related to organism physiological condition rather than to gender. In agreement, no gender-specific responses were observed in the gills of the EMS-exposed crayfish. Overall, current and previous studies demonstrate the difficulty of predicting a gender-specific patterns of DNA damage, which clearly depend on the species. Moreover, the type of genotoxicant appears as a factor that superimpose to gender as determinant of vulnerability to genetic insults.

Alkylating agents (*e.g.*, EMS, the model genotoxicant used in this study) are mutagenic and genotoxic (Drabløs et al., 2004), but they are not primarily DNA strand-breaking agents, although alkylation of phosphodiester bonds may result in some strand breakage (Štambuk et al., 2008). Lesions revealed by the comet assay upon treatment with direct alkylating agents are therefore likely to be apurinic/apyrimidinic sites (AP sites) (Štambuk et al., 2008). Moreover, the DNA base most affected by EMS-like compounds is guanine (Drabløs et al., 2004). Bearing this in mind, and also taking into account the fact that EndoIII removes the altered (oxidized) pyrimidine bases (leaving AP sites that will be converted into breaks) (Azqueta and Collins, 2014), it would be expected that the current $GDI_{EndoIII}$ values for EMS-exposed gills would be significantly elevated. A possible explanation for this not having occurred, and which would also justify the decrease in the $NSS_{EndoIII}$ values, would be an activation of the crayfish DNA repair enzymes, as previously reported on other invertebrate species (Accomando et al., 1989 apud Štambuk et al., 2008). For instance, the repair of DNA alkylation damage [reported in gill and digestive gland cells of *M. galloprovincialis* after *in vivo* exposure to dimethyl sulphate (an alkylating agent)] may have been enhanced by induction of DNA polymerase β (an enzyme directly related to DNA repair) (Accomando et al., 1989 *apud* Štambuk et al., 2008).

In this study no evidence was shown that penoxsulam is an oxidative agent for gill DNA. In the face of an eventual oxidizing potential of Px to crayfish gills, an elevation

of $GDI_{EndoIII}$ level is expected, which didn´t occur. To the authors' knowledge, there are no reports concerning the penoxsulam potential to induce oxidative DNA damage on crustaceans. However, it was reported that it can induce oxidative stress in fish (Cattaneo et al., 2011; Murussi et al., 2014) and mussels, by increasing ROS levels (Patetsini et al., 2013).

The DNA repair mechanisms (*e.g.,* base excision repair or nucleotide excision repair) function to correct DNA damage arising spontaneously or after exposure to environmental genotoxicants (Hoeijmakers, 2001; Hook and Lee, 2004). Therefore, after a prolonged post-exposure period, unless those molecular pathways have been affected, it would be expected that there would be a decrease on the magnitude of DNA lesions. This is exactly what was observed in gills of male crayfish following 70 days of recovery in Px-free medium, in contrast to females, reinforcing the higher vulnerability of this gender to the tested herbicide. This gender-specific profile can reflect a compromised DNA repair capacity in females, but also differences in other processes, such as damage tolerance, cell cycle checkpoints and cell death pathways.

The cellular turnover of gills' epithelial cells, occurring in a short-term scale (hours) (Goss et al., 1995), may result in the elimination of cells with DNA lesions, also contributing to the emergence of recovery signals perceptible in males. This factor is present in crayfish of both genders and exposed to both agents tested, and thus, it could be hypothesized that it would affect recovery equally in the different contexts. However, it cannot be overlooked that Px and EMS can also modulate (increase or decrease) the cellular turnover rate.

Although it is not surprising that organisms, particularly males, are affected by the exposure to the model genotoxicant (EMS), it should be noted that there was persistence of genetic damage (assessed by the comet) after a long period of time past. In future investigations, it will be critical to assess the DNA repair ability in similar exposure/post-exposure scenarios.

The hepatopancreas cells, of both crayfish genders, appear to be less susceptible to DNA damage than gill cells, if one considers the absence of non-specific DNA breaks (GDI data) for the period of exposure both to penoxsulam and EMS. Likewise, when specific DNA damage (*i.e.*, damage measured as $GDI_{EndoIII}$) was considered in the same period, the hepatopancreas DNA showed no pro-oxidant vulnerability following exposure to penoxsulam. However, in regard to the EMS exposure (for both genders), the elevation of $NSS_{EndoIII}$ levels provides divergent evidence. Contrary to what was observed in gill cells during the exposure period (where data suggested an efficient action of the antioxidant system as a whole or, specifically, the repair system in relation to the oxidant injury to DNA), in the hepatopancreas cells the oxidative damage emerged, suggesting that the exposure to EMS didn´t trigger, in a first stage, antioxidant defenses (upstream and/or downstream to the DNA injury) in an extent proportional to the challenge. It is therefore important to integrate a complete set of information concerning, for instance, the type of damage and tissue-specificities, prior to forming firm conclusions.

Penoxsulam was shown to have a late genotoxic effect (as non-specific DNA damage) on male hepatopancreas. A similar profile was detected for EMS, for both genders. Since hepatopancreas is the main site for detoxification of xenobiotics (cytochrome P450, glutathione peroxidase, and glutathione S-transferase may play a significant role) (Vogt, 2019). The increased non-specific DNA damage currently detected in the post-exposure period could be indicative of a time-related accumulation of penoxsulam and EMS active metabolites with DNA damaging potential, eventually coupled with an exhaustion of defense mechanisms.

Kogan et al. (2011) performed a 3-year study on the dynamics and principal dissipation mechanisms of penoxsulam in water. It was stated that, in the first 6 hours after application, 45–55% of the initially applied amount was dissipated and the DT_{50} (dissipation time, which is the time required for a concentration decline to half of the

initial value) values in field water varied from 1.28 to 1.96 days (Kogan et al., 2011). In mammalian tissues, according to the US-EPA report, most (>90%) of the penoxsulam administered was excreted within 36-48 hours (US-EPA, 2004). To the authors' knowledge, there is no information regarding the toxicokinetics of penoxsulam in fish or aquatic invertebrates. In any case, the current research shows that 70 days after the end of a direct uptake of the tested agents from the external milieu, hepatopancreas have their DNA damaged due to past exposure to penoxsulam (in males), as well as to EMS (males and females). Furthermore, the present data highlighted the importance of studying prolonged post-exposure periods to thoroughly evaluate the real hazard posed by pesticides, detecting late effects, and reducing the risk of false negative results.

The post-exposure results also revealed a gender-related profile, as hepatopancreas showed to be more affected by the penoxsulam in males than in females, contrary to what was observed in the gill cells. These contrasting profiles show that the identification of gender-specificity in this context must consider the tissue/organ. It has been suggested that fish bioaccumulation is rather due to gender-specific contaminant elimination (through gametes emission) rather than to differential accumulation (Mounier et al., 2020). Bodiguel et al. (2009) used a mechanistic model to study the dynamics of PCB bioaccumulation in the European hake (*Merluccius merluccius*) and observed that female concentrations decreased suddenly during spawning, due to the loss of PCBs (polychlorinated biphenyls) contained in the eggs. Also, the female fish *Acipenser sinensis* showed a propensity to bioaccumulate more PFAs (perfluorinated acids) in the oocytes and in liver, although the former presented 2.5-fold more concentrations than liver (Peng et al., 2010). So, it is plausible that the hepatopancreas' high DNA integrity in the female exposed to penoxsulam may also rely on an ability to transfer contaminants to oocytes and subsequent elimination during spawning. This strategy achieved by females can allow an easier recovery from contaminant exposure and, thus, a protective action in regard to the hepatopancreas. On the other hand, it can

result in gender-specific reproductive and early development disruptions, and, consequently, be translated into effects at population level.

Overall, the integrated analysis of exposure and post-exposure data for hepatopancreas disclosed antiparallel patterns for temporal variation in regard to non-specific and oxidative DNA damage. This interesting divergence, illustrative of the complexity of DNA damaging/protective processes, is expressed in the late action of penoxsulam and EMS in terms of non-oxidative damage and an early and recoverable effect in terms of oxidative damage.

Response profiles of spermatozoa

The effects of Px on *P. clarkii* spermatozoa was already demonstrated in an *ex vivo* study of our research group, where a concentration (2.3 μg L^{-1}), which was 10-time lower than the one currently tested, was found to be genotoxic (Marçal et al., 2020). Despite involving a different experimental approach, the results of this previous study (*ex vivo* exposure) support those obtained here (*in vivo*). This reinforces the agreement between our previous (Marçal et al., 2020) and current studies, and it should be emphasized that there is a lack of evidence on the penoxsulam potential to oxidatively damage DNA on crayfish spermatozoa. This observation contrasts with what was presently demonstrated for EMS. The elevated concentration of unsaturated fatty acids in the spermatozoa (Subramoniam, 1993) makes them highly susceptible to reactive oxygen species (ROS) (Luño et al., 2015). Adding to this, the lack of efficient DNA repair machinery was described in human spermatozoa (González-Marín et al., 2012), but also in invertebrates (Dixon et al., 2002), and in crustaceans (Erraud et al., 2019). Therefore, in the presence of DNA integrity challenge, namely those with a pro-oxidant potential, considerable levels of oxidative DNA damage would be expected (as can be seen in spermatozoa of crayfish exposed to EMS). Thus, due to the lack of information concerning the penoxsulam oxidative action on the spermatozoon DNA, the present

results suggest that penoxsulam does not exert an oxidative action on the crayfish male gametes.

No signs of recovery were perceptible on DNA integrity of spermatozoa after a period of 70 days without contamination, both for crayfish previously exposed to penoxsulam and EMS. As mentioned above, unlike somatic cells and female gametes, spermatozoa lack the ability to repair their DNA (Mai et al., 2013), being thus this result somewhat expected. Nevertheless, this outcome also raises a question towards the possibility of an early effect of penoxsulam on spermatogonia, the primordial germ cells, rather than on the mature spermatozoa sampled. To the authors' knowledge, the life span of the crayfish mature spermatozoa is still unclear, either inside the vas deferens or inside the spermatophore (when it has not yet been released). In Cambaridae, it is known that the female can carry the spermatophore up to 28 weeks until they are oviposited in the spring or early summer (Cumberlidge et al., 2015), but, to the authors' knowledge, there is no information regarding how long the Cambaridae males can retain the spermatophore until release. So, without the information concerning the crayfish spermatozoa lifespan, it is difficult to discern whether these gametes showed DNA damage due to exposure 70 days previously or if damage corresponds to recently formed cells under the effect of a persistent tissue burden of Px and EMS (in the parental form or, most likely, as metabolites). Furthermore, once the DNA damage on sperm cells could compromise not only the reproductive success (in the exposed generation), but also the viability of embryo and subsequent stages in the succeeding generation(s). As such, the impact of the penoxsulam in the crayfish wild populations could be associated with an increased risk of a transgenerational impacts.

3.5 Conclusions

Px was shown to be genotoxic to the crayfish *Procambarus clarkii*. The exposure to this herbicide induced non-specific DNA damage in the somatic tissues and spermatozoa, but no clear indications of oxidative injury were found.

In an attempt to identify the most vulnerable gender, it was pointed out that the pattern deeply depends on the tissue. Female crayfish were more vulnerable to penoxsulam in the gills, where the DNA damage was not reversible following the post-exposure period. The male crayfish demonstrated to be more vulnerable in what concerns internal organs (*i.e.,* hepatopancreas and gonad), which showed an inability to recover from the DNA damage induced by penoxsulam. Crayfish were unable to recover from the DNA damage in gills and hepatopancreas (of both genders), and in spermatozoa induced by the EMS.

It should be noted that genotoxicity in the hepatopancreas was only perceptible in the post-exposure period. Hepatopancreas and spermatozoa recover from the oxidative DNA damage induced by EMS exposure. For hepatopancreas, it may be that there is an improvement in antioxidant shielding, while processes able to prevent non-oxidative damage were increasingly weakened over time.

The spermatozoa proved to be the cell most vulnerable, which, from an ecological perspective, represents a worrying finding due to the prospective threat to future generations.

Finally, it became clear that a conclusion on the genotoxic hazard of a given agent must consider a complete set of information, including different types of DNA damage, as well as tissue- (somatic and germ) and gender-specificity, and a long-term assessment of DNA damage.

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Chapter IV

Intergenerational patterns of DNA methylation in *Procambarus clarkii* following exposure to genotoxicants – a conjugation in past simple or past continuous?

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4. Intergenerational patterns of DNA methylation in *Procambarus clarkii* following exposure to genotoxicants – a conjugation in past simple or past continuous?

Keywords

Crustacean; epigenetics; methylome; intergenerational; genotoxic; pesticides; penoxsulam; ecoepigenotoxicology.

Abstract

Epigenome is susceptible to modulation by environmental pressures, namely through alterations in global DNA methylation, impacting the organism condition and, ultimately, reverberating on the phenotype of the subsequent generations. Hence, an intergenerational study was conducted, aiming to clarify the influence of genotoxicants on global DNA methylation of the crayfish *Procambarus clarkii*. Two subsequent generations were exposed to the herbicide penoxsulam (Px; 23 µg L^{-1}) and to the genotoxicant model ethyl methanesulfonate (EMS; 5 mg L^{-1}). Px didn't induce changes in DNA methylation of adult crayfish (F_0). However, the hypomethylation occurring in unexposed F_1 juveniles demonstrated that the history of exposure per se can modulate epigenome. In F_1 descendants of the Px-exposed group, males' methylome (hypermethylated) was more affected than in females. EMS induced hypomethylation in adult females (F_0), also showing gender-specificity. In addition, hypomethylation was also observed in the unexposed F_1 crayfish, pointing out an intergenerational epigenetic effect. The modulatory role of past exposure to penoxsulam or to EMS showed also to depend on the crayfish developmental stage. Overall, this research showed that indirect

experiences (events occurring in the predecessor generation) can have an impact on the epigenetic dynamics even greater than direct experiences (present events).

4.1 Introduction

Ecotoxicological research has been mostly centered on temporally restricted assessments at individual and sub-individual levels, which can represent a limitation in terms of representativeness, keeping in view the requirement to predict the actual ecological impact of contamination. In this context, the implementation of inter and transgenerational studies can represent a valuable advance towards the elucidation of processes able to produce deleterious effects at higher organizational levels (*e.g.* population), thereby increasing the ecological relevance. This approach has been settled mainly through reproductive (*e.g.* developmental abnormalities, reproductive success) (Sun et al., 2014) and growth/survival endpoints (Meyer and Di Giulio, 2003). More recently and following the conceptualization of epigenetic inheritance (Skinner, 2008), the use of epigenetic markers emerged as a novel and promising strategy, offering suitable information on the diagnosis and prediction of ecotoxicological impacts. This is substantiated by the assumption that epigenetic changes can be triggered by environmental factors, such as exposure to contaminants, modulating gene expression, which may have repercussions at organism- and population-level (Vandegehuchte and Janssen, 2011). This embodies an environmental epigenetic perspective, allowing critical progresses concerning the knowledge on resistance and adaptation as well as disease and variability processes (Head et al., 2012).

DNA methylation was the first epigenetic marker described (Compere and Palmiter, 1981; Hotchkiss, 1948) and is still the most studied nowadays in the field of environmental toxicology (Vandegehuchte and Janssen, 2014), among a set of parameters also including histone modifications, chromatin remodeling and non-coding RNA

expression (miRNA) (Luzhna et al., 2013). Methylation is the only epigenetic process that directly targets the DNA, where a methyl group replaces the hydrogen atom in the cytosine base, creating thus a new covalent bond (Luzhna et al., 2013), whose effects depend on the genome location where it takes place (Brevik et al., 2018). DNA methylation is involved in many cellular regulation processes, including chromatin condensation, chromosome stability, X-chromosome inactivation, genomic imprinting, and gene transcription (Collotta et al., 2013), playing a crucial role in determining cell normal development, proliferation, and genome stability (Luzhna et al., 2013).

DNA methylation is susceptible to environmental pressures, leading to alterations in gene expression (Roberts and Gavery, 2012), being passible to be translated into the whole organism condition and, ultimately, if this epigenetic mark resists to erasure waves during embryogenesis (Stenz et al., 2018), could have repercussions on the phenotype of the subsequent generations (Vandegehuchte and Janssen, 2014). Studies addressing the induction of changes in the global DNA methylation by environmental toxicants, encompassing different taxa, were revised by Vandegehuchte and Janssen (2011). For instance, the exposure to Zn induced hypermethylation in the fish *Carassius auratus* (Zhou et al., 2001) and hypomethylation in the crustacean *Daphnia magna* (Vandegehuchte et al., 2009a).

Another challenging topic in this context is the identification of the concomitant occurrence of events affecting the epigenome and DNA integrity. Sargsyan et al. (2019) reported that, in the presence of metals that induce genotoxicity, the lizard *Darevskia armeniaca* also displayed DNA hypomethylation. Moreover, it has been hypothesized that some pesticides, despite not increasing cancer risk directly via a genotoxic process, may operate through epigenetic mechanisms (Collotta et al., 2013).

Most of the studies regarding the modulation of DNA methylation by environmental contaminants was carried out in vertebrates (Anway et al., 2005; Head, 2014; Nilsen et al., 2016; Pilsner et al., 2010; Yauk et al., 2008), and only a few addressed

invertebrate species (Gavery and Roberts, 2010; Vandegehuchte et al., 2010; Vogt et al., 2015). Though DNA methylation appears to be associated with gene regulation and expression in both vertebrates and invertebrates (Gavery and Roberts, 2017), their patterns may differ between those animal groups. In the latter, for instance, the genome can have longer sections of methylated DNA interspersed by unmethylated DNA (Head, 2014). In particular, the methylated cytosines tend to be part of the gene bodies, while non-coding regions are less methylated (Gavery and Roberts, 2017; Head, 2014).

While some environmentally-induced epigenetic changes are perishable, DNA methylation may be inherited mitotically along with the genetic code from cell to cell [thereby through cell lineage development, persisting during organism's lifetime (Gavery and Roberts, 2017)], but also meiotically from parent to offspring (intergenerationally) and then to grand-offspring (transgenerationally) (Collotta et al., 2013; Faulk and Dolinoy, 2011; Head et al., 2012; Vandegehuchte and Janssen, 2014). Thus, the convergence of evolutionary developmental biology, environmental toxicology and epigenetics is particularly important at the earliest stages of development when epigenetic modifications are more vulnerable to perturbation resulting in lifelong and possibly inter/transgenerational effects (Faulk and Dolinoy, 2011). One of the first studies on epigenotoxicity of environmental contaminants reported a reduced spermatogenic capacity associated with an abnormal DNA methylation pattern in sperm of rat descendants from breeders exposed to the fungicide vinclozolin (Nilsen et al., 2016).

Inherited epigenetic memory can thus determine the responses to a present environmental scenario, either corresponding to a repeated exposure (in terms of agent and duration, relative to the precedent generation) or to a new context resulting from the exposure to another agent or to uncontaminated media (possible to occur, for instance, if the habitat was restored or the animal moved to a contamination-free local).

So, applying an epigenotoxicity approach, the present study addressed the methylation patterns in the muscle of *Procambarus clarkii*, intra- and

intergenerationally, under present and past scenarios of exposure to the genotoxicants penoxsulam [a new post-emergence herbicide widely used on dry-seeded and water-seeded rice crops in order to control broadleaf weeds, aquatic plants, and certain grasses, approved in the US since 2004 (US-EPA, 2004) and in the EU since 2010 (EU, 2010b); its genotoxic potential for aquatic species has already been demonstrated (Murussi et al., 2014; Patetsini et al., 2013) including to the *P. clarkii* (Costa et al., 2018; Marçal et al., 2020)] and ethyl methanesulfonate [EMS, an alkylating agent known for its genotoxic and mutagenic potential on fungi, plants, insects and human cells (Amini, 2014; Sega, 1984)]. The choice of these epigenetic challengers relied on the hypothesis that events affecting DNA integrity may concomitantly affect the epigenome.

The aims of the present work were: (i) to study the influence of penoxsulam and EMS on DNA methylation of *P. clarkii*, also seeking for gender-related patterns; to pursue an intergenerational approach, evaluating the epigenetic memory in (ii) unexposed crayfish (juveniles and adults) representing the off-spring (F_1) of a genotoxic-exposed generation (F_0), and in (iii) juvenile crayfish (F_1) subjected to an exposure corresponding to the same and to a different genotoxicant relative to the stressful scenario experienced by the predecessors (F_0); (iv) to clarify whether the dynamics of epigenetic changes are determined by direct and indirect (events occurring in the predecessor generation) experiences, thus contributing to the consolidation of an epigenotoxic perspective as a critical element in the ecotoxicology field and risk assessment approaches.

4.2 Materials and Methods

4.2.1 Chemicals

Penoxsulam (Px; CAS No 219714-96-2) and ethyl methanesulfonate (EMS; CAS No 62-50-0) were obtained from Sigma-Aldrich Chemical Company (USA). The NZY tissue gDNA isolation kit (NZYtech) was obtained from NZYtech (Portugal). The

MethylflashTM global DNA methylation (5-mC) ELISA Easy Kit (colorimetric) (Epigentek Group Inc.; USA) was obtained from bioNova cientifica s.l. (Spain).

4.2.2 Animal maintenance

In order to obtain the initial lot of animals (F_0), adult crayfish specimens (*Procambarus clarkii*), with an average length of 11.22 ± 0.91 cm, were collected at Minho River (Vila Nova de Cerveira, NW Portugal), a low impacted area concerning the presence of pesticides (Dagnac et al., 2012) as well as other inorganic and organic contaminants (Capela et al., 2016). In the laboratory, crayfish were kept in individual aquaria, during two weeks for acclimation before starting sub-trial 1, with the following water conditions: constant temperature (20 ± 1°C), freshwater (dechlorinated tap water; salinity 0) with aeration (dissolved oxygen 8.1 ± 0.5 mg L^{-1}), daily UV disinfection, weekly control of nitrites (0.2 ± 0.05 mg L^{-1}), nitrates (25 ± 6.0 mg L^{-1}), ammonia (< 0.1 mg L^{-1}) and pH (7.5 ± 0.2). Animals were daily fed ad libitum with crustacean feed, Caridina Vita, produced by Sparos® (Olhão, Portugal).

4.2.3 Experimental set-up

Fig. 22 exhibits the schematic representation of the experimental set-up of the intergenerational trial. In sub-trial 1 and in sub-trial 2.2, each crayfish was individually placed in 1L aquaria to be exposed to a genotoxicant (penoxsulam or EMS), for 7 days. A control group was kept in uncontaminated water. During the exposure period, animals were fed *ad libitum* in the first 6 days of exposure and fasted the day before sampling. Crayfish of sub-trial 2.1 were maintained in the same conditions as described for acclimation period (please see section 2.2).

The selection of the Px concentration (23 µg L^{-1}) to test relied on its environmental relevance (Murussi et al., 2014), while the EMS concentration (5 mg L^{-1})

was selected according to its use as a positive control on genotoxic assays in fish (Cavas, 2011) and crayfish (Costa et al., 2018).

At the end of each sub-trial, every crayfish was sacrificed with a single cut in the rostrum and the striated muscle (a portion from the ventral-anterior area) was collected (approx. 1 g) and preserved in ethanol absolute until epigenetic analysis.

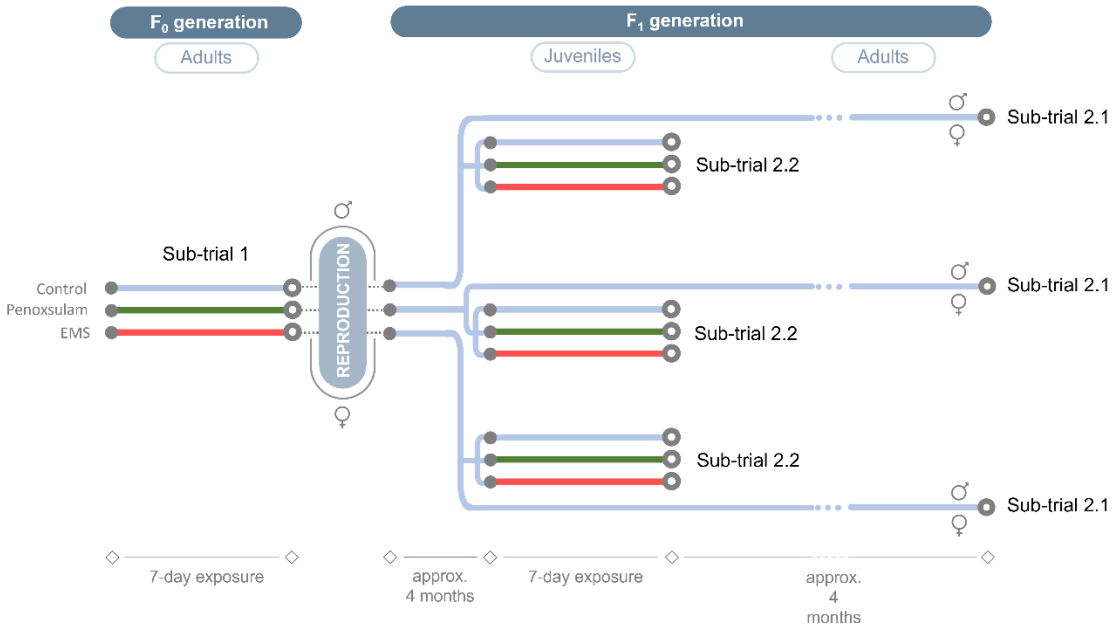

Fig. 22. Schematic representation of the experimental design, depicting an intergenerational trial involving the exposure of the red swamp crayfish (*Procambarus clarkii*) to the genotoxicants penoxsulam (Px; green line) and ethyl methanesulfonate (EMS; red line). Sub-trial 1: exposure (7 days) of male (♂) and female (♀) adults, from F_0, to Px and EMS, in comparison with a control group (blue line). After reproduction of the F_0 organisms (intra-group crosses), F_1 offspring was divided into two sub-trials (2.1 and 2.2). Sub-trial 2.1: the progeny (F_1) of each F_0 group was allowed to grow in uncontaminated water until adulthood (blue line); global DNA methylation was analyzed in both stages (juvenile and adult). Sub-trial 2.2: the progeny (F_1) of each F_0 group was allowed to grow in uncontaminated water only until the juvenile stage, and then exposed to Px and EMS for 7 days, and thereafter analyzed in comparison with a control group. o represents the sampling moments.

Sub-trial 1: direct exposure of adult crayfish (F_0)

Sub-trial 1 aimed to explore the influence of penoxsulam and EMS on DNA methylation of adult *P. clarkii*, from F_0, also seeking for gender-related patterns. So, adult crayfish, forming experimental groups of fourteen animals (n = 7 of each gender), were individually exposed to 23 µg L^{-1} of Px or to 5 mg L^{-1} of EMS, for seven days (Fig. 22), in 1L aquaria (water and room conditions were the same as the acclimation period; please see section 2.2). A control group (n = 7 of each gender; C) was maintained in uncontaminated freshwater. Genotoxic and control medium were daily renewed. Afterward, animals were sacrificed, and muscle samples extracted as described above.

Sub-trial 2.1: indirect exposure of juvenile and adult crayfish (F_1)

This sub-trial was established to pursue an intergenerational approach, evaluating the epigenetic memory in unexposed juvenile and adult crayfish from F_1, representing the offspring of a genotoxic-exposed generation (F_0).

Following the exposure described in the sub-trial 1, crayfish (F_0) were paired and allowed to reproduce (intra-group crosses). During the mating period, the temperature was set for 24.0 ± 1.0°C. Females with eggs were relocated in individual aquaria, and the water temperature was maintained. After hatching, juveniles (F_1) were transferred to a new aquarium (offspring were separated according to the provenience group F_0; Fig. 1) to grow in uncontaminated water until reach the adult stage. Freshwater medium was weekly renewed; temperature and water conditions were similar to the acclimation period (see section 2.2). This sub-trial had two sampling moments: the first, when juveniles reached 4 months of age (n = 6, from each independent group; average length of 4.02 ± 0.17 cm), and the second, when adult crayfish reached 8-month-old (n = 4; 2 of each gender, from each independent group; with an average length of 7.35 ± 0.16 cm). In each sampling moment, every crayfish was sacrificed, and muscle sample extracted as described above.

Sub-trial 2.2: direct exposure of juvenile crayfish (F_1) under the influence of F_0 (indirect) exposure

This sub-trial aimed pursuing an intergenerational approach to evaluate the epigenetic memory in juvenile crayfish (F_1) subjected to an exposure corresponding to the same or to a different genotoxicant, relative to the stressful scenario experienced by the previous generation (F_0). Due to sexual immaturity, no gender discrimination was carried out. Briefly, F_1 4-month-old juveniles [with an average length of 4.02 ± 0.17 cm; n = 54; 6 (animals) x 3 (treatment) x 3 (F_0 groups)] descendants from the F_0 crossings (*i.e.*, males mated with females both exposed to Px; males mated with females both exposed to EMS; unexposed males mated with unexposed females) were exposed to 23 µg L^{-1} of Px, 5 mg L^{-1} of EMS and uncontaminated freshwater for 7 days, at 20 ± 1.0°C (Fig. 22). Genotoxic and control medium were daily renewed. At the end, animals were sacrificed, and muscle samples were extracted, as described above.

4.2.4 DNA extraction

DNA from each muscle sample was extracted using the NZY tissue gDNA isolation kit, according to the manufacturer's instructions. Briefly, a 20 mg piece of tissue was cut into small pieces and placed overnight in the microcentrifuge tube at 56°C with proteinase K and buffer solution (NT1; kit component). After this incubation, 200 µL of lysis solution were added to each sample, and the mixture was vortexed for 10 s. Next, 210 µL of ethanol absolute were added and the mixture immediately vortexed. The mixture was transferred into an NZYSpin tissue column, placed in a 2 mL collection tube, and centrifuged for 1 min at 11 000 *g*. Then, the silica membrane of the NZYSpin tissue column was washed and dried. Thereafter, each DNA sample was eluted with 60 µL of sterile distilled water (at 70°C). The genomic DNA was then stored at 4°C, until further analysis.

4.2.5 DNA methylation analysis

The global DNA methylation of each sample was quantified using MethylflashTM global DNA methylation (5-mC) ELISA easy colorimetric kit, in accordance with manufacturer's instructions. Briefly, 100 µL of a binding solution were added to each well (of a 96-well plate) followed by 2 µL of DNA sample (samples were diluted to obtain 100 ng in a volume of approximately 2 µL, as suggested by the manufacturer's protocol instructions). Also, negative (NC) and positive (PC) controls were considered to the plate to generate the standard curve (as represented in Fig. 23).

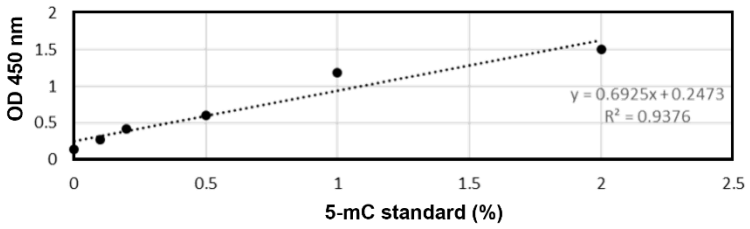

Fig. 23. Standard curve for methylated DNA, built using the kit-provided positive controls for validation of the measurement method (ELISA Easy Kit EpiGentek).

Samples were incubated at 37°C, for 60 minutes. Then, each well was washed three times, and samples were incubated with 50 µL of the 5-mC detection complex solution for 50 minutes. Each well was rewashed five times, and samples were incubated with 100 µL of developer solution for 3 min. The developer solution turned blue in the presence of sufficient methylated DNA. The color in the NC wells remained unchanged. When the PC samples became blue (indicating the presence of methylated DNA), the enzyme reaction was stopped with the stop solution, and the color of each sample changed to yellow. The absorbance was immediately read at 450 nm.

4.2.6 Statistical analysis

Statistical analysis was performed with the software Statistica 7.0. Data were first tested for normality and homogeneity of variance to meet statistical demands by Shapiro-Wilk's W test and Brown-Forsythe (HOV) test, respectively, to apply parametric tests. Two-way ANOVA (gender x treatment) followed by Fisher's LSH post hoc test was used to compare F_0 adults (sub-trial 1). Males *vs.* females, within each treatment, were compared using t-test. A one-way ANOVA, followed by Dunnet's post hoc test, was used to compare the F_1 juveniles of sub-trial 2.1. Also, a one-way ANOVA followed by Fisher's LSH post hoc test, was used to compare F_1 adults of the same gender (sub-trial 2.1). A t-test was used to compare genders within the same treatments of F_1 adults. Another two-way ANOVA (history x treatment), followed by Fisher's LSH post hoc test, was used to compare F_1 juveniles from treated groups with the corresponding group with different history profile (the exposure in the F_0 group corresponds to a past; history) exposure in F_1 (sub-trial 2.2). Differences between groups were considered significant when $p < 0.05$ (Zar, 2010).

4.3 Results

4.3.1 Sub-trial 1: direct exposure of adult crayfish (F_0)

Males' global methylation (5-mC content) presented a similar pattern in all treatments, and no significant differences were observed in the exposed groups with respect to the control (C) (Fig. 24). Regarding females, the 5-mC levels in the group exposed to EMS was significantly lower (4.82-fold; $p < 0.001$) in relation to the unexposed group (C), while no significant differences were observed between crayfish exposed to Px and C (Fig. 24).

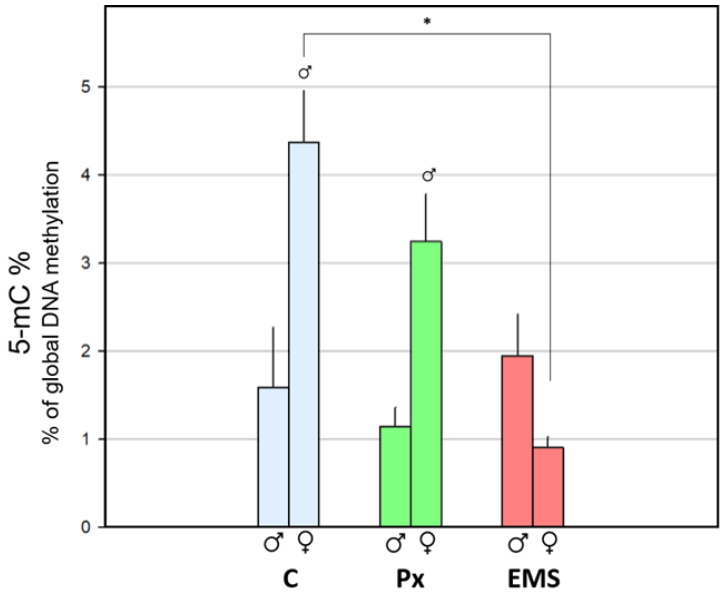

Fig. 24. Sub-trial 1 - Global DNA methylation measured in muscle of F_0 *Procambarus clarkii*, both adult males (♂) and females (♀), following exposure to 23 µg·L^{-1} of penoxsulam (Px; green) or 5 mg·L^{-1} of ethyl methanesulfonate (EMS; red), in comparison with the corresponding control groups (C; blue). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between treatments, within the same gender; (♂) *vs.* male group, within the same treatment.

The females from C and Px groups displayed higher 5-mC content when compared with the respective male groups ($p = 0.009$ and $p = 0.006$, respectively) (Fig. 24). In the group exposed to EMS, despite the absence of statistical differences, males showed a tendency of a higher 5-mC levels (2.14-fold, in relation to females).

4.3.2 Sub-trial 2.1: indirect exposure of juveniles and adult crayfish (F_1)

Concerning juvenile stage, data point out a significantly lower DNA methylation in the groups descending from F_0 exposed groups (both Px and EMS) when compared with the offspring of the unexposed group. Specifically, 3.40-fold ($p = 0.002$) and 2.17-fold ($p = 0.02$) lower 5-mC levels were detected in descendants from the Px-exposed and EMS-exposed groups, respectively, in comparison with the offspring of the unexposed group (Fig. 25).

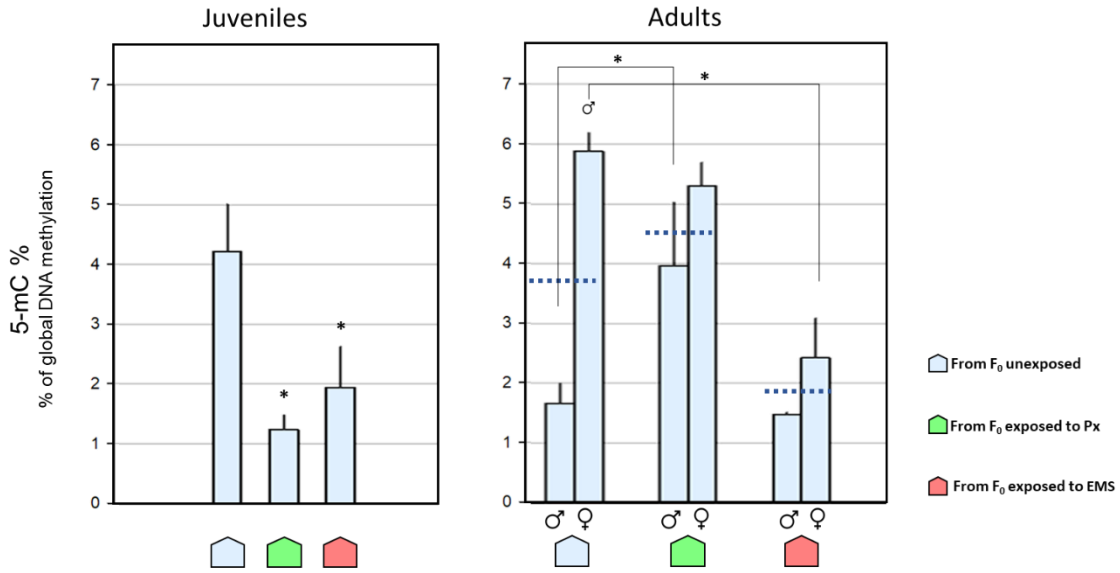

Fig. 25. Sub-trial 2.1 - Global DNA methylation measured in muscle of unexposed F_1 *Procambarus clarkii*, in juveniles (left) and adults (right; males: ♂; females: ♀), descendants from F_0 unexposed (⬠), penoxsulam-exposed (⬠), and ethyl methanesulfonate-exposed (⬠) groups. Dark blue dashed-lines represent the 5-mC% mean values for the corresponding adult groups combining both genders. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) *vs.* descendants from F_0 unexposed group; (♂) *vs.* male group, within the same exposure background.

Adult males descending from Px group presented significantly higher (2.38-fold; $p = 0.03$) 5-mC levels than those descending from the unexposed group (Fig. 25). Regarding females, crayfish descending from EMS group presented significantly lower global DNA methylation (2.42-fold; $p < 0.001$) when compared with offspring of the unexposed group (Fig. 25).

Comparing genders, females descending from the unexposed group presented higher 5-mC levels of (3.5-fold; $p < 0.001$) than the corresponding males. The crayfish descendants from EMS-exposed groups did not present any differences between genders (Fig. 25).

Contrarily to juveniles [sexually immature (Anastácio, 1993; Vogt et al., 2008)], the quantification of DNA global methylation in adult crayfish considered gender separation. In Fig. 25, dark blue dashed lines represented correspond to the 5-mC mean values for each adult group combining both genders. It is visible in all groups that the % global methylation in females is higher than the average of the two genders together, and, on the other hand, the 5-mC level in males is lower than the determined average.

4.3.3 Sub-trial 2.2: direct exposure of juvenile crayfish (F_1) under the (indirect) influence of F_0 exposure

No significant differences were detected in global DNA methylation when the experimental groups (C, Px and EMS) were compared within the same exposure history (Fig. 26).

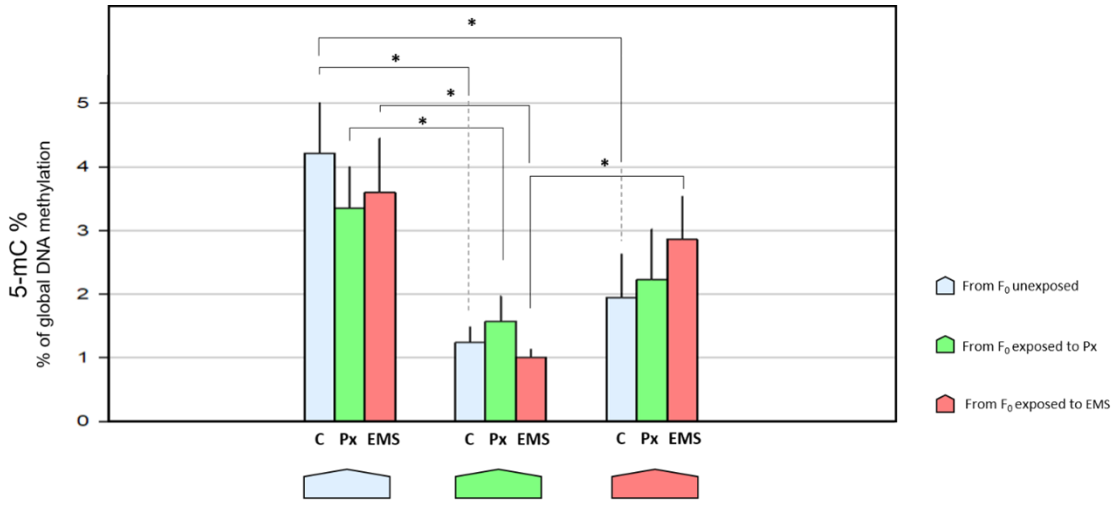

Fig. 26. Sub-trial 2.2 – Global DNA methylation measured in muscle of F_1 juvenile *Procambarus clarkii*. Descendants from F_0 unexposed (⌂), penoxsulam-exposed (⌂), and ethyl methanesulfonate-exposed (⌂) groups were currently exposed to 23 µg·L^{-1} of penoxsulam (Px; green) or to 5 mg·L^{-1} of EMS (EMS; red) and compared to the control groups (C; light blue; it should be noted that these data are also represented in Fig. 4). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between different historical backgrounds, within the same current treatment.

On the other hand, significant differences were detected when comparing different historical backgrounds within the same current treatment. Globally, it was discernible a variation pattern with lower global DNA methylation in crayfish descendants from Px- and EMS-exposed groups in comparison with those descending from unexposed parents. Detailing, juveniles with a history of exposure to penoxsulam showed a decrease in the percentage of methylated cytosines of 2.14-fold ($p = 0.04$) when exposed to penoxsulam and 3.59-fold ($p = 0.003$) when exposed to EMS, compared to juveniles exposed to the same compounds but with a history of no contamination. On the other hand, juveniles with a history of exposure to EMS showed an increase in methylated cytosines when exposed to EMS, compared to juveniles with a history of penoxsulam (2.86-fold; $p = 0.02$) also exposed to this compound (Fig. 26).

4.4 Discussion

The present work aimed to understand how two different genotoxicants (Px and EMS) affect the DNA methylation pattern in both genders of the species *Procambarus clarkii* as well as in what extent the methylation pattern of a given offspring is influenced by the genotoxic exposure of the progenitors, contributing to predict the ecological impact of the complex interactions of past and present exposures affecting wild populations under real field scenarios.

P. clarkii can adapt very quickly to environmental stressors, presenting also high tolerance to environmental heterogeneity (*e.g.*, regular periods of drought, omnivorous diet) (Geiger et al., 2005), features that contribute to its success as an invasive species. These characteristics may also justify why this species can be found in inhospitable environments, such as those impacted by pesticides (*e.g.* rice fields) (Geiger et al., 2005). Since *P. clarkii* reaches maturity maintaining a small body size, has a rapid growth, large number of offspring and a relatively short life span (Gherardi, 2006), is regarded as a

suitable non-model organism for intergenerational studies. To date, there are no studies regarding DNA methylation in *P. clarkii*, though Vogt (2008) assessed this epigenetic process in muscle of the congener species *P. fallax*, the marbled crayfish. Thus, global DNA methylation was the marker chosen to study in the epigenome of the F_0 and F_1 generation of *P. clarkii*, within the framework above enunciated.

4.4.1 DNA methylation in *P. clarkii* (F_0) after direct genotoxic exposure

The methylation pattern can be altered by external factors, such as the presence of contaminants (Wang et al., 2009; Zhou et al., 2001). A study with *D. magna* showed that exposure to the fungicide vinclozolin (Sargsyan et al., 2019) induced hypomethylation, which may affect gene regulation and expression. In the present study, no changes were observed in the DNA global methylation following a direct exposure to the herbicide penoxsulam, in both genders. In the work developed by Akcha et al. (2021), it was demonstrated a positive correlation between the presence of oxidative DNA damage, as measured by the level of 8-oxodGuo, and DNA methylation, as measured by human DNMT1 (DNA methyltransferase involved in methylation maintenance) activity. In line with these findings, it can be inferred that penoxsulam, probably, has a negligible pro-oxidant potential, which was corroborated in *P. clarkii* spermatozoa by Marçal et al. (2020).

Under exposure to EMS, DNA of female crayfish became hypomethylated. This could be supported by the fact that EMS induces base replacements of guanine-cytosine (C/G) to adenine-thymine (A/T) (Griffiths et al., 2000). So, once EMS may reduce the cytosines, the amount of 5-mC may also be diminished. However, global DNA methylation in male crayfish was not diminished by the EMS, which could be related to a naturally less methylated epigenome in the striated muscle cells of this gender (as recurrently observed in the different components of the current study), limiting the

margin for a reduction. To the authors' knowledge, the present study provides the first results regarding EMS effects on the crayfish epigenome.

Small differences in global DNA methylation can have great consequences for the phenotype (Vogt et al., 2008). In an *in vitro* study performed by Hiendleder et al. (2006) with bovine fetuses, phenotypic features as fetal overgrowth and endocrine changes were related to only 11.2% deviation from normal methylation values in the liver. In the present study, females exposed to EMS presented 60% deviation from the normal/basal global DNA methylation profile. Although it was not the focus of this study, considering that the deviation currently described is almost 6 times that reported by Hiendleder et al. (2006), important phenotypic changes in crayfish can be hypothesized due to this shift in the global methylation. Nevertheless, it is not expectable that the measured changes were fully and proportionally mirrored in the loss of DNA homeostasis and genomic instability. Future studies are required in this direction.

Gender is an important variable to consider when assessing global DNA methylation. In this study, it was disclosed, for the first time, the global methylation basal values in the striated muscle of the crayfish *P. clarkii* with gender discrimination. Specifically, males presented less methylated cytosines than females. Considering previous research with invertebrates, and particularly with the insect *Acyrthosiphon pisum* (Walsh et al., 2010) and the crustacean *D. pullex* (Kvist et al., 2020), it was described that males presented higher levels of global DNA methylation than females. Contrary to the present study, in which there was only one tissue analyzed, the striated muscle, the studies mentioned above (Kvist et al., 2020; Walsh et al., 2010) analyzed the methylome in the whole body. Therefore, it can be only concluded that males of *A. pisum* and *D. pullex* have globally more methylated cytosines than females; it remains unknown the tissue specific ratio of methylated cytosines for each gender. Therefore, regarding the current results, gender-dependent differences in adult crayfish' epigenome suggest a higher basal global DNA methylation on muscle of females, which represents an

innovative aspect. To the authors' knowledge, no solid scientific information is available on the mechanistic understanding of gender-specificity, namely applicable to DNA methylation in crustaceans.

Gender-related methylation patterns have been reported also in ecotoxicological studies (mostly in vertebrates) in association to contaminants exposure. It has been reported that compounds with the ability to modulate the DNA methylation may affect differently males and females. For instance, male zebrafish presented greater changes in the DNA methylation patterns in the brain and eyes, after chronic exposure to depleted uranium, than females (Gombeau et al., 2016); also, males polar bear presented their DNA methylation in the brain more affected than females after mercury exposure (Pilsner et al., 2010).

4.4.2 DNA methylation in unexposed crayfish descendants (F_1) from a genotoxic-exposed generation (F_0)

The history of exposure to penoxsulam showed to have an impact on the global methylated cytosines of the offspring (F_1). The F_1 young crayfish grown in an uncontaminated environment showed a decrease in 5-mC content (DNA hypomethylation). DNA hypomethylation is the most consistent epigenetic alteration observed in cancer studies (Counts and Goodman, 1995), while genes involved in development, tissue-specific functions or response to environmental stimuli are poorly methylated and could be associated to higher phenotypic plasticity (Akcha et al., 2021). Therefore, although not being possible to identify the consequences for F_1 generation from the contact to penoxsulam in the previous generation, it was evident the modulating effect on the DNA methylation in the offspring, highlighting an intergenerational impact of penoxsulam. Interestingly, it should be recalled that the direct exposure to penoxsulam (F_0 generation) did not alter DNA methylation (see Fig. 24). In agreement, though testing a different agent, a study with *D. magna* reported that

the epigenetic effects of Zn were only observed in the F_1 generation, where the global DNA methylation was also diminished (Vandegehuchte et al., 2009). These authors suggested that Zn reduced the substrate for DNA-methylation, since the concentration of metallothioneins (which interacts with homocysteine to form conjugates) increased after the exposure; this conjugation caused a decrease in the availability of free homocysteine, used as a substrate to form methionine (which is converted to S-adenosyl-methionine, an important methyl donor for DNA methyltransferases) (Vandegehuchte et al., 2009). Furthermore, when detoxification processes are favored, homocysteine, that could be also needed for DNA methylation, will be exploited for glutathione synthesis (Oppold et al., 2015).

Adult males (F_1) showed a significant increase in the global DNA methylation (hypermethylation) in relation to those descending from the unexposed group. DNA hypermethylation is considered the default epigenetic state and serves in maintaining genome integrity (Weber and Schübeler, 2007). The DNA hypomethylation observed before in F_1 juveniles, along with the hypermethylation observed in adult F_1 males, reinforces the suggestion that penoxsulam can change the DNA methylation pattern across generations. Studies with the European honeybee *Apis melifera* (Elango et al., 2009; Foret et al., 2009) reported that genes predicted to be hypermethylated are associated with housekeeping functions, while those predicted to be hypomethylated are associated with general immune functions. Hypermethylation of intragenic regions of housekeeping genes is consistent in the invertebrate species *Crassostrea gigas* (Gavery and Roberts, 2010) and *A. mellifera* (Elango et al., 2009). Therefore, the present data concerning muscle give plausibility to the hypothesis that penoxsulam can induced different phenotypic changes depending on the developmental stage. In future studies, it will be important to considerer the evaluation of gene expression, since it highlights the genes that could be vital to cell metabolism, including pathways related to the immune system.

The data obtained for the descendants from F_0 Px-exposed group show that the epigenome of females (adults) does not seem to be affected by penoxsulam. A similar pattern (*i.e.*, the percentage global methylation of Px-exposed females similar to the unexposed females) was also observed on their offspring F_1 (*i.e.*, females F_1 Px-exposed descendants presented a percentage of methylated cytosines similar to females F_1 unexposed descendants). Hence, it was demonstrated that penoxsulam induced changes indirectly in the crayfish offspring epigenome as well as a gender-specific intergenerational epigenetic effect.

As observed in the descendants from the F_0 Px-exposed group, the history of exposure to EMS showed to have an impact on the juveniles grown in an uncontaminated medium, where DNA hypomethylation was observed. In adults, the EMS intergenerational effect in unexposed male crayfish was not evident, contrary to what was observed in Px-descendant males. However, the hypomethylation observed in the unexposed F_1 females descended from the EMS-exposed group, representing a similar pattern to the F_0 females exposed to EMS, suggests that F_1 females had an epigenetic memory (*i.e.*, it was observed a hypomethylation like in the progenitors) of the F_0 exposure. Accordingly, in a study with *D. magna*, an exposure to 5-azacytidine (a DNA methyltransferases inhibitor) induced epigenetic changes in the F_0 generation, which were transmitted to the unexposed F_1 and F_2 generations (Sargsyan et al., 2019).

The current outcomes suggest that EMS had a potential gender-specific intergenerational epigenetic effect. Moreover, the exposure to this genotoxicant affected more the females methylome (since F_0 generation), contrary to what was observed with penoxsulam exposure (in this study) as well as with uranium (Gombeau et al., 2016) and mercury (Pilsner et al., 2010) exposures. This highlights the gender as an important variable that cannot be overlooked when studying this type of parameters in sexually mature animals.

The outcomes of this section, where animals displayed epigenetic changes, despite they only had contact with the genotoxicants in the previous generation (indirect exposure), confirmed the importance of the incorporation of the first exposed generation as well as the subsequent generations on risk assessment (Vandegehuchte and Janssen, 2014).

4.4.3 DNA methylation in juvenile crayfish (F_1) submitted to a current exposure

Exposure to the same genotoxicant

The juvenile crayfish (F_1) were subject to an exposure corresponding to known genotoxicants (*i.e.*, the same genotoxic agent experienced by the predecessors). From the present data, it was possible to understand that the exposure to Px in the F_0 generation greatly influenced the methylation pattern on the next generation (F_1). When juveniles, descendants from the F_0 Px-exposed group, were exposed to penoxsulam, they presented a hypomethylation, compared to the crayfish that was in contact with penoxsulam for the first time. This eventual epigenetic memory, transmitted by the progenitors, was also observed in the unexposed juveniles, descendants from the F_0 Px-exposed group. Since this group did not contact directly with penoxsulam, this outcome supports the potential of this herbicide to induce generational epigenetic changes, specifically on DNA methylation. To the authors' knowledge, there are no scientific studies addressing this aspect, *i.e.*, what happens to the methylation pattern of the offspring (with a history of exposure from the previous generation) when facing an exposure to a genotoxicant, since most research focus on the effects caused by parental exposure on subsequent unexposed generations.

Considering the descendants from F_0 EMS-exposed group, F_1 juveniles appear to have also inherited the memory of their parents' exposure. The similarity of DNA methylation profile between the unexposed F_1 crayfish and the F_1 EMS-exposed suggests that a tolerance to this compound may have been acquired. It should also be noted that,

while the F_0 EMS-exposed groups had less methylated cytosines (in both crayfish genders) when compared to the unexposed group, their descendants after being exposed to the same compound, *i.e.*, EMS, tended to display higher DNA methylation than the unexposed group (despite without statistical significance). This reinforces the theory that the memory of the genotoxic exposure in F_0 was transmitted to F_1, strengthening the stability of methylation processes when F_1 is exposed to the same genotoxicant as in F_0.

Exposure to a different genotoxicant

Juveniles (F_1) were exposed to a different genotoxicant (*i.e.*, distinct from that experienced by the ancestors). The decrease in % of methylated DNA in the unexposed F_1 crayfish derived from F_0 genotoxic-exposed groups (Px and EMS F_0 groups) revealed that the offspring suffered an indirect impact from the genotoxic pressure in the F_0 generation. These results are particularly important since there is a lack of scientific information elucidating what happens at the level of DNA methylation when offspring is subjected to a new genotoxic exposure. Oppold et al. (2015) exposed a F_0 generation of the Asian tiger mosquito (*Aedes albopictus*) to vinclozolin (fungicide) and observed a decrease in the offspring (F_1) sensitivity to the insecticide imidacloprid (an hypomethylation was observed). Their results suggest that the epigenetic marker DNA methylation may be involved in the mechanisms that allow adaptation (*e.g.*, lower vulnerability) to insecticides (Oppold et al., 2015). Although it remains to be elucidated how organisms acquire toxic resistance, Bates et al. (2005) reported that low doses generally provide the best opportunity for its development. It is important to recall here that the species used in the present study (*P. clarkii*) is known for being an invasive species in European, African, and Asian ecosystems, with successful physiological strategies (phenotypic characteristics) even in inhospitable environments. This raises a key question of whether the observed changes in methylome due to exposure to an environmentally relevant concentration of penoxsulam, specifically in juveniles and male adults, may be behind an adaptive strategy for this species.

Juvenile crayfish from the Px-exposed F_0 group presented hypomethylation after exposure to EMS, indicating that, in the presence of a different genotoxic challenge, the memory of F_0 does not seem to prevent global methylation changes. In contrast, the % of DNA methylation increased in Px-exposed juveniles descended from EMS-exposed F_0, which may indicate the emergence of mechanisms that will permit to the exposed organisms to tolerate the stress and survive (Boothby, 2019).

Again, without data regarding gene expression information (*i.e.*, what genes in the crayfish are over- or under-expressed due to penoxsulam and EMS exposure), it is not possible to understand if the induced epigenetic inheritance was a burden or a gift. Therefore, in future work, in addition to be interesting to analyze the gene expression, it will also be of interest to see what phenotypic changes are occurring, for example from possible shifts in metabolic pathways to changes on reproductive and morphological features. It is noticeable, however, that these compounds induced changes in the methylation pattern, and this probably had consequences for the organisms and, consequently, for their populations.

4.4.4 The legacy of a parental exposure - an overview

Epigenetic transmission enables parents to influence the phenotypes of their offspring, providing, thus, a mechanism by which the parental environment can influence the offspring's performance (Donelson et al., 2018).

The impact of the parental exposure to penoxsulam on offspring appears to vary with the crayfish stage of development. Accordingly, juveniles (F_1) presented hypomethylation whereas hypermethylation occurred in the sexual maturity phase (adult males of F_1). This represents further evidence that methylome is not stable throughout the life cycle.

The present study showed that, in the juvenile stage, when crayfish face a new exposure to the same (penoxsulam) or to a different genotoxicant (EMS), no significant

changes were observed in the level of methylated cytosines (in relation to the unexposed crayfish also with an historical exposure to penoxsulam). However, comparing the offspring resulting from the herbicide-exposed group with the offspring from the non-exposed group, it was possible to perceive that, in general, penoxsulam induced a strong decrease in juveniles' global methylation.

Concerning the impact on offspring of a parental exposure to EMS, it was pointed out an identical pattern (when compared to penoxsulam groups). Thus, the unexposed offspring presented hypomethylation in juveniles (F_1) and hypomethylation in adults females (F_1). When offspring from the EMS-exposed group were compared with the non-exposed group, it was shown that the parental exposure to EMS also induced a decrease in global methylation, but only considering the unexposed juveniles. Moreover, juveniles exposed both to the same (EMS) and to a different (penoxsulam) genotoxicant did not have their global DNA methylation changed. Contrary to what happened in EMS-exposed F_0 adults (both genders), the level of methylated cytosines in F_1 juveniles did not decrease following exposure to EMS. Again, this could indicate that the historical impact of the parental exposure to EMS may provide to juvenile specimens some mechanisms to better tolerate stress and survive.

Bearing all this in mind, and considering future research in this thematic, it will be important to address the intergenerational effects when only one parent is exposed (*e.g.,* F_0 exposed male x F_0 unexposed females and *vice-versa*). Moreover, it would be also interesting, to consider the different organism's tissues/organs, as it has been reported that different cell types respond differently to the modulating action of xenobiotics on DNA methylation (*e.g.*, Akcha et al., 2021). Akcha et al. (2021) observed that the epigenetic effect of diuron (an herbicide) seemed to be tissue-specific in *C. gigas*, where DNA hypermethylation occurred in the digestive gland but not in gills and gonads. So, it will be important to investigate in future studies which tissues, other than muscle, may be susceptible to penoxsulam-induced changes on global DNA methylation. In fact, in

future works it would be particularly interesting to study the penoxsulam effect on the methylome of germ cells, since it is this epigenome that will be transmitted to the offspring.

Epigenetic changes, including in DNA methylation, constitute the basis for long-term adaptations (Suarez-Ulloa et al., 2015). In line, the present study suggests that the modulation of epigenome may partially explain the *P. clarkii* success as invasive alien species (Gherardi, 2006; Souty-Grosset et al., 2016), "conquering the world" and inhabiting from pristine to highly impacted environments. Overall, current data confirmed the occurrence of an intergenerational epigenetic memory, evidencing that the consequences of a given exposure to environmental stressors are not confined to the respective generation, which, using a grammatical analogy, can be translated into a conjugation in past continuous rather than past simple.

4.5 Conclusions

The present findings demonstrated, for the first time, the presence of DNA methylation in the species *Procambarus clarkii*, specifically in the striated muscle. Moreover, it was demonstrated that the global DNA methylation in this tissue differs naturally between genders, with females showing higher levels.

The herbicide penoxsulam did not induce changes in DNA methylation of adult crayfish (F_0). However, the hypomethylation occurring in unexposed F_1 juveniles demonstrated that the history of exposure per se (indirect exposure) can modulate epigenome. In the F_1 descendants of the penoxsulam-exposed group, males methylome (hypermethylated) was more affected than in females, showing a gender-specificity.

The genotoxicant model EMS induced hypomethylation in *P. clarkii* adult females (F_0), also showing to be gender specific. In addition, hypomethylation was also

observed in the unexposed F_1 crayfish, pointing out an intergenerational epigenetic effect.

The modulatory role of the historic exposure to penoxsulam or to EMS showed also to depend on the crayfish developmental stage.

Overall, this work showed that indirect experiences (events occurring in the predecessor generation) can have an impact on the epigenetic dynamics even greater than direct experiences (present events).

Finally, it is strongly recommended to consider epigenotoxic approaches as a critical element to thoroughly identify hazards and risk factors associated to environmental contaminants.

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Chapter V

Unveiling the nexus between parental exposure to toxicants and heritable spermiotoxicity - is life history a shield or a shadow?

Environmental Toxicology and Pharmacology, 95:103955, 2022

5. Unveiling the nexus between parental exposure to toxicants and heritable spermiotoxicity - is life history a shield or a shadow?

Keywords

Intergenerational impact; *Ex vivo*; Ecogenotoxicity; Pesticide; Reprotoxicity; Freshwater crustacean.

Abstract

The knowledge on parental experiences is critical to predict how organisms react to environmental challenges. So, the DNA integrity of *Procambarus clarkii* spermatozoa exposed *ex vivo* to the herbicide penoxsulam (Px) or ethyl methanesulfonate (EMS; model genotoxicant) was assessed with and without the influence of *in vivo* parental exposure to the same agents. The parental exposure alone did not affect the DNA of unexposed spermatozoa. However, the history of Px exposure increased the vulnerability to oxidative lesions in Px-exposed offspring. Otherwise, parental exposure to EMS allowed the development of protection mechanisms expressed when F_1 was also exposed to EMS, unveiling life history as a shield. The parental exposure to a different agent adverse and decisively affected Px spermiotoxic potential, pointing out life history as a shadow to progeny. Given the complexity of the aquatic contamination scenarios, involving mixtures, the spermiotoxicity of Px to wild *P. clarkii* populations emerged as probable.

5.1 Introduction

One of the most important works reporting the transmission of heritable toxic effects was the transgenerational study performed by Anway et al. (2005). Their outcomes were recognized as ground-breaking in the field of ecotoxicology, highlighting that contaminants entering the environment could alter the basis of future toxicological inheritance. Since then, research on this context aimed to address the heritance transmitted by the F_0 generation to the descendants, assuming that intergenerational approaches focus on F_1 generation (Perez and Lehner, 2019), while transgenerational studies focus on the F_2 and succeeding generations (Cleary et al., 2019). Although the intergenerational approaches can be considered essential for ecotoxicological research, there is a limited number of studies focusing on the influence of parental exposure on offspring responses to environmental stressors. The scarce literature available demonstrated that the knowledge on parental experiences is critical to predict how organisms react to environmental conditions (Plautz et al., 2013; Perez and Lehner, 2019).

There are several factors contributing to determine the profile of intergenerational effects, and thus, it is crucial to design full factorial experiments to investigate them (Donelson et al., 2018). For instance, the works developed by Donelan and Trussell (2015) and Luquet and Tariel (2016) showed that the same parental experience (*i.e.,* exposure to predator odors) induced opposite reactions in two gastropods species (*Nucella lapillus* and *Physella acuta*). In *N. lapillus*, the parental exposure to crab odors influenced the offspring phenotype only when it was exposed to the same cue; differently, in the experiment with *P. acuta* the predator-cue parental environment influenced the offspring phenotype only when it was raised in control conditions.

Notwithstanding the remarkable contribution of pesticides to the agricultural productivity in the last 50 years, their unpreventable ubiquity throughout the environment has resulted in a severe contamination, negatively impacting the

ecosystems health (Tang et al., 2021). Intergenerational studies concerning the impact of a parental exposure to pesticides are, nevertheless, uncommon and provided divergent indications. Oppold and co-workers (2015) demonstrated a positive intergenerational effect, as the descendants of mosquitoes exposed to the fungicide vinclozolin showed a decreased sensitivity to the insecticide imidacloprid. Occasionally, the repeated (or continuous) exposure to low levels of pesticides may provide an opportunity for the development of pesticide-resistance mechanisms (Bates et al., 2005; Brausch and Smith, 2009; Oppold et al., 2015). Still, the long-term effects of pesticides and the mechanisms involved in adaptation to chemical stress are questions that need to be unraveled (Farida Akcha et al., 2021). On the other hand, negative impacts (*e.g.*, hatching impairment) were reported following the exposure of parents and developing snails (*Lymnaea stagnalis*) to the fungicide pyraclostrobin (Fidder et al., 2016). Summarizing, intergenerational effects may be beneficial, providing a reinforced shield for upcoming challenges, but also a shadow for the future, due to an accumulation of negative consequences on the subsequent generations (Byrne et al., 2020). This bimodal profile is in line with epigenetic intergenerational effects described in mollusks, displaying to be disruptive but also adaptive, impairing or enabling organisms to cope with fast-changing environment (Fallet et al., 2020).

One of the most serious consequence of pesticides as water contaminants is the occurrence of DNA damage, mutations or chromosomal alterations, since various ingredients of commercial formulations present genotoxic properties (Bolognesi and Hayashi, 2011). In addition, unrepaired DNA damage has been associated with growth impairment, abnormal development, reduced fitness (*e.g.*, reproductive success) and embryo survival, which will affect populations and, subsequently, ecosystems (Anderson et al., 2014; Canty et al., 2009; Lee and Steinert, 2003; Sahlmann et al., 2017). Thus, and keeping in view the population level, the possibility of a parental exposure to genotoxic agrochemicals affect the genomic integrity of the offspring is a foremost concern,

although it remains underexploited. Barranger et al. (2014) reported evidence of vertical transmission of DNA damage in the *Crassostrea gigas* exposed to the herbicide diuron.

Genotoxic pressures are suspected to drive adverse effects at the population level (Jha, 2008), which are more likely when DNA damaging events occur in germ cells (Lacaze et al., 2010), comparing to somatic cells. The loss of DNA integrity on gametes could cause reproduction impairment, low fertilization rate, abnormal sperm morphology, embryo abnormalities and decrease offspring number (Barranger et al., 2016; Carroll and Marangos, 2013; Kumar et al., 2017; Lacaze et al., 2010; Shaw et al., 2017). Thus, the direct assessment of DNA integrity in germ cells, such as spermatozoa, is relevant and could allow understanding how toxicant-induced changes in the genome may affect the long-term success of the next generations. Male gametes of aquatic species with external reproduction may be directly exposed (after mating or during fertilization) to aquatic contaminants with genotoxic properties, including pesticides, which can impact the descendants (Arizza et al., 2009). Therefore, with the intuit of protecting aquatic populations, namely in what regards the mitigation of reproductive impairments, there is an increasing environmental concern related to spermiotoxicity. Considering aquatic invertebrates, the spermiotoxicity has been assessed through functional impairments, such as motility (Esposito et al., 2020) and metabolic disorders (Favret and Lynn, 2010), as well as through DNA integrity (Lacaze et al., 2011). It is, therefore, reinforced the need to develop intergenerational approaches coupled with pesticide spermiotoxic assessment.

The central hypothesis of the present study concerns the possibility of a memory of parental (F_0) exposure influence the offspring (F_1) responses to a chemical challenge, either corresponding to a repeated exposure (same agent relative to F_0) or to a new context resulting from the exposure to a different agent or the permanence in an uncontaminated milieu (*e.g.*, simulating events on the wild such as habitat restoration or organisms' displacement to a pesticide-free area). Hence, an intergenerational

approach was carried out addressing the spermiotoxic effects of the herbicide penoxsulam (potential environmental genotoxicant; see Marçal et al., 2020 and Patetsini et al., 2013) and ethyl methanesulfonate (EMS; model genotoxicant). Penoxsulam is a post-emergence herbicide belonging to the triazolopyrimidine sulfonamide class, in the market since 2005 (DGAV, 2015) and widely used against a broad-spectrum of annual and perennial weeds (Sondhia et al., 2016), that can reach the aquatic environment through leaching and runoff processes. The EMS is an alkylating agent that can induce DNA strand-breaks through the alkylation of phosphodiester bonds (Štambuk et al., 2008), known for its genotoxic and mutagenic potential on insect, crustacean, and human cells (Amini, 2014; Costa et al., 2018; Sega, 1984). This study included an *in vivo* F0 (parents) exposure and a subsequent evaluation of F1 (offspring' gametes) following *ex vivo* spermatozoa exposure, therefore building an intergenerational approach where only the spermatozoa of F1 organisms were assessed. So, the present study aimed to evaluate the DNA integrity in F1 spermatozoa exposed to penoxsulam or to EMS, with and without the influence of parental exposure (to penoxsulam or EMS).

5.2 Materials and methods

5.2.1 Chemicals

Penoxsulam (Px; CAS: 219714-96-2) and ethyl methanesulfonate (model genotoxicant; CAS: 62-50-0) were obtained from Sigma-Aldrich Chemical Company (Spain). The DNA lesion-specific repair enzyme, namely formamidopyrimidine DNA glycosylase (FPG) was obtained from New England Biolabs (USA). All other chemicals were obtained from Sigma-Aldrich Chemical Company (Spain).

5.2.2 Organisms' maintenance

The red swamp crayfish (*Procambarus clarkii*) analyzed in this study (and belonging to the F_1 generation) were born in the laboratory, descending from an early generation (F_0) collected (2017) in the Minho River (Vila Nova de Cerveira, NW Portugal), a low impacted area in terms of inorganic and organic contaminants, including pesticides (Rocha et al., 2021; Vera et al., 2021), with a good water quality (Castro-Valencia et al., 2019). The initial set of adult crayfish specimens (F_0 generation) presented an average length of 11.22 ± 0.91 cm.

In the laboratory, F_0 crayfish were kept in individual aquaria, during two weeks, for acclimation before starting the parental exposure trial (see section 2.3), with the following water conditions: constant temperature (20 ± 1°C), freshwater (dechlorinated tap water; salinity 0) with aeration (dissolved oxygen 8.1 ± 0.5 mg L^{-1}), daily UV disinfection, weekly control of nitrites (0.2 ± 0.05 mg L^{-1}), nitrates (25 ± 6.0 mg L^{-1}), ammonia (< 0.1 mg L^{-1}) and pH (7.5 ± 0.2). Animals were daily fed *ad libitum* with crustacean feed, Caridina Vita, produced by Sparos® (Olhão, Portugal).

5.2.3 Intergenerational experimental design

In Fig. 1 is represented the intergenerational trial performed. Briefly, F_0 adult crayfish, kept individually in 1 L aquaria, were exposed to 23 µg L^{-1} of penoxsulam [Px; concentration based on its environmental levels (Murussi et al., 2014)] or to 5 mg L^{-1} of EMS (model genotoxicant; concentration used as a positive control in genotoxic studies, *e.g.*, Cavas, 2011; Costa et al., 2018), for seven days (water and room conditions were the same as for the acclimation period). Groups of 10 males and 10 females were used for each treatment. Px is water soluble, hydrolytically stable and non-volatile, with a half live in the aquatic system between 1.28 and 1.96 days (Kogan et al., 2011). A control group (NC) was maintained in contaminant-free freshwater. Penoxsulam, EMS and control media were daily renewed (100%) in the exposure period. Afterward, F_0 crayfish were paired (10

couples per treatment) and allowed to reproduce (intra-group crosses) in uncontaminated freshwater. During the mating period, the temperature was set for 23.0 ± 1.0°C to provide the proper conditions for reproduction. Females with eggs were relocated in individual aquaria, and the water temperature was maintained. After hatching, F_1 specimens were transferred to a new aquarium (offspring were separated according to the provenience group F_0) to grow in a contaminant-free water, until reach the adult stadium. This medium was weekly renewed; water conditions were similar to acclimation. Two F_1 adult males (8-month-old, with an average length of 7.35 ± 0.16 cm) were randomly selected from each F_0 descendant group (*viz*. NC, Px, and EMS; in a total of six males) as sperm donors to perform the *ex vivo* assay. For sperm extraction, the vas deferent of each animal was excised and placed into 1.5 mL of cooled PBS, in a petri dish. Then, the distal section was gently pressed to release the sperm into the PBS medium, making a cell suspension that was immediately used. The individual sperm samples from each F_1 group were combined into a pooled sample, and then, divided into 18 sub-samples (microtubes), corresponding to 6 per treatment (n=6).

Ex vivo incubations were carried out in 2 mL microtubes, for 2 hours, at 18 ± 1°C, with constant rotational movement (0.9 rpm; 150 mm radius) to avoid cells deposition. Briefly, 200 µL of cell suspension in PBS (approx. 3 x 10^5 cells) were added to 1.8 mL of the proper medium, *i.e.*, PBS (control condition) or test solution (*i.e.,* Px: 23 µg L^{-1} of Penoxsulam; EMS: 5 mg L^{-1}, both dissolved in PBS). A total of nine groups of the F_1 spermatozoa were formed (see Fig. 27):

(i) descendants from the unexposed group, *viz*. NC-NC (unexposed spermatozoa), NC-Px (penoxsulam-exposed spermatozoa) and NC-EMS (EMS-exposed spermatozoa);

(ii) descendants from the penoxsulam-exposed crayfish, *viz*. Px-NC (unexposed spermatozoa), Px-Px (penoxsulam-exposed spermatozoa) and Px-EMS (EMS-exposed spermatozoa);

(iii) descendants from the EMS-exposed crayfish, *viz*. EMS-NC (unexposed spermatozoa), EMS-Px (penoxsulam-exposed spermatozoa) and EMS-EMS (EMS-exposed spermatozoa).

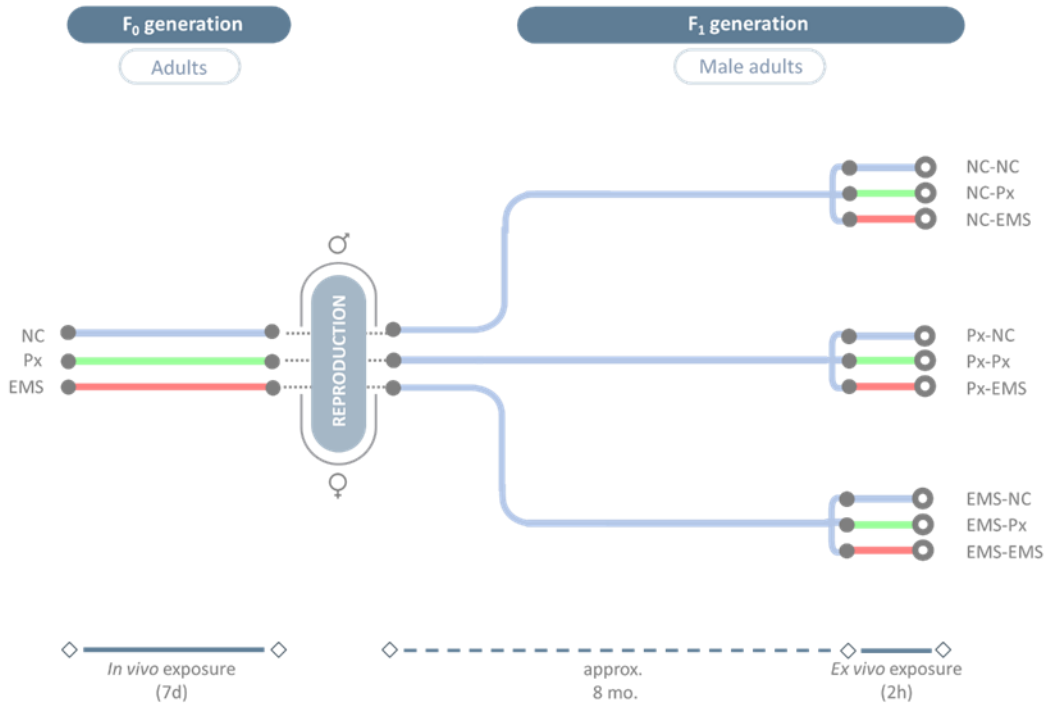


Figure 27. Schematic representation of the intergenerational experiment involving the exposure of the red swamp crayfish (*Procambarus clarkii*) to penoxsulam (Px; green line) and ethyl methanesulfonate (EMS; red line). Male (♂) and female (♀) adults (F_0) were exposed during 7 days to Px or EMS. During this period, a control group (NC; blue line) was maintained in contaminant-free freshwater. After reproduction of the F_0 organisms (intra-group crosses), F_1 offspring was allowed to grow in uncontaminated water until adulthood (blue line). Spermatozoa samples of F_1 male adults were exposed in an *ex vivo* trial (2 h) to Px or EMS, and thereafter analyzed in comparison with a NC group (n=6 per treatment). o represents the end of the *ex vivo* exposure (sampling corresponding to data currently shown). Code references of the *ex vivo* exposed groups have the following interpretation: the first letters, before the hyphen, translate the parental exposure condition (F_0), *i.e.*, the history; the letters after the hyphen translate the condition of *ex vivo* spermatozoa exposure (F_1).

Cell viability was determined at the end of the assay by using the trypan blue exclusion method (Anderson et al., 2003; Strober, 2001). Thus, following 2 hours exposure, the sperm cell viability levels were above 90% in all experimental groups (Tice et al., 2000).

5.2.4 Cell preparation and genetic damage evaluation

At the end of the *ex vivo* exposures, the 2.0 mL microtubes containing cell suspensions were centrifuged at 200 *g*, for 5 min, at 4°C. Then, the supernatant was removed, and the pellet was resuspended in 2.0 mL of glycerol (10%) (Kwok et al., 2013) to carry out the freezing step (-20°C), for 48 h, to facilitate the cell lysis. This procedure is required since crayfish' spermatozoa is covered by an extracellular capsule, the spermatophore (Niksirat et al., 2013a, b). At the end of this step, samples were rapidly thawing at 37°C, the cryoprotectant solution was replaced by PBS, and the comet assay procedure was initiated.

The comet assay alkaline version was performed based on the method presented by Collins (2004) with slight modifications according to Lacaze et al. (2010) and Shaposhnikov et al. (2010). The incorporation of enzymes in the comet assay provided the opportunity to detect not only breaks but also specific lesions, such as oxidized bases (Collins, 2004). Specifically, FPG detects alkylated and oxidized bases (Azqueta and Collins, 2014; Speit et al., 2004) and has been used in several ecotoxicology studies to detect oxidative DNA damage in aquatic invertebrates (Braga et al., 2021; Costa et al., 2018; Gielazyn et al., 2003).

Briefly, 60 µL of cell suspension were resuspended in 70 µL low melting point agarose (1%; dissolved in PBS). Twelve drops (gels) with 6 µL of cell suspension were placed on a pre-coated glass slide with 1% of normal melting-point agarose (1%; dissolved in distilled water), as two rows of 6 gels (6 groups of two replicates), without coverslips, containing approximately 2.2×10^3 cells/gel. Gels were left for 5 min at 4°C to let agarose polymerize and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) overnight. To improve lysis step, slides with gels were washed for 5 min in cooled PBS to remove lysis solution and then incubated with proteinase K (40 µg mL^{-1}; dissolved in ultrapure water) in the dark, at 37°C, for 60 min.

Two sets of slides were prepared, after PK incubation: one was incubated with the endonuclease FPG, which converts oxidized purines into DNA single-strand breaks (Azqueta et al. 2009). Another set of slides was incubated only with endonuclease buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8). Slides were washed with this buffer for 3 times, 5 min each, at 4°C. Thirty µL of FPG (diluted in endonuclease buffer) were applied in each gel. Slides were then immediately incubated in the dark at 37°C, for 30 min, and then, gently placed in a horizontal electrophoresis tank, filled with freshly electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), for alkaline treatment. DNA was then allowed to unwind for 40 min. Electrophoresis was performed under 0.6 V cm^{-1} for 24 min. Lysis, DNA unwinding, and electrophoresis were carried out in the dark, at 4°C. Once finished the electrophoresis, slides were washed in PBS (10 min), distilled water (10 min), and then fixed for 15 min in absolute ethanol.

Gels were stained with ethidium bromide (20 g L^{-1}). Fifty nucleoids per gel were scored, using an Olympus BX 41 fluorescence microscope (400× magnification).

The DNA damage was quantified by visual scoring, where nucleoids were grouped into five discrete comet intensity and length classes (ranging from class 0, collecting nucleoids with no tail, to class 4 for nucleoids with almost all DNA in the tail; Collins 2004). A genetic damage indicator (GDI) was calculated as a major endpoint for each assay as follows:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where i is the number of each defined class (ranging within 0-4) and GDI values were inherently expressed as arbitrary units, in a scale of 0–400 per 100 scored nucleoids. The difference between GDI_{FPG} (genetic damage indicator of slides treated with FPG) and GDI, corresponding to NSS_{FPG}, was calculated to indicate additional DNA breaks, which occur in net enzyme-sensitive sites solely (Azqueta et al. 2009).

5.2.5 Statistical analysis

Statistical analysis was performed with the software SigmaPlot® 14.0. Normality and homogeneity of variances were confirmed by Shapiro-Wilk's W and Brown-Forsythe (HOV) tests, respectively, to meet the required statistical demands. Two factors were considered: history (factor 1, reflects the parental exposure – F_0); treatment (factor 2, contemplates the treatment to which the F_1 gametes were submitted). Consequently, a two-way ANOVA were applied to assess the effects of each factor and the interaction of the factors in DNA' response. A two-way ANOVA on ranks was applied when the normality assumption failed. When the two-way ANOVA revealed a significant interaction between factors, simple main effects were assessed for each factor. Then, each ANOVA was followed by a post hoc Tukey test to compare groups within the same history factor, and by a post hoc Dunnett test to compare groups with the respective control within treatment factor. Differences between groups were considered significant when $p < 0.05$ (Zar, 2010).

5.3 Results

All the parameters (*i.e.,* GDI, GDI_{FPG}, NSS_{FPG}, GDI individual classes and GDI sub-total) showed a statistically significant interaction between the factor's "history" and "treatment" (Table 1). Therefore, the results presented below are based on the simple main effects analysis of each factor.

Table 1. Summary of the two-way ANOVA relative to the genetic damage indicators [including the analysis of the individual classes of GDI, the genetic damage indicator with an extra step of digestion with endonuclease FPG (GDI_{FPG}), and DNA breaks corresponding to net FPG sensitive sites (NSS_{FPG})] measured in the spermatozoa of *Procambarus clarkii* following an *ex vivo* exposure. Two factors were considered: history (reflects the parental exposure); treatment (contemplates the treatment to which the gametes were exposed). When the interaction between factors was significant the p value was marked bold.

Parameter	Source of Variation	df	MS	F	p
GDI	History	2	14326.718	6.713	0.003
	Treatment	2	51877.377	24.307	<0.001
	History x Treatment	4	36440.566	17.074	**<0.001**
	Residual	38	2134.255		
	Total	46	7090.122		
GDI_{FPG}	History	2	4559.528	2.979	0.062
	Treatment	2	58265.53	38.064	<0.001
	History x Treatment	4	11795.796	7.706	**<0.001**
	Residual	42	1530.706		
	Total	50	4742.389		
NSS_{FPG}	History	2	1645.89	1.299	0.284
	Treatment	2	1287.798	1.016	0.371
	History x Treatment	4	4907.553	3.872	**0.009**
	Residual	40	1267.435		
	Total	48	1601.789		
GDI Class 0	History	2	714.171	10.62	<0.001
	Treatment	2	1538.889	22.884	<0.001
	History x Treatment	4	352.371	5.24	**0.002**
	Residual	41	67.248		
	Total	49	188.969		
GDI Class 1	History	2	417.418	1.792	0.179
	Treatment	2	2811.401	12.07	<0.001
	History x Treatment	4	2052.828	8.813	**<0.001**
	Residual	41	232.922		
	Total	49	471.211		
GDI Class 2	History	2	275.849	2.128	0.132
	Treatment	2	265.932	2.052	0.141
	History x Treatment	4	530.518	4.093	**0.007**
	Residual	41	129.603		
	Total	49	173.755		
GDI Class 3	History	2	498.848	3.357	0.045
	Treatment	2	1882.825	12.671	<0.001
	History x Treatment	4	1458.766	9.817	**<0.001**
	Residual	41	148.59		
	Total	49	319.774		
GDI Class 4	History	2	297.263	2.821	0.071
	Treatment	2	1176.004	11.161	<0.001
	History x Treatment	4	808.378	7.672	**<0.001**
	Residual	41	105.372		
	Total	49	200.827		
GDI Subtotal (2+3+4)	History	2	448.559	5.891	0.006
	Treatment	2	1003.6	13.181	<0.001
	History x Treatment	4	1190.6	15.637	**<0.001**
	Residual	41	76.142		
	Total	49	212.439		

5.3.1 Genotoxicity assessment on spermatozoa with no influence of parental exposure

This section presents the results concerning the descendants of the F_0 unexposed group (NC) (Fig. 28: bars above blue arrow; Table 2: data aligned with the blue arrow), thus translating, simply, an unigenerational effect of the tested agents.

Non-specific DNA damage

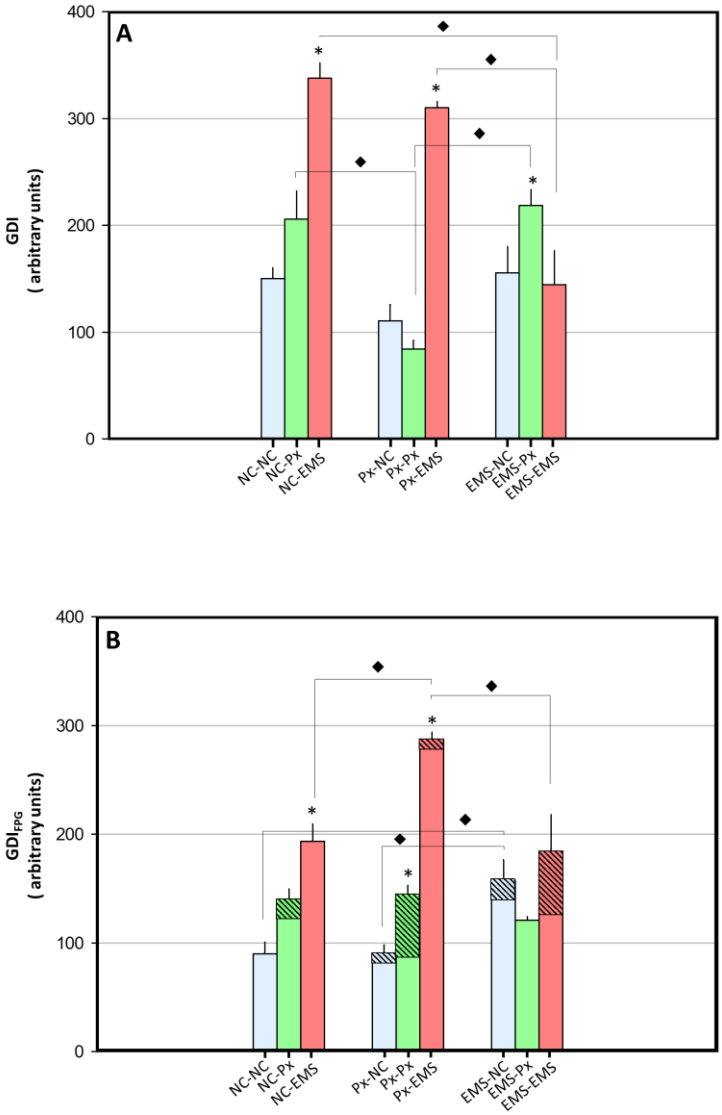
No significant differences were observed in GDI levels between the spermatozoa exposed to Px and the negative control group (NC-Px = NC-NC; Fig. 2A). Considering the GDI individual classes (Table 2), the spermatozoa exposed to Px presented less nucleoids in the class 0 (no-damage) than the control group (NC-Px < NC-NC; 2.98-fold; $p < 0.05$). Moreover, the predominant classes of the Px group were class 1 (low damage) and class 2 (medium damage) (Table 2).

Spermatozoa exposed to EMS (model genotoxicant) presented higher GDI values when compared to the negative control group (NC-EMS > NC-NC; 2.25-fold; $p < 0.001$; Fig. 28A). The analysis of the individual classes of damage on the spermatozoa exposed to EMS showed that the classes 0 and 1, from the gametes belonging to the control group, were significantly lower (NC-EMS < NC-NC; $p < 0.05$, $p < 0.01$, respectively; Table 2); the classes 3 and 4 were predominant, while the sum of nucleoids corresponding to the damaged classes (represented by the sub-total) was significantly higher than the control group (NC-EMS > NC-NC; $p < 0.01$; Table 2).

Oxidative DNA lesions

Concerning the oxidative DNA damage resulting from the exposure of F_1 spermatozoa (Fig. 28B), a pattern similar to that above described for GDI parameter was observed comparing the exposed groups and the negative control, *i.e.*, no significant

differences were observed on GDI_{FPG} values (NC-Px = NC-NC), while EMS-exposed gametes presented 2.15-fold higher GDI_{FPG} values than the unexposed spermatozoa (NC-EMS > NC-NC; $p < 0.01$). No statistically significant differences were observed considering the NSS_{FPG} parameter (Fig. 2C).



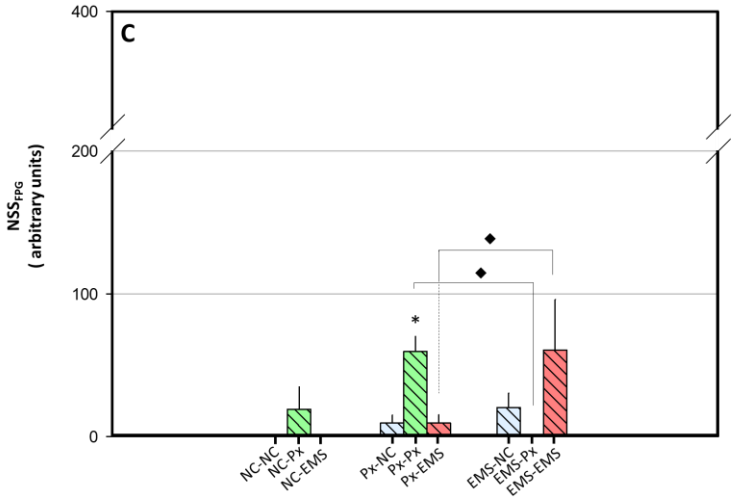


Figure 28. Genotoxic assessment on spermatozoa of F_1 crayfish. Mean values of DNA damage measured by comet assay on male gametes of *P. clarkii*. F_1 spermatozoa of descendants from F_0 unexposed penoxsulam-exposed, and ethyl methanesulfonate-exposed (EMS) groups were currently exposed to 23 µg L^{-1} of penoxsulam (Px; green) or to 5 mg L^{-1} of EMS (red) and compared to the respective negative control group (NC; blue). (A) Genetic damage indicator as non-specific DNA damage (GDI); (B) global and partial DNA damage (GDI_{FPG}), *i.e.*, GDI and additional strand breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; striped area); (C) NSS_{FPG}. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) *vs.* NC, within the same history group; (◆) *vs.* other history group within the same treatment.

5.3.2 Assessment of the parental exposure impact on F_1 unexposed spermatozoa (intergenerational genotoxicity)

This point presents the results regarding specifically the cell groups unexposed in F_1, but with an history of exposure (*i.e.*, F_0 exposed to Px and F_0 exposed to EMS) (NC; Fig. 28 blue bars; Table 2: data highlighted in blue). The statistical analyses here described allow the singly identification of the impact of parental exposure (or history) on the spermiotoxicity expressed on the following generation. As mentioned above, the results from the two-way ANOVA showed that the effects of the treatment in the studied parameters depend on the factor "history" (Table 1).

Non-specific DNA damage

Considering the GDI parameter, no differences were observed between the three NC groups of F_1 (*i.e.*, Px-NC = NC-NC and EMS-NC = NC-NC; Fig. 28A).

Similarly, the analysis of the GDI individual classes, among the unexposed F_1 groups, showed no differences between the descendants of exposed groups and the descendants of unexposed group (*i.e.*, Px-NC = NC-NC and EMS-NC = NC-NC; Table 2).

Oxidative DNA lesions

Spermatozoa of NC group (F_1) descending from F_0 EMS-exposed group presented significantly higher GDI_{FPG} values than those descending from unexposed (EMS-NC > NC-NC; 1.75-fold; $p < 0.05$) and Px-exposed (EMS-NC > Px-NC; 0.99-fold; $p < 0.05$) groups (Fig. 28B). No significant differences were found in the NSS_{FPG} parameter comparing F_1 unexposed spermatozoa (NC) groups (Fig. 28C).

Table 2. Mean frequencies (%) of damaged nucleoid classes (±standard error), measured by comet assay on male gametes of *P. clarkii*. Descendants from F_0 unexposed ( ), penoxsulam-exposed (Px;), and ethyl methanesulfonate-exposed (EMS;) groups were currently exposed to 23 $\mu g \cdot L^{-1}$ of penoxsulam (highlighted green) or to 5 $mg \cdot L^{-1}$ of EMS (highlighted red) and compared to the control groups (NC; highlighted blue). Statistically significant differences ($p < 0.05$) are: (*) *vs.* NC within history group; (◆) *vs.* groups within same treatment with different history.

	GDI - DNA damage classes					
	0	1	2	3	4	Sub-total (2+3+4)
NC-NC	11.17 ± 3.44	43.86 ± 2.93	25.17 ± 4.09	14.19 ± 1.14	5.61 ± 2.11	44.97 ± 5.61
NC-Px	3.75 ± 2.55*	36.37 ± 9.01	34.38 ± 5.89	17.59 ± 7.79	7.92 ± 3.91	59.88 ± 11.05
NC-EMS	0.00 ± 0.00*	2.22 ± 0.22*	4.07 ± 0.93	47.40 ± 15.40*	46.31 ± 14.69*	97.78 ± 0.22*
Px-NC	24.24 ± 5.27	49.39 ± 2.36	19.78 ± 3.78	4.54 ± 1.57	2.06 ± 1.42	26.37 ± 6.11
Px-Px	29.53 ± 6.80	60.16 ± 8.26	7.64 ± 2.06	1.66 ± 0.71	1.00 ± 1.00	10.30 ± 3.42
Px-EMS	0.00 ± 0.00*	4.71 ± 2.67*	21.60 ± 7.96	45.33 ± 6.80*	28.36 ± 6.85*	95.29 ± 2.67*
EMS-NC	11.67 ± 4.01	35.50 ± 6.78	29.17 ± 2.95	16.83 ± 7.40	2.83 ± 1.60	48.83 ± 9.68
EMS-Px	0.67 ± 0.67*	31.33 ± 5.10	21.33 ± 2.09	18.67 ± 2.81	22.17 ± 2.82*	62.17 ± 4.67
EMS-EMS	0.33 ± 0.33*	43.33 ± 8.69	21.83 ± 5.53	7.83 ± 3.66	8.50 ± 4.10	38.17 ± 12.73

5.3.3 Genotoxicity assessment on exposed spermatozoa under the influence of parental exposure

This point presents the results of the exposed spermatozoa (Px and EMS) of F_1 crayfish descending from the F_0 exposed groups (*i.e.*, F_0 exposed to Px and F_0 exposed to EMS). The results here putted on the spotlight allow the identification of the combined effect of parental (indirect) and current (direct) exposures. Again, the results of the two-way ANOVA showed that the effects of the treatment in the studied parameters depend on the factor "history" (Table 1).

Non-specific DNA damage

Px-exposed spermatozoa of F_1 crayfish descendants from F_0 Px-exposed group (Px–Px) presented lower GDI values (2.59-fold) than those descending from F_0 unexposed (Px–Px < NC–Px; 2.44-fold; $p < 0.01$) and EMS-exposed (Px–Px < EMS–Px; $p < 0.01$) groups (Fig. 28A). The F_1 descendants from the F_0 Px-exposed group presented a higher GDI value (2.80-fold) following EMS exposure when compared to the unexposed group (Px-EMS > Px-NC; $p < 0.01$). The analysis of the DNA damage individual classes (Table 2) also showed that the Px-exposed group, from crayfish descendants of F_0 Px-exposed group, presented more nucleoids in the class 1 (Px-Px > EMS-Px), and less gametes in the damaged classes (classes 2, 3 and 4), than the groups Px-exposed spermatozoa from F_0 EMS-exposed group (Px-Px < EMS-Px) and the Px-exposed spermatozoa from F_0 unexposed group (Px-Px < NC-Px).

Considering the descendants of F_0 EMS-exposed group (Fig. 28A), data showed that the Px-exposed spermatozoa presented a GDI value significantly higher than the unexposed spermatozoa (EMS-Px > EMS-NC; 1.40-fold; $p < 0.05$). The EMS-exposed spermatozoa from crayfish descendant of F_0 EMS-exposed group presented lower GDI values (2.15-fold) than the EMS-exposed spermatozoa from F_0 Px-exposed group (EMS-EMS < Px-EMS; $p < 0.01$; Fig. 28A) as well as than the EMS-exposed spermatozoa from F_0

unexposed group (EMS-EMS < NC-EMS; 2.34-fold; $p < 0.01$; Fig. 28A). In Table 2 (highlighted red data), it is possible to observe in the results of the analysis of the GDI individual classes that the EMS-exposed spermatozoa from descendants of F_0 EMS-exposed group presented less gametes in the damaged classes than the spermatozoa of crayfish from F_0 Px-exposed group (EMS-EMS < Px-EMS; 2.15-fold; $p < 0.01$) and the EMS-exposed spermatozoa from F_0 unexposed group (EMS-EMS < NC-EMS; 2.50-fold; $p < 0.01$).

Oxidative DNA lesions

No significant differences in the GDI_{FPG} parameter were observed between the Px-exposed gametes from groups with different exposure histories (Fig. 28B). However, the Px-exposed spermatozoa of crayfish descendants of F_0 Px-exposed group presented higher NSS_{FPG} values than the Px-exposed spermatozoa from crayfish descendants of F_0 EMS-exposed group (Fig. 28C; Px-Px > EMS-Px; $p < 0.05$).

In what regards to the descendants of F_0 Px-exposed group (Fig. 28B), the Px-exposed and the EMS-exposed spermatozoa presented higher GDI_{FPG} values than the unexposed male gametes (Px-Px > Px-NC and Px-EMS > Px-NC; 1.62-fold and 3.22-fold, respectively). Even though, only the Px-exposed spermatozoa presented higher NSS_{FPG} values (Fig. 28C) than the unexposed group (Px-Px > Px-NC; 6.33-fold; $p < 0.05$).

Considering the EMS-exposed spermatozoa (Fig. 28B), statistically significant differences were observed in the GDI_{FPG} parameter between the group descendant from F_0 Px-exposed and the group descendant from the F_0 unexposed (Px-EMS > NC-EMS; 1.48-fold higher; $p < 0.01$) and F_0 EMS-exposed (Px-EMS > EMS-EMS; 1.57-fold higher; $p < 0.01$). The EMS-exposed spermatozoa, from descendants of F_0 EMS-exposed group, presented higher NSS_{FPG} values (6.04-fold; $p < 0.05$) than the EMS-exposed gametes from descendants of F_0 Px-exposed group (EMS-EMS > Px-EMS; Fig. 28C).

No significant differences in the GDI_{FPG} and the NSS_{FPG} were observed between groups descendants from the F_0 EMS-exposed (Fig. 28B and C).

5.4 Discussion

The real impact of genotoxicants on populations can only be predicted by perceiving the consequences on the offspring. Since there is the possibility of the parental exposure to a contaminant inflict a genotoxic effect in the progeny, the knowledge of these effects, and how they might be transmitted (parental exposure memory), is crucial to understand the vulnerability and/or the resistance in the following generation, which might be extrapolated to the actual impact on the population. So, the impact of a parental exposure in the spermatozoa DNA integrity of offspring, considering both non-specific and oxidative DNA damage, was considered in the present work.

During mating in wild, at a given moment and even during a short period of time, the spermatozoa of *P. clarkii* (a species with external fertilization) are in direct contact to the surrounding water, exposing them to the waterborne contaminants (Marçal et al., 2020). Thus, the currently adopted *ex vivo* approach, in addition to represent a cell-based methodology alternative to *in vivo* approaches, can also mimic what happens on the aquatic environment with this particular type of cells. Moreover, it allows a high-throughput toxicological screening, simplifying complex-toxicology trials into low-cost and low-time consuming assays, providing a larger volume of relevant information with a reduced number of organisms (Marçal et al., 2020), thereby minimizing ethical issues related to the use of animals in experimentation.

The above-mentioned principles were central in the planning of this research but an approach exclusively *ex vivo* won´t allow an intergenerational assessment. Hence, the study was designed with the combination of *in vivo* (F_0) and *ex vivo* (F_1) approaches.

5.4.1 How is DNA integrity of spermatozoa affected by a genotoxic challenge in the absence of a parental exposure?

In the present work, groups without parental exposure memory represent a scenario in which a given crayfish population faces, for the first time, a genotoxic pressure.

The genotoxic potential of penoxsulam, at environmental concentrations, to aquatic invertebrates was already reported (Marçal et al., 2020; Patetsini et al., 2013). Patetsini and co-workers (2013) observed a loss of DNA integrity in hemocytes of *Mytilus galloprovincialis* after an *in vivo* exposure (0.05 µg L^{-1} of penoxsulam). Besides, an increase of DNA breaks was observed in crayfish somatic cells (gills and hepatopancreas) and male gametes following penoxsulam *in vivo* exposure (23 µg L^{-1} of penoxsulam) (Marçal et al. 2022). Marçal et al. (2020) also observed a decrease of DNA integrity in crayfish sperm cells after an *ex vivo* exposure to penoxsulam, which was not confirmed by the present observations. The main differences between our current and previous (Marçal et al., 2020) studies that can explain an apparent divergence is that, presently, F_1 generation was born and developed in laboratory conditions, in contrast with a wild population captured from a referenced pesticide-free local. It appears that maintenance in the laboratory for one generation created a different predisposition to cope with the herbicide exposure. Furthermore, this reinforces the idea that parental history influences the DNA susceptibility to penoxsulam. Though no Px-induced DNA damage (including oxidative) was presently detected, the previous data and the current analysis of DNA damage classes individually (showing that almost 60% of the gametes had their DNA damaged) suggest that its genotoxic potential should not be disregarded.

EMS has been used as a model genotoxicant in invertebrate studies (*e.g.* Carmona et al., 2011; Costa et al., 2018; Kumar et al., 2014). Despite none of the studies had evaluated specifically its genotoxic effects on spermatozoa, a previous *in vivo* experiment performed by the authors (Marçal et al. 2022) demonstrated a DNA

damaging action on crayfish spermatozoa. Thus, the DNA damage found on male gametes during the present study (more than 90% of cells had their DNA affected), following an *ex vivo* EMS exposure, was expected.

No oxidized DNA bases were found in the sperm cells after the EMS *ex vivo* exposure. This outcome was somehow surprising, once the oxidative potential of this agent to the sperm cells of the red swamp crayfish was observed in an *in vivo* preceding study (Marçal et al. 2022). However, many variables may be behind this difference in the results of the two studies, namely: (i) the animals have different origins/historical profiles (animals in the previous work were sampled in the field, whereas those used in the present work were born and developed under laboratory conditions); (ii) the mode of exposure, as in the previous work the crayfish were exposed *in vivo* and in the present work the exposure was *ex vivo*; (iii) the enzyme incorporated into the comet assay in the previous work was the endonuclease III (which detects oxidized pyrimidine bases), while in the present work it was the FPG [which detects oxidized purine bases such as 8-oxo-2'-deoxyguanosine (8-oxodGuo), the major DNA oxidation product] (Collins and Azqueta, 2012). The 8-oxodGuo was detected in the male gonads of the Pacific oyster (Barranger et al., 2016) after *in vivo* exposure to diuron.

5.4.2 How is DNA integrity of spermatozoa impacted by a parental exposure to genotoxic pressures?

Response profile of F_1 spermatozoa under a current contaminant-free environment (intergenerational genotoxicity)

The presence of toxic compounds in the aquatic environment may be ubiquitous and frequent, despite not necessarily continuous (temporally and spatially). Adding this to the crayfish behavior (*e.g.*, performing relatively long displacements), the exposure to contaminants could be temporally circumscribed and followed by a period of permanence in non-contaminated areas (Loureiro et al., 2015). In fact, if the

contamination is perceived, organisms may be able to avoid it (Wilding and Maltby, 2006). Moreover, behavior traits like avoidance and burrowing have been proposed as sensitive indicators of contaminant effects (Beiras, 2018). Bearing this in mind, a scenario where the spermatozoa from F_1 crayfish, descending from F_0 exposed organisms, might be released in a nonpolluted environment is realistic. Conceivably because of this, but not only, many of the inter- and transgenerational studies carried out trials with similar frameworks (Anway et al., 2005; Vandegehuchte et al., 2009b).

It has been observed that the herbicide diuron induced structural DNA lesions in spermatozoa of F_0 Pacific oyster (*C. gigas*) with a negative impact in offspring, namely on F_1 recruitment (decreased hatching rate, higher levels of larvae abnormalities and reduced larvae growth) (Barranger et al., 2014; 2016). Unfortunately, no information about DNA integrity of adult' oyster belonging to F_1 was provided by these studies, lacking a link with the following generations in that regard. The assessment of DNA integrity on F_1 organisms is valuable once the quality of the spermatozoon' DNA have a great impact on their reproductive success and also on the F_2 embryos development (Herráez et al., 2017).

In the present study, no genotoxic effects exclusively attributable to parental exposure were observed, indicating that the F_0 exposure to penoxsulam (as well as to EMS) did not affect the DNA integrity of F_1 unexposed spermatozoa. Furthermore, concerning the parental exposure to penoxsulam, the results showed that it did not induce oxidative DNA damage in the F_1 unexposed spermatozoa.

Differently, the parental exposure to EMS increased the oxidized bases on the crayfish' spermatozoa, depicted in the GDI_{FPG} outcomes; it should be noted that this loss of DNA integrity in the spermatozoa was not observed for non-specific DNA damage. As mentioned above, EMS tends to interfere with guanine bases (Drabløs et al., 2004) and the FPG recognizes and removes alkylated and oxidized purine bases (Azqueta and Collins, 2014; Speit et al., 2004). So, this result points to an intergenerational transmission

of a spermiotoxic vulnerability only concerning the oxidative DNA damage associated to the history of exposure to EMS.

Response profile of F_1 spermatozoa under the exposure to an agent coinciding with the parental exposure

In this point, it is approached an environmentally realistic scenario predictive, for instance, of the responses of crayfish inhabiting a rice field where penoxsulam-based formulations are used every year (Costa et al., 2018), thereby provoking the exposure of consecutive generations. In other words, it concerns the assessment of the effect of Px (and EMS) on the spermatozoa of F_1 organisms whose progenitors (F_0) were also exposed to the same agent.

The history of penoxsulam-exposure seems to provide some protection to the offspring also exposed to the herbicide considering non-specific DNA damage, since, although organisms without parental history showed no effects, they still displayed higher DNA strand breaks than those (also exposed to the herbicide) descending from penoxsulam-exposed parents. On the other hand, this group (Px-Px) was the only where an increase of oxidative DNA bases (as NSS_{FPG}) was detected. So, while it appears that parental memory to the herbicide can make DNA less vulnerable to penoxsulam, the observed oxidative DNA damage (detectable here in the presence of FPG) points to a silent danger that could manifest itself later and endanger the F_1 generation. Despite the lack of studies addressing oxidative DNA damage on invertebrate sperm cells, several woks reported a particular vulnerability of spermatozoa DNA to oxidation, as a consequence of their limited capacity for DNA repair [well studied in mammalian spermatozoa (Smith et al., 2013; Xavier et al., 2019), but also reported in invertebrates (Erraud et al., 2019; Lacaze et al., 2011; Lewis and Galloway, 2009)]. Therefore, it may be predicted an instability of the sperm genome endangering Px-exposed populations.

Concerning the acquisition of resistance in pesticide-exposed populations, Rahman et al. (2010) observed that the insect diamondback moth (*Plutella xylostella*), under continuous selection pressure, developed a metabolic resistance to the pesticide emamectin benzoate. Furthermore, in this study it was shown that the first step towards establishment of resistance does not have a genetic background but is rather based on alterations of the immune and metabolic status transmitted as an epigenetic maternal effect (Rahman et al., 2010). Although the mechanisms behind the acquisition of resistance are not known, the invasive pest beetle *Leptinotarsa decemlineata* is often repeatedly subjected to insecticides, and, in spite of the use of high doses that may be lethal and, consequently, induce a strong selection pressure, the exposure to mild and sublethal levels may lead the exposed beetles and their descendants to evolved traits that increase their pesticide-stress management and survival (Margus et al., 2019). Another study points that the exposure to the insecticide imidacloprid induced a sensitivity decrease in two subsequent generations (F_1 and F_2) of the Asian tiger mosquito (*Aedes albopictus*), suggesting that a resistance was acquired (Oppold et al., 2015). However, parental exposure does not always have a beneficial impact, as shown by the study of Ishimota and Tomiyama (2019) where the F_1 neonates of *Scapholeberis kingi* (descendants from a F_0 insecticide-exposed group; pyraclofos and pirimicarb) were more sensitive to the insecticide exposure than the respective control group (descendant from a F_0 non-exposed group).

On the basis of the current data, it is not discernible a clear pattern of heritance, since the influence of Px parental memory on crayfish assumes different facets depending on the type of DNA damage considered. In contrast, the parental exposure to EMS allowed crayfish to acquire the development of DNA protection mechanisms that granted the adaptation to the same genotoxicant stimulus, explaining the lack of DNA integrity loss after a direct exposure to EMS.

Response profile of F_1 spermatozoa under the exposure to an agent different from that of parental exposure

In this point it is discussed what happens to the spermatozoa DNA of F_1 crayfish when exposed to a different agent, comparing to the one to which their parents (F_0) were exposed. This rationale supports the assessment of how a parental exposure may modulate the genotoxic response to an “unknown” (the concept of "unknown" here applied considers the present and precedents generations) DNA integrity challenge. To the authors’ knowledge, there are no studies addressing this topic.

In this representative scenario, the spermatozoa exposed to EMS showed no interference of the Px-exposure history, while those exposed to Px revealed compromised DNA integrity in the presence of an EMS-exposure history, pointing out an increased risk. Moreover, it should be highlighted that this was the only scenario (EMS-Px) where the herbicide induced a significant loss of DNA integrity measured as non-specific DNA damage.

The non-specific damage considered herein, namely single and double strand breaks (SSBs and DSBs) can be a product of the direct or indirect interaction of environmental, physical and/or chemical agents as well as ROS (reactive oxygen species) and/or free radicals and cellular molecules (Chatterjee and Walker, 2017; Tiwari et al., 2017). It is well stablished that these DNA lesions may initiate mutations and other genomic instabilities, carcinogenesis, and ageing (Tiwari et al., 2017). In addition, environmental pollutants, such as pesticides, can change genome function through epigenetic mechanisms involving DNA methylation, miRNA expression, and histone modifications (Collotta et al., 2013). The alteration of DNA methylation patterns will induce destabilizing changes in gene expression patterns, potentially leading to cell transformation and tumorigenesis (Collotta et al., 2013). For instance, in mammals, alterations of DNA methylation patterns, such as global genome hypomethylation and promoter hypermethylation of CpG islands of specific genes, have been increasingly

found in different types of tumors (Laird, 2005). In invertebrates, an intergenerational study with *P. clarkii* also demonstrated that the parental exposure to penoxsulam induced hypomethylation in their prole (Marçal et al., 2021). Brevik et al., (2020) found that the exposure of *Leptinotarsa decemlineata* to an insecticide decreased the global DNA methylation on F_0 and F_2 organisms, also revealing that many methylation changes occur within genes associated with insecticide resistance. miRNA alterations can also be related with insecticide (indoxacarb) resistance in *Spodoptera litura* (Shi et al., 2019).

In mammals, the predisposition to disease can be determined by factors other than genetic background and it has been suggested that it could be transgenerationally inherited through sperm miRNAs (Cruz et al., 2020). miRNA profiles have been studied in sperm cells of mice (Cruz et al., 2020) and rats (Herst et al., 2019). Cruz and her co-workers (2020) observed that the pre-conception paternal exposure to the pesticide DDT modulates the sperm non-coding RNA load of F_1, particularly miRNAs. Herst et al. (2019) observed that the effects of parental exposure to persistent organic pollutants (POP) on sperm miRNA profiles induce effects until F_3 generation. The sperm miRNAs that were affected by POPs alone are known to target genes involved in mammary gland and embryonic organ development in F_1, sex differentiation and reproductive system development in F_2 as well as cognition and brain development in F_3 (Herst et al., 2019).

Histone modifications could change the chromatin structure, impacting the degree of DNA exposure to damaging agents (Williamson et al., 2018). That is, more compact chromatin is more resistant to DNA damage and protected from DSBs (Williamson et al., 2018). A recent study developed by Chen and co-workers (2020) on the identification of histone proteins in spermiogenesis of Decapoda species, including the *P. clarkii*, ponder that the sperm nuclear histone distribution may play a role in the fertilization process. This is because similar nonmotile sperm, as in *Caenorhabditis elegans*, have demonstrated that genome is packaged by a nucleosome structure and carries a histone-based epigenetic memory that can effectively guide embryonic

development in offspring (Tabuchi et al., 2018). These observations demonstrate that epigenetic information carried by sperm histone modifications can regulate offspring gene expression and development (Chen et al., 2020).

The previously invoked mechanisms are likely to be on the basis of the intergenerational inheritance patterns demonstrated in *P. clarkii*. However, the identification of a particular mechanism based on the present data would be purely speculative, so the pathways described in other animal groups and contexts should be understood as clues to be explored in future investigations.

The results of the present study showed that the heritance profile is compound-specific, highlighting the complexity of the "parental memory" concept in aquatic invertebrates, but also the need to be further and deeply explored, namely disclosing the (epi)genetic mechanisms involved. Moreover, pesticides are commonly applied as commercial products, frequently with intricate chemical formulations, rather than as active components individually. Therefore, in upcoming investigations, a penoxsulam-based commercial herbicide, *e.g.* Viper®, might be studied, as well as the potential additive, synergistic, or antagonistic effects of the formulation on the sperm DNA could be determined. Additionally, and considering the experimental methodology chosen, there is a gap in the evaluation of the inherent effects of the corresponding metabolites generated by active compounds decomposition in the environment, which is also an approach ecologically relevant.

The impact of toxicants in descendants through parental exposure can be markedly affected by individual variations. In this direction, the use of a small number of sperm donors (2 per treatment) can not be overlooked when the extrapolation to the whole population is attempted. However, putting on the scales the sample representativeness and the effort towards the reduction of animals' use, it was privileged the latter, trying to maximize the information obtained from each animal, aware that the option does not significantly compromise the relevance of the data. It should be

highlighted that, adopting the current experimental design, a 9-fold reduction on the number of animals sacrificed (6 crayfish instead of 54 that would be required for the equivalent experiment adopting an *in vivo* approach) was achieved. This complies with the reduction goal of the 3R's principles.

Further research exposing spermatozoa with intact spermatophores to environmental stresses is advised to clarify their protective role against potential genotoxins in freshwater crayfish prior to fertilization. This is because spermatozoa in crayfish during and after mating are protected against environmental stresses by the spermatophore layers.

5.5 Conclusions

In an attempt to unveil the nexus between parental exposure to toxicants and heritable spermiotoxicity, the current data demonstrated that a memory of Px (as well as EMS) exposure, by itself, does not pose a risk to spermatozoa DNA integrity in the offspring if exempt from any genotoxic challenge. However, if the following generation (F_1) is targeted by potentially DNA damaging agents, the heritance emerges, though displaying multifaceted profiles. Hence, the heritance of a parental exposure to Px in a Px-exposed offspring showed somewhat contradictory patterns, depending on the type of DNA damage, where the clearest effect was an increased vulnerability to oxidative lesions. Differently, the reverberation of EMS exposure in F_0 and F_1 allowed the development of DNA protection mechanisms to the genotoxic challenge, stalling the propensity to DNA damage in spermatozoa and unveiling life history as a toxicological shield.

The intergenerational impact of a parental exposure to an agent different from that to which the present generation is subjected showed to be adverse and decisively affecting the spermiotoxic potential of Px (only detected with a background of parental

exposure to EMS). This points out life history as a shadow to progeny. Given the complexity of the aquatic contamination scenarios, involving complex mixtures, the scenario here invoked can be assumed as the most realistic among those tested, thereby demonstrating the potential of Px to affect wild *P. clarkii* populations, in particular through a spermiotoxic action.

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Chapter VI

General Discussion and Final Remarks

6. General discussion and Final Remarks

This chapter intends to carry out an integrated and transversal discussion of the main findings of chapters II to V, considering the general and specific objectives previously delineated for this thesis, contributing with new interpretative perspectives.

The present work was divided in two phases, where the first focused on the assessment of the spermiotoxic impact of waterborne pesticides, namely glyphosate and penoxsulam (herbicides), dimethoate and imidacloprid (insecticides), imazalil and pyrimethanil (fungicides), on the macroinvertebrate *Procambarus clarkii* (Chapter II). The second phase (Chapters III to V) addressed the intergenerational (epi)genotoxic impact of penoxsulam (the most genotoxic pesticide elected in the previous phase) to *P. clarkii*. The latter phase started by exploring the ability of the crayfish' somatic cells and spermatozoa to recover from the DNA damage induced by penoxsulam (Chapter III). The following chapters approached the impact of a parental exposure to penoxsulam on the methylome (Chapter IV) as well as on the DNA integrity (Chapter V) of the prole, considering different exposure scenarios. A transversal perspective throughout the work appears as a relevant contribution to the knowledge concerning the pro- and anti-genotoxic inheritance associated to pesticides exposure, suggesting, also, the implementation of the pesticidovigilance concept.

6.1 *Procambarus clarkii* as a non-model organism in ecogenotoxicology: weighing the pros and cons

"For such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied". August Krogh, Nobel Prize winning physiologist

In October 2021, the search engine Google Scholar gave 2320 results considering the associated terms "*Procambarus clarkii*" and "ecotoxicology" (*e.g.*, Alcorlo et al., 2019; Zhao et al., 2019; Frontera et al., 2014; Wei and Yang, 2015). Despite being a high number, it is 12 times lower than the results obtained by the conjugation

of the words was "*Daphnia magna*" and "ecotoxicology", mainly due to *D. magna* be effectively stablished as a model organism in aquatic toxicology research. Notwithstanding, according to Dietrich et al. (2020), from an epistemological perspective, the organism must "match" the entity that it seeks to represent in ways that are relevant for the question(s) being addressed (whether the entity is only organism's own species, a broader group or taxon, or some other target group such as humans). In that regard, the crayfish appears as a more realistic choice as a bioindicator in contaminated ecosystems (Passantino et al., 2021) over traditional model organisms, since the red swamp crayfish (i) is currently considered to be a major freshwater pest in Europe, where it is present in at least 15 countries (Souty-Grosset, 2016), (ii) provides comparable tissues to vertebrates for analysis and similar responsivity to xenobiotics (Stara et al., 2016), (iii) inhabits aquatic areas which can be anthropologically impacted (e.g., agricultural practice) (Anastácio, 1993; Costa et al., 2018), making this species of special interest.

In the ecotoxicology field, bioindicator species must have a set of characteristics, concerning: (i) ecology (*e.g.*, globally distributed, high densities and low mobility), (ii) morphology (*e.g.*, large body size for an easy identification of tissues) and, (iii) physiology (*e.g.*, sensitivity to toxic substances and be prone to toxin accumulation) (Belanger et al., 2017). The crayfish *P. clarkii* has all these attributes, since it is an invasive species in Europe, worldwide distributed (Loureiro et al., 2015), a dioicy species, with an adequate body size, and showing sensitivity to toxic compounds, namely genotoxic as previously demonstrated by Oskoei (2018). In the present work, this species demonstrated to be sensitive to the direct exposure of genotoxic compounds in germ and in somatic cells.

Having an r-selected reproductive strategy (*i.e.*, tending to be small in size, having large broods with high fatalities, and living in unpredictable environments where the probability of long-term survival is minimal) (Reynolds and Souty-Grosset, 2011), and, at the same time, the ability of tolerate sub-optimal habitat conditions (Belanger et al., 2017), *P. clarkii* emerges as an adequate choice to perform a laboratory intergenerational trial during approximately one year, achieving the generation F_1.

Despite this, the extreme mortality observed in juvenile stage (a characteristic of the r-selected crayfish) was a drawback since the number of specimens reaching the F_1 generation was too low, and the possibility of obtaining F_2 specimens was, then, discarded. Another disadvantage of planning *in vivo* experiments with this species, in particular, was the cannibalism, specifically inter-sexual during the mating period (as corroborated by He et al., 2021). Despite attempts to minimize this aggressive behavior in the laboratory, where the crayfish were always separated by gender (unless, obviously, in the reproduction period), the mortality due to intra-sexual aggression was high.

The use of the crayfish for the experimental works meets the 3R's principle (addressed in the section below), specifically the "refinement" principle, since they are considered less sentient than fish, for example, and its use is not subject to strict legislation, contrarily of what is related to vertebrates. Besides, the macroinvertebrate *P. clarkii* can provide differential tissues for analyses, which allowed a physiological perspective to understand the individual toxicological impact on distinct cells/tissues/organs, such as, spermatozoa (analyzed in the Chapters II and V), gills (performs similar functions to vertebrates' gills; analyzed in the Chapter III)(Foyle et al., 2020; Henry et al., 2012), hepatopancreas (performs the functions of storage of nutrients and detoxification equivalent to the liver and pancreas of vertebrates; analyzed in the Chapter III) (Cervellione et al., 2017) and the skeletal muscle (analyzed in the Chapter IV). Overall, crayfish can serve as an alternative to aquatic vertebrates in biological assays, since it can have a central role in the assessment of the impact of environmental pollution and it showed to be a suitable bio-indicator, as pointed out by other researchers (Kouba et al., 2010; Velisek et al., 2013).

6.2 Different approaches in ecogenotoxicology assays

In 1959, the zoologist W.M.S. Russell and the human scientist R.L. Burch published the work "The Principles of Humane Experimental Technique" which gave rise to the principle of the 3R's (Russell and Burch, 1959). The 3R's principle, *i.e.*

"Replacement, Reduction, and Refinement", aimed to be used as a key strategy of a systematic framework to achieve the goal of humane experimental techniques. The Directive 2010/63/EU (revising Directive 86/609/EEC on the protection of animals used for scientific purposes) adopted the 3R's principle with the main intention of protecting animals (*i.e.*, live non-human vertebrates and cephalopods) which were used for experimental and other scientific purposes (EU, 1986, 2010a).

Briefly, and considering the publication of Russell and Burch (1959), "replacement" can be defined as the methods, strategies or approaches (*e.g.*, *in vitro* systems using tissues, whole cells or part of cell systems, based on biochemical approaches) which do not involve the use of living animals. The "reduction" concept aims to decrease the number of animals used in the original procedure, including the maximization of the information obtained per animal. Currently, the term "refinement" implies the modification of any procedures or husbandry and care practices, from the time the experimental animal is born until its death, minimizing the pain, suffering and distress experienced and enhancing its well-being. "Refinement" can also be achieved by moving from species that are considered less sentient (*e.g.,* substituting the use of fish with aquatic invertebrates). For instance, daphniids and other crustaceans as crayfish, are not included in the "animal" definition in the Directive of 2010 (EU, 2010a). Even though, new studies suggest that it is likely that Decapoda organisms experience nociception (Appel and Elwood, 2009; Elwood, 2019; Kawai et al., 2004). Notwithstanding, although nowadays there is no European legislation to protect the use and handling of crayfish, animal welfare organizations, such as UFAW (Universities Federation for Animal Welfare; UK) and RSPCA (Royal Society for the Prevention of Cruelty to Animals; UK), provide some guidelines to proceed with crustaceans, where some methods to induce the minimum level of pain and distress (*e.g.*, chilling in air and then kicked by splitting or spiking to destroy their nervous system) should be adopted (EFSA, 2005). Thus, bearing all these ideas in mind, the implementation of an *ex vivo* approach (addressed in the next paragraph), a simple alternative to *in vivo* methods, proved to be a valuable contribute to the practical implementation of the 3R's. Considering this, in Chapters II and V,

where the *ex vivo* approach was performed, the 3R's were applied, *i.e.,* the outcomes were obtained promoting a great decrease in the number of animals (*Reduction*), since exposures performed using cells (male gametes) instead of animals (*Replacement*), and the few that were used were killed by spiking to minimize the pain, distress, and suffering (*Refinement*).

The *ex vivo* approach represents a strategy somewhat between *in vitro* and *in vivo*. It can be applied to: (i) the whole solid tissue, being the cytoarchitecture retained, as well as many of the intercellular connections and interplays, maintaining the metabolic processes more closely to the *in vivo* situation (Dusinska et al., 2012); (ii) tissues with circulating cells (*e.g.*, erythrocytes, lymphocytes, hemocytes), and (iii) gametes (in which this approach, considering species with external reproduction, can mimic a realistic scenario). Taking male gametes as an example, in the present thesis, the *ex vivo* approach allowed to disclose the spermiotoxic potential of the six pesticides with dose-response assessment (Chapter II), and the spermiotoxic evaluation of the alkylating agent EMS (Chapter V). Despite the advantages of the *ex vivo* approach, it is still not widely applied in studies with living organisms (*e.g.*, Sahlmann et al., 2017; Santos et al., 2013; Valant and Drobne, 2012). In fact, and despite being relevant, this approach presents a few limitations, such as (i) the short lifespan of cells, which only allows–short exposure length (2 h) of Chapters II and V experiments [this choice was based on the work of Oskoei (2018)], and (ii) the unrealism of direct exposures of internal tissues/cells, while in the *in vivo* exposure all biological process from the pesticide exposure (from uptake, to distribution, metabolization, and excretion) are taken into consideration.

In vivo experiments allow the exposure of the whole organism, where in the same experiment is possible the assessment of several tissues. In this regard, the experiment of Chapters III and IV was performed *in vivo*, where it was observed the herbicide effects on two somatic tissues (*i.e.*, gills and hepatopancreas) and germ cells (spermatozoa). The work developed in the Chapter V (a follow-up of Chapter III) showed that these two different approaches (*in vivo* and *ex vivo*) can be strategically combined and integrated. Additionally, an intergenerational assay started with a

parental (F_0) *in vivo* assay (Chapter III) and ended with the *ex vivo* exposure of offspring' male gametes (Chapter V). The combination of the two approaches answered the question whether parental exposure affects the DNA of the offspring's gametes and whether it influences their sensitivity to the herbicide exposure (Chapter V). Therefore, the combination of these two approaches allowed the obtention of relevant information at the level of pesticide genotoxicity at intergenerational level.

6.3 Pesticide risk assessment relying on (epi)genotoxic data

Chapter II met the first main goal of the thesis, where the genotoxicity of 6 currently used pesticides was studied. The spermatozoa were directly exposed to each pesticide (namely, glyphosate, penoxsulam, dimethoate, imidacloprid, pyrimethanil and imazalil), for a short period. To the author's knowledge no information regarding penoxsulam and dimethoate reprotoxic effects were known. Direct *ex vivo* exposure, albeit short, allowed to disclose the spermiotoxic potential of each pesticide in the *P. clarkii*. The highest concentration (environmental relevant level) of glyphosate, penoxsulam, dimethoate, pyrimethanil and imazalil was found genotoxic to the crayfish spermatozoa. Imidacloprid was the only pesticide that was shown to have a pro-oxidant effect. Penoxsulam and dimethoate were the only where the lowest concentration (10-times lower than the highest) induced DNA breaks on sperm, and therefore they were considered the most spermiotoxic. To approach the remaining goals of the thesis the penoxsulam was chosen, since, despite the lack of information regarding specifically the use of each pesticide, in Europe the utilization of herbicides is approx. 2.38 times higher than insecticides (Eurostat, 2022), and penoxsulam expiration of approval will be 2023 while dimethoate was 2020 (ECHA, a, b) suggesting higher environmental occurrence of the former in the near future.

6.3.1 The specific case of penoxsulam

Penoxsulam demonstrated to be one the most aggressive genotoxicant for the crayfish male gametes (Chapter II), among the analyzed pesticides, and therefore it was the pesticide selected to fulfill the remaining main aims of the thesis. Penoxsulam hazard was not limited to gamete cells, as it also proved to be dangerous for somatic cells. Furthermore, this herbicide showed to be gender-specific in somatic tissues of the crayfish, with long-lasting effects, even after the exposure cessation (Chapter III), enhancing the hazard risk of this compound. Moreover, at the epigenetic level, penoxsulam showed to have an intergenerational effect. In fact, this outcome may act as an alert to the risk related to the use of penoxsulam, since effects last in time and affect the forthcoming generations (see Chapter III and IV). The methylation pattern of male adults (F_1) was also changed, suggesting that the transmitted effects follow the development of the organism, at least to the next generation. The data from Chapter IV highlight the need to consider several parameters in the evaluation of the impact of a compound, even if the effects studied are only at the DNA level. These data should be complemented with future studies where, for example, phenotypic changes (*e.g.*, at the cellular and metabolic level) are considered. In the present study, no changes were observed in morphological characteristics visible to the naked eye. Bearing this in mind, it will be mandatory to study the transgenerational effects of penoxsulam, contributing to the understanding of: (i) up to which unexposed generation the epigenetic and phenotypic effects can be observed; (ii) how long the altered epigenetic and phenotypic states will last in an uncontaminated environment, in the case of acquired tolerance to the herbicide, as well as whether the epigenetic profiles will be reversed and the acquired tolerance will be lost, in the absence of the herbicide (Herrera and Bazaga, 2011; Kille et al., 2013; Morgan et al., 2007; Vandegehuchte and Janssen, 2014).

The present work intended to study the intergenerational effects of pesticides on DNA, namely its integrity and associated methylome. Parental exposure to penoxsulam induced a negative impact, suggesting that the probability of any

acquired tolerance by the offspring is reduced (Chapter V). The fact that no signs of acquired tolerance to the herbicide were observed on the descendants highlights the threat of this compound to the crayfish. Moreover, also in Chapter V, it was shown that the genotoxic action of penoxsulam in the F_1 crayfish was, undoubtedly, detected in organisms with parental exposures to the genotoxicant model EMS. These data reinforce the risk of the penoxsulam occurrence in the environment, since the aquatic ecosystems are the ultimate receptacle for a mixture of anthropogenic substances. In fact, in a real environmental scenario, where several compounds occur simultaneously and interacting, a parental exposure to penoxsulam may enhance the genotoxic pressure of other contaminants. Therefore, and taking all the outcomes into account, it becomes clear the real existence of a risk posed by the penoxsulam to aquatic species.

An active substance is approved by the European Commission only after a rigorous and lengthy (> 3 years) science-based assessment to ensure that its use is safe. Penoxsulam is accepted in EU since 2010 until 2025 however genotoxic side effects were detected in aquatic organisms. This reveals that the tests carried out for the pre-certification may not be sufficient. Besides, the control of the compounds and the knowledge of their actual risk could improve if pesticides, after approval and market entry, were studied and monitored in the environment during their approval period (approximately 10 years).

6.4 The environment calls for pesticidovigilance

Pesticides can only be accepted by regulatory agencies, such as the European Food Safety Authority (EFSA) with the fulfilment of the approval criteria laid down in Regulation (EC) No 1107/2009a, where the evaluation of the product (*e.g.*, the genotoxicity of the active substance) is required. According to EFSA, the genotoxic assessment requires the detection of three genotoxic endpoints, such as gene mutations, structural chromosomal aberrations (clastogenicity), and numerical chromosomal aberrations (aneugenicity and polyploidy) (More et al., 2021). The

alkaline comet assay, performed in the present thesis to detect the genotoxic potential of pesticides, is not an accepting test by EFSA Panels, but there is an internationally agreed protocol (OECD TG 489) for this technique (OECD, 2016). After obtaining permission to enter the market, this authorization is valid for up to 10 years, after which it can be renewed for up to 15 years (EC, 2009a). As seen in previous chapters, where the exposure to lower concentrations of penoxsulam, a pesticide approved by US-EPA in 2004 (US-EPA, 2004) and by EC in 2010 (EU, 2010b), compromised the integrity of the DNA of male gametes and induced changes in the methylome of the unexposed offspring.

In the pharmacovigilance, it is monitored the risk/benefit ratio of drugs as well as the improvement of the patients' safety and their quality of life (Fornasier et al., 2018). This long-term monitoring involves the collection, detection, assessment, monitoring, and prevention of adverse effects of pharmaceutical products, and it continues throughout the lifetime of each product, building a well-developed safety database for that product (Milner and Boyd, 2017). The pharmacovigilance consists in collecting and managing data on the safety of medicines, looking at individual case reports to detect new "signals" and a pro-active risk management to minimize any potential risk associated with the use of medicines, communicating and informing stakeholders and patients (Fornasier et al., 2018). So, since the implementation of pharmacovigilance drug tests is carried out, not only before the compound is approved, but also during its lifetime (*i.e.*, the time that it is in circulation on the market), crucial information regarding emerging impacts, such as secondary effects (*i.e.*, subsequent or less predictable effects), is obtained.

The long-lasting genotoxic impact of penoxsulam to the crayfish cells/tissues/organs, which may affect not only the exposed generation but also the following, warns that a continuous and more effective evaluation of pesticides is mandatory, as above described for pharmaceuticals, to guarantee the safety of organisms. Milner and Boyd (2017) suggest the concept pesticidovigilance to long-term monitor pesticides, determining unexpected and unknown effects, allowing to act in a timely manner, with the major intuit of protecting the environment.

According to European Commission, data on approved substances and authorized products are reviewed periodically, for instance, to reflect scientific progress. Moreover, a thorough EU review involving the European Commission, EFSA and Member States has been performed in the past 25 years on all the substances used in Europe. As a result, the number of approved active substances of pesticides has been cut down by more than 50%, and 25% were considered of low risk (microbial pesticides, insect pheromones and plant extracts). However, the last special report concerning pesticides, *viz.* "Sustainable use of plant protection products: limited progress in measuring and reducing risks" (European Court of Auditors, 2020), shows that a protocol/method for common use by EU member states for the surveillance of active substances (pesticidovigilance-like report) is not well defined, resulting into insufficient efforts which will be not enough to have the knowledge of the real risk of using pesticides.

Bearing the pesticidovigilance in mind, were the ecotoxicological biomarkers currently chosen (*i.e.*, DNA integrity and DNA methylation) sufficient to assess the danger of a compound at the intergenerational level? Will these be enough to carry out evaluations of the effects of pesticides in a population? Each DNA is a life project, and the preservation of its integrity is extremely important. Therefore, the preservation of the genetic entity must have its due importance in ecotoxicological studies. The lack of DNA integrity in somatic and germ cells, if not repaired will have effects on the immediate fitness as well as the reproductive success of exposed organisms (Jha, 2008). This will eventually lead to adverse effects on the long-term survival of the population and, therefore, as a consequence of the deterioration of the ecosystem health and sustainability. The preservation of DNA integrity against environmental insults is one of the most important adaptive traits in animals. Over time, animals evolved complex and finely tuned mechanisms to repair DNA (the first line of defense against genotoxicants). Impairing the defense responses to DNA damage leads to the accumulation of genomic lesions that can lead to cell death, mutagenesis and even teratogenesis and neoplasia (Costa, 2022).

So, for pesticidovigilance, it will be interesting start with the assessment of DNA integrity (that gives a first warning signal), followed the DNA repair, DNA methylation and genetic expression, followed by the reproduction impairment (*e.g.*, gametes viability, fecundity, embryo viability), and behavior (*e.g.*, matting, predation, avoidance) in the exposed and following generations. It will be of greater value to study the 2nd and 3rd generations (transgenerational effects). The pesticidovigilance will be a most valuable path to predict the real impact of pesticides on populations and ecosystems.

6.5 Final Remarks

The present thesis brought new insights regarding the intergenerational genotoxic potential of pesticides to non-target organisms, where it was demonstrated that a parental exposure can affect the offspring's DNA integrity, as well as their DNA methylation pattern.

Concerning the pesticide exposure, the two experimental approaches, *viz. in vivo* and *ex vivo*, displaying different advantages and the possibility to answer distinct questions, proved to be able to complement each other in the intergenerational study.

At environmental relevant concentrations, the waterborne pesticides, namely glyphosate, penoxsulam, dimethoate, pyrimethanil and imazalil, showed to induce DNA damage in spermatozoa of *Procambarus clarkii*. In addition, imidacloprid induced a pro-oxidant effect on male gametes.

The direct exposure of penoxsulam showed to be highly genotoxic, not only for germ cells (spermatozoa) but also for somatic cells (gills and hepatopancreas) of *P. clarkii*. Moreover, this herbicide presented cells- and gender-specificities, with females showing to be more vulnerable in the gills (where the unspecific DNA damage was not reversible following the post-exposure period), while males demonstrated higher susceptibility in what concerns to cells of internal organs, *i.e.,* hepatopancreas cells and spermatozoa (showing an inability to recover from DNA injuries). The

spermatozoa proved to be the most vulnerable cell type (among the cells analyzed), which, from an ecological perspective, represents a worrying finding due to the prospective threat to future generations.

Indirect influences (*e.g.*, parental exposure) showed to be able to have an impact on the DNA integrity and on epigenetic dynamics even greater than direct experiences (*e.g.*, current exposure). Thus, parental exposure to penoxsulam showed to affect the DNA integrity and the global DNA methylation pattern of offspring. The spermatozoa of crayfish, with a parental exposure to the penoxsulam, presented a higher DNA integrity following the herbicide exposure. Nonetheless, this effect did not occur when the spermatozoa were exposed to a different compound (*i.e.*, EMS), where it was detected an increase of the DNA damage. In this sense, the parental memory seems to be a crucial factor in the maintenance of the DNA integrity. Concerning the DNA methylation, the herbicide showed to have epigenotoxic properties. The offspring's methylome was affected due to the parental exposure (*i.e.*, juveniles presented hypomethylation while adult males hypermethylation).

The results of this thesis showed that an effort remains to be done in pesticides development, so that they are more specific in their target, and can be safe for the environment. Once the offspring of a generation exposed to a different chemical faces the exposure to penoxsulam in concrete, the loss of DNA integrity was visible and no evidence of activation of protective DNA mechanisms was seen.

Studies characterizing pesticides need to integrate more data, namely addressing different types of DNA damage, tissue- (somatic and germinative) and gender-specificities, a long-term appraisal of temporal progression of damage, and be extended over the "lifetime" of the chemicals (*i.e.*, during the time they are in circulation on the market, and where several generations may be affected), similar as it is done for drugs in pharmacovigilance.

Overall, this thesis contributed to the knowledge of the real impact of pesticides on the *P. clarkii*, as a non-target species. Penoxsulam damaged the DNA in several tissues, and the damage still persisted following the herbicide removal (*i.e.*,

females' gills and males' hepatopancreas and sperm). On the other, the intergenerational assay allowed to disclose that the invasive species *P. clarkii* seems to be able to develop favorable strategies, as detected in descendants from EMS-exposed parents (*e.g.*, mechanisms to protect the DNA integrity).

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