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Tracking the Ghost of the Genome: The epigenetics of pollution adaptation in an environmental sentinel

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Abstract

Understanding the long-term impacts of pollution and pesticides is important for environmental protection and management of chemical risks. Such knowledge can also provide a better understanding of evolutionary adaptation to stress. In this project, the transgenerational effects of toxicants and underlying epigenetic molecular mechanisms of effects within and between generations in earthworms are explored. Long-term chronic transgenerational exposures and field assessments were used to establish the phenotypic, genetic and epigenetic consequences of long-term exposure to a trace metal (Cd), a metalloid (As) and a pesticide (the neonicotinoid imidacloprid). Results from exposures of the earthworms *Eisenia fetida* and *Eisenia andrei* over three generations show that exposure to environmentally realistic concentrations of arsenic causes a mild hormetic effect on reproduction after one generation of exposure followed by a detrimental effect on growth after further continuous exposure and on survival regardless of whether worms are later kept in a control or spiked environment. Similar exposure to cadmium resulted in a detrimental impact on growth during the first generation, after which the earthworms adapted to this exposure in subsequent generations. Continuous Imidacloprid exposure reduced growth rates for three generations. Some degree of cumulative effect on rates of survival were seen (i.e. effect increasing in each generation) up to three generations of exposure. No transgenerational effects were observed. To further understand the mechanistic and epigenetic effect of long-term exposure, a study was undertaken to investigate global gene expression responses in a population of the earthworm species *Lumbricus rubellus* resident at a long-term heavily polluted site associated with the Avonmouth zinc and lead smelter compared to *L. rubellus* from an unpolluted reference population. Results from a common garden transplant experiment for the two populations indicated that many basic cellular processes, such as cytoskeleton reorganisation, protein phosphorylation and gene expression were affected as a result of the exposure to metal pollution from the smelter source. Genes important in chromatin reorganisation were shown to be involved in adaptation to the heavy metal exposure and that a difference in global DNA methylation was present in *L. rubellus* adapted to living in heavily polluted soil compared to control individuals. In a laboratory study, a difference in DNA methylation was also found in *L. rubellus* after a life-time exposure to fluoranthene, but not arsenic or cadmium. This may point to a role for other epigenetic mechanisms (e.g. histone modification) in the chromatin remodelling response seen in the field.

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Chapter 1. Introduction

1.1. General introduction

Human caused alterations to environments, such as climate change and pollution, are major stressors for other organisms in the biosphere (Demnerová *et al.*, 2014). The manner in which organisms respond to these environmental perturbations can illicit profound alterations in their behaviour and physiology (Calfapietra *et al.* 2015; Wong & Candolin 2015) . Pollutant toxicity may also cause changes in population structure, and it can have important downstream effects on other species, such as change in availability of food for organisms at higher trophic levels and effects on the community structure in an ecosystem. These may have vital implications for biodiversity maintenance and survival of endangered species as well as evolutionary processes via genetic drift, bottleneck events and other population genetics events (Deng *et al.* 2007; Brown *et al.* 2015). Toxic stressors may also have a more direct effect on the prevalence of particular genetic mutations which help exposed organisms to survive under stress, a phenomenon that has in particular been observed in pesticide exposed species (Enayati *et al.*, 2005). It has also been observed that species are able to adapt to stressors like pollution in a quick way that has not been the results of new genetic mutations (Ayyanath *et al.*, 2013).

It has long been known that random genetic mutations underpin the process of evolution and are responsible for much of diversity between individuals and adaptation to stressors in the environment (Campbell, 1971). However, while it was understood that genetic variation could account for differences in phenotypes between individuals, the manner in which the uniform DNA sequence of an individual could give rise to the myriad of specialised cells with different characteristics has been a subject of extensive study for some time. This resulted in the discovery of differentiation of cell phenotypes during development and mechanisms by which genes can be switched on and off during development which ultimately led to the establishment of the field of gene regulation (Griffiths *et al*. 2012).

Painstaking work in developmental biology and gene expression analysis has begun to identify the cascade of change that leads from a single totipotent cell to the development of a complexly differentiated individual. However, there is an ever-growing body of evidence that shows that the pathways guiding development are not fully ridged and that environment can contribute to the variations in phenotypes of the resulting cells. Research in development and gene regulation has shown that individuals respond to their environment within their life time and develop accordingly

(Colborn *et al.*, 1994). A similar possibility in the development of a whole phenotype was suggested as early as 1942 (Waddington, 1942). Because organisms or the cells they arise from are directly affected during their development within their parents and, in mammals, grandparents, there is potential for the developmental phenotypes of individuals to be influenced by the environments experienced by their ancestors, giving rise to multiple generational effects (Bale, 2014). A theoretical model of inheritance in which gene regulatory mechanisms may be inherited meiotically has also been proposed (Klosin *et al.*, 2017). This has now lead to the discovery of evidence of environmentally induced (e.g. diet, stressor exposure) phenotypic effects on development sometimes persisting for generations after the exposure (Feil and Fraga, 2012).

The possibility that the experiences of a forbearer can have phenotypic consequences for individuals in later generations raises a number of interesting questions on the nature of adaptation. For example, experiences that facilitate stress tolerance in offspring may have a direct role on the adaptation of organisms which may develop resistance to stressors without DNA sequence change which they may then pass on to their offspring. This does not change the fundamental role of DNA mutations but provides an understanding of a much more plastic adaptation to stress. While the potential for generational transfer of stress phenotypes may provide some benefit to offspring in an increasingly more polluted world (Agrawal *et al.*, 1999), it may also mean that pollution has a more long term effect on populations. For example, if the organisms' mechanism of responding to a stressor is to reduce reproduction in order to preserve energy (as in, for example, a study on mussels exposed to temperature stress (Petes *et al.*, 2007)) this is likely to have direct effects on population resilience resulting in increased extinction risk. Alternatively, adaptation to one stressor may cause higher sensitivity or higher resilience to other stressors, thus having an effect on populations subject to specific multi-stressor scenarios (Cleuvers 2004; Fonte *et al.* 2016). Finally, adaptation by such mechanisms may mean that one species can adapt to stressors at a faster rate than another, giving it a competitive advantage which may result in a loss of ecological balance and lower species diversity (Sun *et al.*, 2013). Given their possible importance in understanding adaptive processes and their effect on populations, it is therefore important to understand how organisms' gene regulatory mechanisms and phenotypes respond to pollution, how this response changes over more than one generation and how it may have affected the evolution of species adapted to extreme and changing environments.

1.2. Epigenetics

The term epigenetics directly translates as “above genetics” (Szyf, 2015), it is a research field which explores a) the non-genetic inheritance of phenotypes either via mitosis (from cell to cell and organism to organism) or meiosis (from parent to offspring in sexually reproducing organisms) and b) the processes via which this inheritance can occur. The chemical changes involved in these processes are often called epigenetic marks, a term which is most commonly used to refer to DNA methylation, histone modifications and non-coding RNAs (Feil and Fraga, 2012). Epigenetic marks may be products of the DNA sequence (small RNAs) or modifications of the chemistry of a nucleic acid sequence (DNA/RNA methylation) or confirmation changes of DNA structure resulting from histone marks being placed by proteins (Griffiths *et al.* 2012). All types of epigenetic marks can change in response to signals from the environment and when these changes are inherited through cell division they are referred to as epimutations (Chan *et al.*, 2005). Histone modifications, DNA methylation and non-coding RNAs are the mechanisms most explored in epigenetic research, but other forms of non-genetic inheritance are also abundant in nature. These can be in the form of prions and other cellular mechanisms like metabolic loops and cilia positions in ciliates (Namy *et al.*, 2008) as well as cultural inheritance (Lindenfors *et al.*, 2016).

1.2.1. Histone modifications

In most cells, very long strings of DNA need to be kept compact in a very small amount of space, which has resulted in the requirement to package DNA into tight complex organisational structures termed chromatin. The basic unit of chromatin is the nucleosome, which contains about 150 base pairs of DNA wrapped around histone proteins. Histones undergo different posttranslational modifications, which can control how accessible DNA is to the gene expression machinery and therefore whether the underlying genes are available for expression or not (Peterson, 2004). These modifications include methylation, acetylation of lysines and arginines, phosphorylation of serines and threonines as well as sumoylation, ubiquitylation of lysines and ribosylation. An exceedingly large number of different types of these modifications is being discovered, with some combinations having a more or less clear role in a certain type of gene expression regulation (silencing or activating). However, the role of each particular mark combination is context-specific. Histone modification pathways are generally well conserved between different taxa (Yan *et al.*, 2014). Histone modifications are involved in imprinting (Trerotola *et al.*, 2015) and can affect gene expression in combination with DNA methylation and non-coding RNAs (ncRNAs) (Griffiths *et al.* 2012) as well as underlying DNA sequence (Peterson and Laniel, 2004).

1.2.2. Non-coding RNAs

NcRNAs are ubiquitous and vital in gene regulatory mechanisms (Peschansky and Wahlestedt, 2014). They include the housekeeping rRNAs (ribosomal RNAs) and tRNAs (transfer RNAs) which are involved in mRNA translation (Mattick and Makunin, 2006), these, however, tend not to be a focus in epigenetically relevant studies, which generally look at regulatory ncRNAs which have an impact on altering gene expression. NcRNAs can also be classified according to size: long ncRNAs (lncRNA) if they are of more than 200 bp in length and short ncRNAs if they are shorter. Both types have many diverse functions and further subdivisions (Romano *et al.*, 2017).

LncRNAs are loosely defined as long RNA transcripts that do not encode a protein sequence, they are present in all types of organisms and are involved in such processes as imprinting, chromatin shape formation and gene silencing, however, most lncRNAs have no known function. Sometimes lncRNAs are translated similarly to mRNAs, however, the resulting protein sequence is destroyed. Interestingly, the complexity of lncRNAs is correlated with organismal complexity more strongly than that of mRNA transcripts. Also, their mutations are implicated in many diseases (Quinn and Chang, 2016). LncRNAs are often transcribed from protein coding genes, but can also have a different genomic origin, they can include introns or consist of antisense mRNA transcripts (Peschansky and Wahlestedt, 2014).

Short ncRNAs include microRNAs which modulate target RNAs, as do endogenous short interfering RNAs (which are formed from cleaved lncRNAs) and piwi-interacting RNAs (Peschansky and Wahlestedt, 2014) as well as small nuclear RNAs which are involved in splicing (Mattick and Makunin, 2006) and short nucleolar RNAs, which are involved in rRNA regulation and have also been implicated in gene regulation (Romano, 2017). These are the most well-known short ncRNA types, however, new types are being discovered and their functions being explored, as in the case of tRNA-derived fragments, which consist of, as the name suggests, cleaved tRNA fragments, and are thought to be involved in gene silencing, apoptosis and epigenetic inheritance (Romano *et al.*, 2017).
As in the case of histone modifications, ncRNAs play different, context specific roles in different organisms. They were first discovered and are also very important in, for instance, *Caenorhabditis elegans ,*which do not contain cytosine methylation (Greer *et al.*, 2015). They can be involved in epigenetic inheritance of DNA methylation by being inherited through cell division and guiding *de novo* methylation after meiosis (Calarco et al., 2012) and are thought to be important in guiding sequence specific epigenetic modifications generally (Chan *et al.*, 2005; Chan, Henderson et al.

2005). Non-coding RNAs have been definitely proven to cause transgenerational epigenetic inheritance in *C. elegans* (Houri-Ze'evi *et al.*, 2016).

1.2.3. DNA methylation

DNA methylation usually refers to the addition of a methyl (CH3-) group to a DNA base, most commonly on the fifth position of the cytosine ring (5-mC) (Yan, 2015) by an enzyme called DNA methyltransferase (DNMT), which transfers a methyl group from the universal methyl donor s-adenosyl-L-methionine onto the 5th position of a cytosine ring (Bestor, 2000). This is the chemical process that is generally (and within this thesis) referred to as DNA methylation, however, DNA methylation can also occur in the exocyclic NH_2 group on adenine. This has been observed in *C. elegans* (Greer *et al.*, 2015) and may also be relevant for other species, although the extent to which this is the case still remains to be clarified.

In currently explored adult animals DNA methylation usually occurs within CpG sites (where cytosine and guanine are next to each other in the genome), although in *Drosophila* it is present mostly in CpT nucleotides (Feil and Fraga, 2012) and in plants and embryonic stem cells it can also be present in CHG and CHH sites (H=A,T or C) (Feil & Fraga, 2012; Cingolani et al., 2013). The type of cytosine methylation sites can differ depending on where in the genome they are located, for example, in honey bee cytosine methylation is enriched in CpG sites in exons, but is also present in non-CpG sites in introns (Cingolani *et al.*, 2013).

Deamination of a methylated cytosine molecule produces thymine, resulting in a C to T mutation. This type of mutation is very common in human cancer and between closely related mammalian species, therefore it is likely that DNA methylation is a noticeable burden on genetic stability (Schübeler, 2015). Possibly because of this, the amount of CpG sites within genomes is smaller than other combinations of nucleotides, which is particularly prevalent in mammals, although less so in the frog *Xenophus laevis* and zebrafish (Schübeler, 2015). The exceptions are sites called CpG islands, which have a relatively high abundance of this nucleotide pair and are often placed within gene regulatory regions. The methylation pattern in these islands has been related to the expression of genes in the 3' direction. Highly methylated islands are often associated with lower expression and *vice versa*, although these sites can stay unmethylated even when their associated genes are silent, particularly if they are within the heterochromatin (Griffiths 2012). This has mostly been observed in vertebrates, however, promoter methylation and associated gene repression has also bene observed

in invertebrates (Gavery and Roberts, 2014). Unlike histones, DNA methylation pathways are not very strongly conserved between different taxa (Yan *et al.*, 2014).

In vertebrates, methylation is dispersed globally throughout the genome, in contrast to invertebrates and plants (Schübeler, 2015), where a characteristic of DNA methylation is the so-called mosaic distribution with large regions of methylated DNA , mostly in DNA repeat regions and coding regions of genes, interspersed with unmethylated regions. Genic DNA methylation has been suggested as helpful for ensuring DNA stability in heavily transcribed regions of DNA (Schübeler, 2015) as it likely prevents transcription occurring from intragenic transcriptional start sites thus reducing transcriptional noise (Gavery and Roberts, 2014). DNA methylation within genes may also aid alternative splicing (Gavery and Roberts, 2014). Gene body methylation is broadly associated with a high or medium level of gene expression (Yan *et al.*, 2014). The presence of DNA methylation in repeat regions is likely because it supresses transposable element activity (Gavery and Roberts, 2014).

1.2.3.1. DNA Methyltransferases

Methylation of DNA is managed by DNA methyltransferases, which can either maintain existing methylation patterns or add *de novo* methylation. Specific methyltransferase isoforms have been shown to perform distinct functions with DNMT1 being associated with methylation maintenance by ensuring that the DNA methylation pattern present in the template strand is copied during DNA replication while DNMT3 methyltransferases carry out *de novo* DNA methylation on unmethylated cytosines (Yan *et al.*, 2014).

In mammals, there is a single DNMT1 isoform whilst there are multiple DNMT3 paralogues, including DNMT3a, DNMT3b and DNMT3L (Trerotola *et al.*, 2015) as well as DNMT3c in rodents (Barau *et al.*, 2016). Another member of the DNA methyltransferase gene family is DNMT2, which is thought to be less involved in DNA methylation, but rather in RNA methylation (Schaefer and Lyko, 2010), including tRNA methylation (Khoddami and Cairns, 2013) and possibly RNA modification-mediated epigenetic inheritance (Kiani *et al.*, 2013). In other species, orthologous genes are classified according to their similarity to the mammalian DNMTs.

As genome sequencing is becoming more ubiquitous and less expensive, more and more gene families are being explored in different species, therefore there is more and more information on DNA methyltransferases in different animals. In zebrafish, there are six DNMT3-related genes (named DNMT 3- DNMT 8), which show similar expression patterns to either DNMT3a or DNMT3b

(Smith *et al.*, 2011) and in the killifish *Kryptolebias marmoratus*, five DNMT3 orthologs were found (Kim, 2016). In *Drosophila melanogaster*, the only DNMT expressed is orthologous to DNMT2 (Mandrioli, 2007).

In the copepod *Tigriopus japonicus*, three DNMT1 orthologs were identified, while no DNMT3 ones (Kim, 2016). The rotifer *Brachionus koreanus* was found to contain a DNMT2 ortholog, but no DNMT1 or DNMT3 ones (Kim *et al.*, 2016).

1.2.3.2. DNA hydroxymethylation

Demethylation of DNA can occur passively, when DNMT1-dependent maintenance processes are blocked, or actively, when the methyl group is transformed and then removed from the cytosine molecule. This process can be directed by Tet (ten-eleven translocation) proteins, which transform the methyl group into a hydroxymethyl group (Yan *et al.*, 2014) and is likely involved in gene expression control. The abundance of DNA methylation is correlated with DNA hydroxymethylation (Branco, Ficz, & Reik, 2012;Tellez-Plaza et al., 2014). The cycling between methylation and hydroxymethylation has been identified as a dynamic process, the balance of which controls both local and global DNA methylation levels (Cingolani *et al.*, 2013). This process has been well described in mammals, less is known about hydroxymethylation in invertebrates, however, tet protein homologs have been found in eusocial insects and hydroxymethylation has been found to occur in honeybees (Yan et al., 2014; Hackett & Azim Surani, 2013).

1.2.4. DNA methylation in vertebrates

In vertebrates, DNA methylation has been shown to be involved in imprinting, processes like X chromosome inactivation and repetitive DNA silencing as well as DNA transcription regulation. It is therefore considered that cytosine methylation is generally a way of repressing DNA expression. This is further supported by the fact that, generally, regulatory regions of genes are unmethylated when the genes are active (Schübeler, 2015).

Mammalian genomes are generally highly methylated, for example, in humans, about 1% of overall DNA and about 70-80% of cytosines within CpGs are methylated (Bird, 1980). However, DNA methylation levels can be altered in different stages of development, for instance, in mice it drops at the early stages of embryonic development and then goes up again due to *de novo* methylation (Monk *et al.*, 1987) and in humans it decreases with age and, interestingly, a decrease in general methylation levels is also associated with many age-related illnesses (Baccarelli and Bollati, 2009). In

other types of vertebrates, methylation changes during embryonic development are either less prominent (as in the case of *Xenopus laevis*) or have not been detected (as in the case of zebrafish) (Macleod, Clark, & Bird, 1999;Suzuki & Bird, 2008).

1.2.4.1. DNA methylation in invertebrates

Most of what is known on DNA methylation has been discovered in vertebrates, particularly mammals, however, as invertebrate methylation is being explored to a greater extent the heterogeneity of methylome patterning is being revealed (Kim *et al.*, 2016).

In invertebrates, the overall amount of DNA methylation can vary considerably between taxa, being a fraction of a percent in some specious taxa such as the arthropods, but higher (reaching even 5-10%) in others such as molluscs and annelids (Regev and Lamb, 1990). Initially it was thought that *C. elegans* and *Drosophila melanogaster*, the two major invertebrate model organisms for molecular analysis, contain no functional DNA methyltransferases and, thus, that DNA methylation was not important as a mechanism of gene regulation in these two widely investigated species (Yan *et al.*, 2014). However, it has now been discovered that *C. elegans* contains methyltransferase DAMT-1 and demethylase NMAD-1 which regulate adenine methylation (Greer *et al.*, 2015) and *D. melanogaster* contains small levels of cytosine methylation and a single DNMT2, which, however, as mentioned above, is likely not involved in DNA methylation (Kunert, 2003). However, the absence of large numbers of methylated cytosines in some species in comparison to others raises interesting questions on the evolution of cytosine methylation. It has been suggested, for instance, that, while cytosine methylation can have positive impacts, such as helping silence unwanted transposable elements within the genome, its likely impact on unwanted mutations has made it beneficial for some species to lose the mechanism (Yan *et al.*, 2014). Within species that do contain DNA methylation, it has been suggested that reduced methylation in gene bodies may facilitate a greater diversity in gene expression by providing access to alternative start sites, exon skipping and transient methylation, thus it may be important for genes/organisms that need to rapidly evolve (Roberts and Gavery, 2012).

A growing body of research has expanded the knowledge on DNA methylation in invertebrates by looking at different species within the taxa, below are some examples of it across different groups. The basal metazoans ctenophores have been found to contain DNMT1 and both cytosine and adenine methylation, with higher levels of 5-mC in promoters than gene bodies (Dabe *et al.*, 2015). DNA methylation has also been observed in multiple bivalves, among those, the pacific oyster, *Crassostrea gigas* containing roughly 2% of total cytosine methylation. Similarly to *Xenopus laevis*

and mice, they have also been found to have different levels of methylation at different developmental stages (Gavery and Roberts, 2014). In bivalves, majority of cytosine methylation has been found within the gene bodies (Gavery and Roberts, 2014).

While in *C. elegans* cytosine methylation is lost, other nematode species have retained it and its levels are related to the DNA methyltransferases found within their genomes. In nematodes that have DNMT1 and/or DNMT3 cytosine DNA methylation was detectable, it was enriched in CG sites and within transposable elements within the genome. *Nippostrongylus brasiliensis*, which contains DNMT2, showed trace amounts of cytosine methylation, but these were below the significant rate of conversion. This study states that the preferred nucleotide after CpG for methylation site for DNMT1 was conserved between invertebrates and mammals, while this was not the case with DNMT3. This study found that these DNMTs introduced 3mC (methylation at the 3rd position of the cytosine ring) at low rates, which is a chemical addition subject to alkylation and repaired by ALKB enzymes, which were also found to possibly coevolve with DNMTs 1 and 3, implying that DNA methylation may be costly in terms of DNA instability and the necessary repair mechanisms, which is possibly why *C. elegans* has lost the mechanism altogether (Rošić *et al.*, 2018). In the water flee *Daphnia magna*, global DNA methylation levels have been observed at roughly 0.5% (Asselman *et al.*, 2015). Many insects have sparse methylation in only a subset of transcribed genes (Rošić *et al.*, 2018), however, it can still be highly important. In eusocial insects, where most individuals within a hive are genetically identical, DNA methylation plays a particular role in differentiating phenotypes and distinguishing caste-specific characteristics (Yan *et al.*, 2014). DNA methylation has also been found to play a role in alternative splicing in different types of eusocial insects. This is likely due to methylation affecting the accessibility to splice sites by enzymes involved in transcription (Yan *et al.*, 2014). Some hymenopteran insects display exonic methylation enrichment (Yan *et al.*, 2014). Monallelic methylation has been observed in an ant species (Yan *et al.*, 2014) and parental impact on behavioural specialization has been detected in ants and honeybees (Yan *et al.*, 2014), which imply genetic imprinting as a possible mechanism in eusocial insects .

1.2.5. Epigenetic inheritance

1.2.5.1. Mitotic (somatic) epigenetic inheritance

Mitotic inheritance of epigenetic marks is a well-documented process in multicellular organisms; it is why tissues and the cell lineages they comprise of show and retain different properties in organisms throughout their lifetime. As they differentiate, cells inherit certain epigenetic marks from their respective stem cells (Sharif *et al.*, 2007). This has been documented in, for example, human

hematopoietic tissue where different types of stem cells, progenitor cells and resulting tissue cells show characteristic DNA methylation patterns that are associated with gene expression patterns, histone modifications and chromatin structure (Farlik *et al.*, 2016).

The process of differentiation through cell division is mediated by epigenetic processes as well as various signalling pathways within the cell. Such control is vital as incorrect differentiation can cause long term damage resulting in the production of daughter cells which are dysfunctional and/or can develop into a tumour. Thus mitotic epigenetic inheritance and the epigenetic mechanisms controlling mitosis play an important role in carcinogenesis (Knoblich, 2008).

1.2.5.2. Meiotic (germ line) and transgenerational epigenetic inheritance

This form of inheritance occurs when daughter cells arising from meiosis retain epigenetic marks from the mother cell. Because meiosis occurs when gametic cells are produced, this process may result in the cells of the next generation of offspring in sexually reproducing species retaining similar, non-genetic, properties as their parent (Griffiths *et al.* 2012). The process of meiotic epigenetic inheritance is an important concept in evolutionary research as it provides an alternative source of stably inherited new variation in organisms to genetic mutations. Most research in the meiotic inheritance of epigenetic marks has been done in vertebrates, especially mammals, with basic questions being addressed in both yeast (Grewal and Klar, 1996) and plants (Mirouze and Paszkowski, 2011). Different organisms, from plants to mammals, have been observed to maintain DNA methylation marks for multiple generations (Feil and Fraga, 2012), however, whether this is direct inheritance of DNA methylation or one mediated via other mechanisms in the cell, such as ncRNAs, is unknown.

In mammals, global DNA demethylation, including in imprinted loci, also occurs during germ cell production (Heard and Martienssen, 2014). Therefore, it is considered unlikely that DNA methylation can get directly inherited throughout meiosis in mammals. However, in zebrafish, only the maternal methylome undergoes extensive changes at this point and paternal methylation is inherited by early embryos (Jiang *et al.*, 2013).

Non coding RNAs and histone marks may interact in causing transgenerational inheritance, for example, dsRNA was used by Gu et al to induce changes in H3 lysine 9 trimethylation (H3K4me3) patterns in *C. elegans* genomes and these were inherited for at least another two generations (Gu *et al.*, 2012). The knockdown of genes in a pathway that methylates the H3K4me3 histone mark in the parental worms caused the transmission of longevity for another three generations, implying that

alterations in the histone mark may be transgenerationally inherited (Greer *et al.*, 2012). In *C. elegans*, piRNAs (Piwi-interacting RNAs) have been observed to cause epigenetic transmission for 20 generations (Ashe *et al.*, 2012) .

There is mounting evidence for the inheritance of histone marks and ncRNAs throughout meiotic cell division and/or throughout multiple generations, however, the specific mechanisms remain to be elucidated (Wang and Moazed, 2017).

1.2.5.3. Parental (maternal/paternal) effects

Parental effects, often referred to as either maternal or paternal effects in sexually differentiated organisms, are the non-genetic impacts of parents on the phenotypes of their offspring. These can involve an effect on the plasticity of the offspring development and adaptation to stress and can be a result of the environment and the phenotype of the parent (Uller, 2008). Parental effects are considered a transgenerational form of phenotypic plasticity. They can be both advantageous and detrimental to the offspring and result from a presence of particular types of food or a predator as well as other environmental impacts and even a parent's "selfishness" when producing less fit offspring results in long term advantage to the parent (Burgess and Marshall, 2014).
Research in parental effects is a growing field in environmental biology. For example, *Daphnia cucullata* offspring whose mothers have been exposed to predators grow a "helmet" addition to their carapace as a protection against predation, even if they have not been exposed to the predators themselves (Agrawal *et al.*, 1999). The offspring of wild radish plants that had been exposed to caterpillars also appear to be less palatable/nutritious to caterpillars; this being indicated by a 20% reduction in caterpillar weight for animals feeding on the progeny of herbivore grazed plants compared to unaffected plants (Agrawal *et al.*, 1999). Parental feeding of polyunsaturated fatty acids by *Daphnia magna* caused an increase of bacterial infection levels in offspring (Schlotz *et al.*, 2013). In the absence of any particular stress, brown trout which had hatched from parentally affected smaller eggs experienced competitive disadvantages in body size and survival (Einum and Fleming, 1999). The abundance of examples of parental effects and the variety of species in which it has evolved shows the importance of this type of phenotypic plasticity and as an adaptation that has arisen in very different organisms, implying its great importance in survival.

1.2.5.4. Epigenetic marks and evolution

The impact of epigenetics on evolution can be difficult to study because of time constraints. However, some studies have looked at the effect DNA methylation may have on genomic evolution as well as the impact evolution has on DNA methylation patterns. For example, Keller and Yi looked

at duplicate gene methylation in human cell cultures and found a correlation of promoter hypomethylation and relative methylation pattern divergence with evolutionary time since duplication. This work also correlated DNA methylation with gene expression, older duplicates were more likely to be expressed. Gene body methylation showed no correlation with the age of the duplicate. Most of the duplicate pairs showed a consistent difference in methylation across all tested tissues and this correlated with heterochromatin presence in these sites, while the genes whose methylation fluctuated also had fluctuating heterochromatin patterns. This may indicate that the chromatin environment of the genome has an effect on the duplicate gene methylation patterns (Keller and Yi, 2014) . One investigation looked at copy number variations (CNVs) and DNA methylation changes in a group of related species of Darwin's finches. The researchers found epimutations to be more closely correlated with the phylogenetic distance than genetic mutations, no significant overlap between the DNA methylation changes and CNVs and a higher overlap in epimutations between species than CNVs. Interestingly, signalling pathways previously linked to finch evolution showed an overrepresentation of epimutations, indicating a possible link between the finch differentiation and DNA methylation (Robinson and Barron, 2017; Guerrero-Bosagna et al., 2010).It has also been suggested that invertebrates compensate for their lack of lymphocyte – based immunological memory similar to that of vertebrates using epigenetic mechanisms (Emilia, 2015).

Epigenetic alterations may even have a direct effect on genetic mutations if the epigenetic mark is DNA methylation (or other direct chemical alteration of DNA) as methylation has been suggested as one factor that can increase rate of mutations in the underlying DNA (Bird 1980). Alternatively, for non-coding RNA/histone marks that silence genes there is the possibility that long-term suppression of expression may result in the accumulation of mutations in sequences not necessary for the organism's survival (Griffiths *et al.* 2012).

The role of epigenetics in evolution has made evolutionary theorists reconsider the ideas of Lamarck, who proposed in 1802 that the environment can directly influence the inheritance of phenotypic traits. While the particular way the effects occur may be different from Lamarck's ideas, the concept appears to be a possibility. This has resulted in the proposition of a unified neo-Lamarckian and neo-Darwinist theory in which random DNA mutations retain their role in long term adaptation, but environmentally induced epigenetic changes provide a short term adaptive mechanism and may also have a role in causing new genetic mutations (Skinner 2015a; Skinner 2015b). This idea, however, is controversial and, while transgenerational epigenetic inheritance has

been observed in multiple species, whether this results in an actual effect on natural selection is not known (Wang *et al.*, 2017).

1.2.5.5. Inbreeding and epigenetic changes

Some research appears to show a link between DNA methylation and a lack of genetic diversity. A group of researchers looked at populations of the house sparrow (*Passer domesticus*), one in Tampa, Florida, USA, the other in Nairobi, Kenya. The American sparrows had been introduced 150 years ago and possessed as much genetic diversity at different microsatellite loci as native populations in Europe, while the Kenyan ones had been introduced only 50 years ago and were less genetically diverse (Schrey et al., 2011). The researchers used msAFLP to detect diversity in DNA methylation between individuals in each population and found a similar level of variation within both groups and more methylation in Nairobi sparrows despite their lower levels of genetic diversity (Schrey *et al.*, 2012). Another study on earthworms *Lumbricus rubellus* adapted to living in highly polluted soils near mining sites found that a more genetically diverse cryptic lineage of the worm showed no difference in DNA methylation between animals collected in highly polluted or nearby clean soils (as detectable using the methylation sensitive amplified fragment length polymorphism(msAFLP) method). Meanwhile, a more inbred lineage showed an alteration in methylation profiles between polluted and control environments. It was proposed that the more genetically diverse lineage contained genetic changes that may be linked to adaptation to surviving arsenic exposure (Kille *et al.*, 2012). Research on the invasive alligator weed (*Alternanthera philoxeroides*) (Gao, Geng *et al.* 2010) and the European house sparrow (*Passer domesticus*) (Liebl *et al.*, 2013) also showed a correlation between cohort low genetic diversity and higher changes in DNA methylation when the organisms are exposed to new environments. These findings indicate a possible evolutionary importance to DNA methylation as a mechanism that improves an inbred species' ability to adapt in response to stress. These are, however, very speculative conclusions, due to the small number of studies and the limitations of both msAFLP and microsatellite techniques.

1.2.5.6. Transgenerational inheritance of adverse stress effects

Inheritance of stress related phenotypes across multiple generations, via epigenetic marks or otherwise, is an interesting and potentially important phenomenon as it may cause effects on populations long after the cause of the particular form of stress, such as temperature or pollution, is no longer present. It may also cause differences in the responses of later generations that continue living under toxic exposure.

Whole organism studies in transgenerational epigenetic inheritance are complicated by the fact that it can be difficult to distinguish between maternal, transgenerational and meiotically inherited effects. Thus, as the cellular environment of the developing egg/sperm/embryo/embryo's eggs is likely to be affected, producing a different phenotype in the offspring, this is then likely to be having a further effect on its own offspring. Because of this, the complexity of cellular processes involved and the varied ways in which they may be adversely affected by stressors, as well as the different ways in which a species and even individuals within a species may react to it, similar experiments can give inconsistent results (Wang *et al.*, 2017). However, despite these challenges, a number of transgenerational effects have been reported in the literature (Wang *et al.*, 2017). The results reflect the difficulty of consistent proof in this area of research. For example, in mice, early life stress induced depressive symptoms and associated methylation patterns in certain DNA sequences in both brains and germline cells have been shown to be, at least partially, transmitted through three generations via paternal inheritance (Tamara *et al.*, 2010). Meanwhile, another study in mice on paternal transmission of social defeat stress induced symptoms showed little inheritance of these symptoms in IVF produced offspring (Nestler *et al.*, 2011).

While most research in this field has been done on rodents, invertebrate studies have also produced some interesting results, for example, the broadcast spawner (*Styela plicata*, a solitary ascidean) showed a paternal transmission of phenotype when the male gamete producing parents were kept in either dense or more solitary environments (Crean *et al.*, 2013). Some evidence of a very particular mechanism of transgenerational epigenetic inheritance resulting in increased risk of tumorigenicity has also been found in *Drosophila melanogaster* (Xing *et al.*, 2007).

It is possible that transgenerational epigenetic inheritance of stress induced phenotypes may only appear if the individual is exposed at a particular time of development (Bale, 2014) or for multiple generations, accumulating a stress response over time, which then becomes fixed in the following generations. Stress related altered epigenetics may affect the evolution of organisms even without meiotic transmission if the organism is asexual and thus doesn't go through meiosis. Also, continuous presence of a toxicant and/or related phenotypes may affect the newly forming gametes for many generations, causing DNA mutations.

Chemical stress has also been explored in this context. For instance, the fungicide vinclozolin appeared to reduce fertility and cause a variety of health defects in rodents three generations after removal of the chemical and this effect was linked to particular methylation marks in their DNA

(Guerrero-Bosagna *et al.*, 2010). This and the findings that other toxic chemicals like methoxychlor, dioxin and bisphenol A have also been observed to cause similar effects in rodents (Vandegehuchte *et al.*, 2015) , suggests that toxicants can cause transgenerational effects in mice. These and other chemicals can be prevalent in the environment, where they affect different organisms and ecosystems, therefore transgenerational impacts and epigenetics in general should be a consideration in ecotoxicological research.

1.2.6. Epigenetics and ecotoxicology

1.2.6.1. Toxicants and epigenetics

Toxic pollution can have major negative impacts on animals, causing reduction in growth and reproduction, death and at times even wipe out a species from a polluted area (Spurgeon *et al.* 2008; Spurgeon & Hopkin 1996). Therefore exploring the impacts of toxicants on species, especially environmentally relevant ones, is a vital task for environmental scientists.

When looking at environmental impacts on the epigenome, it is important to differentiate between the effects on the whole organism and the individual cells, which depends on the type of tissue exposed. When looking at toxicant effects on the epigenome, it is also important to understand how the organism reacts to chemicals exposed through different routes. The initial effects can be observed in the cells directly encountering the toxicant. These are usually epidermal and endodermal cells from which the chemical may be further absorbed and distributed to other tissues within the organism, as well as cells in organs that are involved in chemical handling like the chloragogenous tissue of earthworms (Roots, 1960). Chemical transport in organisms may be either passive through diffusion (Daughton, 2004), or active, such as by transport to detoxifying organs (e.g. liver) (Verma *et al.*, 1978). Earthworms ingest and live in soil, therefore they are exposed to soil pollution both via their digestive system and their skin. In one experiment, the uptake via ingestion was negligible for some heavy metals (copper and lead), while it had a significant effect on zinc and cadmium accumulation in some types of soil (Vijver *et al.*, 2003). When it comes to organic chemicals, an increased hydrophobicity increases the fraction of absorbed chemicals via ingestion, rather than the skin (Jager *et al.*, 2013). Therefore, exposures via both routes can be avenues for research in the area, but ingestion may be a safer bet for exploration of cellular impacts of organic chemicals.

Exposure likely affects a range of cell types and results in an uptake of the toxicant. This can stimulate changes in the epigenome that facilitate gene expression change as a response to

exposure (Luo *et al.*, 2014). On cessation of exposure or after detoxification, it is possible for tissue to return to a normal unstressed state. However, if stem cells are affected, particularly at an early stage of development, there may be long-term effects on cell function, differentiation and development that may lead to adverse phenotypes. An infamous example is thalidomide, the glutamic acid derivative, which caused limb deformities in children of women who consumed the drug during pregnancy (Shortt *et al.*, 2013). Other toxicants can also affect this process, for example, cadmium has an effect on histone mark regulation and cell cycle of mitotically dividing embryonic stem cells (Gadhia *et al.*, 2015).

While some toxicants may alter cellular homeostasis with effects on the epigenome, others may directly affect the epigenome itself. Such epigenetic alterations may in turn lead to effects like the release of stress hormones (Crews and McLachlan, 2006) and epigenetic deregulation of cell gene expression, which has been suggested as the likely primary cause of cancer (Nephew and Huang, 2003). It may also cause latent toxicity of the pollutant, e.g. exposure to arsenic can cause methylation changes which may result in a tumorigenesis later in the animal's life (Reichard and Puga, 2010). Via this mechanism even small concentrations of chemicals with no initial observed effect may cause damage to the organism exposed. Therefore, due to these detrimental effects, individuals may evolve mutations that allow normal function of cells in toxic environments (Van Straalen *et al.*, 2011).

Epigenetics has only relatively recently become a tool for understanding the impact of toxicants on the natural environment and individual species within it. It can help explain the cellular mechanisms which lead to toxicity induced by pollution and may provide an explanation for ecological problems like the lack of recovery of species even after the removal of a toxicant and the rapid rate at which a species can adapt to new and changed environments (Head *et al.*, 2012).

1.2.6.2. Research on the effects of pollution on epigenetic mechanisms and multiple generations of organisms

There is a growing body of research on the effects of pollution on the epigenetics in organisms, mostly on DNA methylation, with some studies looking at the inheritance of phenotypes and/or epigenetic marks in the following generations (Head *et al.*, 2012). Presented here are example studies across different taxa covering different chemicals and approaches to analysis.

1.2.6.2.1. Plants

Scots pines (*Pinus sylvestris*) in the highly toxic environment around the Chernobyl nuclear reactor have been found to have hypermethylated DNA and this hypermethylation seems to be dependent on the dose of the radiation received (Kovalchuk *et al.*, 2003)(Kovalchuk, Burke et al. 2003). In *Brassica napus* L. potassium dichromate caused dose depended hypermethylation of DNA (Labra *et al.*, 2004). A study on industrial hemp (*Cannabis sativa*), which shows resistance to heavy metal pollution and white clover (*Trifolium repens*), which does not, found that the amount of 5methyl-Cytosine methylation was higher in control hemp than white clovers. The amount of methylated DNA reduced after exposure to cadmium and nickel (Aina *et al.*, 2004). In *Arabidopsis thaliana*, nickel chloride caused a change in DNA methylation patterns and some of this was inherited by the following generation (Li *et al.*, 2015). Dandelions (*Taraxacum officinale)* show altered DNA methylation when exposed to different stressors which is largely inherited by the next generation of the asexually reproducing plants (Verhoeven *et al.*, 2010).

1.2.6.2.2. Mammals

Tobacco smoke, asbestos, bisphenol A, cadmium, nickel, methylmercury, arsenic, silica, benzene and other toxicants have all been found to alter DNA methylation in mammals, usually humans or mice. These have also been associated with various detrimental health problems, including cancer and fertility issues (Feil and Fraga, 2012) and cancer associated changes in the cell DNA methylome (Brocato and Costa, 2013). A number of environmentally relevant studies have been carried out in toxicants in mammals, for example, nickel factory workers were found to contain significant changes in histone methylation, namely an increase in H3K4 trimethylation and a decrease in H3K9 dimethylation (Arita *et al.*, 2012). It is thought to act by inhibiting the histone demethylase JMJD1A (Chen *et al.*, 2010).

1.2.6.2.3. Fish

A study on the common carp (*Cyprinus carpio*) found that a 40 day laboratory exposure of different concentrations of atrazine or chlorpyrifos or a mixture of the two caused a decrease in global DNA methylation in the livers, kidneys and gills of the fish, with some of the effects remaining present even after 40 days of recovery. This work also looked at the mRNA expression levels of DNA methyltransferases, which were mostly underexpressed. Further, the expression of MBD2 (methyl-CpG-binding protein DNA- binding domain protein 2, which can bind directly to methylated DNA and may act as a demethylase and activate DNA expression (Fuks *et al.*, 2003), was increased in most of the treated groups and in some cases stayed overexpressed even after recovery (Wang, Zhang et al. 2014). In a study using zebrafish (*Danio rerio*) altered DNA methylation was found when individuals

were exposed to arsenic, benzo[a]pyrene and tris(1,3-dichloro-2-propyl) phosphate (Kamstra *et al.*, 2015) and altered DNA methyltransferase expression when exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Aluru *et al.*, 2015). In adult female zebra fish exposure to dioxin and methylmercury, however, altered global DNA methylation differences in the livers were not detected, in either the exposed fish or their F1 and F2 offspring, while altered DNA methylation in some individual genes was found present in the methylmercury exposed females and their F1 offspring (Olsvik *et al.*, 2014).

1.2.6.2.4. Invertebrates

As stated previously, the nature of epigenetic mechanisms relevant to different invertebrate taxa can vary considerably which means that it may be difficult to relate effects seen in one taxa to other species. Most environmentally relevant studies to date have focussed on work with arthropods, with a limited number of studies also for other taxa (e.g. nematodes, annelids) (Head *et al.*, 2012). In a study on green peach aphids (*Myzus persicae*), imidacloprid affected patterns of global DNA methylation in two continuously exposed generations of the invertebrate, indicating an interesting generation- and developmental stage- specific epigenetic response to the pollutant, as the global methylation amounts differed between the two generations and the adult and second instar larval stages (Ayyanath *et al.*, 2014). Another study on the brown plant hopper (*Nilaparvata lugens*) researchers continuously exposed the insect to imidacloprid for multiple generations and observed changes in msAFLP signatures in each generation and multiple methylation polymorphisms (DeZhen and YanFei, 2013). It has been proposed that in insects pesticide resistance accumulated over multiple generations may affect competition between species. In one study, the silverleaf whitefly (*Bemisia tabaci)* has outcompeted the greenhouse whitefly (*Trialeurodes vaporariorum)* in recent years in Northern China. Seven generations of exposure to LC25 of the neonicotinoid nitenpyram resulted in six-fold resistance to the chemical and 3.1 and 5 fold cross resistance to imidacloprid and acetamiprid in *B. tabaci*, while *T. vaporariorum* developed a smaller, 3.7 fold resistance to nitepyram and low cross resistance to imidacloprid. Short term (24 hour) exposure to nitepyram also resulted in increased sensitivity to nitepyram and imidacloprid in *T. vaporariorum* (Liang *et al.*, 2012).

1.2.6.2.5. Caenorhabditis elegans

A study in *C. elegans*that exposed the worms to uranium for 16 generations found a decrease in fecundity after 2 and 3 generations of exposure, the effect being present in both the group placed in control and exposed conditions during the fertility testing stage (Goussen *et al.*, 2013). In a multigenerational exposure of *C. elegans* to ionic silver and PVP nanoparticles for ten generations, egg production was found to be more adversely affected by high concentrations of silver in the exposed generations. Furthermore, after 5 generations of exposure and then five generations of

recovery in a control environment, the effect on reproduction was still present. However, the exposed generations showed higher levels of egg production in low concentrations of the toxicant and were only more sensitive to higher concentrations (above 0.8 mgAg/litre). In this experiment it was also found that development slowed down accumulatively after multiple generations of exposure and one population stopped reproducing altogether (Schultz *et al.*, 2016).

1.2.6.2.6. Daphnia

A study on the global DNA methylation levels in *Daphnia magna* in response to various environmental cues found no effect of cadmium, arsenic, or lead, while it was affected by diet, predation cues and salinity. The exposure occurred over 48 hours with a single concentration for each exposure (Asselman *et al.*, 2015). After a 14 generation exposure to sublethal levels of cadmium, *D. magna* offspring continued to show lower levels of reproduction even when grown in a clean environment as well as lower levels of stress (cadmium and temperature) tolerance in the exposed *Daphnia* in comparison to controls (Kimberly and Salice, 2015).

1.2.6.2.7. Earthworms

Some research on the epigenetics of earthworms has been carried out. In a study of the earthworm *Lumbricus rubellus* populations in and around a mine site found that exposure to arsenic resulted in differences in DNA methylation patterns in a more inbred lineage of *L.rubellus*, but not in a more genetically diverse lineage (Kille *et al.*, 2012). At the same time, using similar sampled populations, multigenerational studies have been proven difficult to be carried out, with contaminated populations unable to produce offspring in a laboratory setting beyond one generation (Langdon *et al.*, 2003). This study also found no residual levels of arsenic in the tissues of offspring from field-contaminated populations and the cocoons they produced, which lead to the researchers to suggest that the resistance to pollution observed in these populations was due to genetic adaptation. However, this effect could also result from epigenetic adaptations. This area of study clearly requires expansion.

1.3. Experimental considerations

Exploration of the epigenomes of wild, non-model species can be problematic due to a lack of sequenced genomes and higher variation of DNA polymorphisms than clonal organisms as well as possible, more complex genomic structures than seen in most genomic model organisms. This and

monetary constraints are amongst the reasons why most of this kind of research uses techniques that are not as precise and informative as bisulphite sequencing or chromatin immunoprecipitation. Instead methods such as the analysis of global DNA methylation levels using liquid chromatograph mass spectrometry or biochemical analysis kits or more simple molecular methods such as methylation sensitive AFLP (amplified fragment length polymorphism) or slot blot techniques are used (Laird, 2010). These global analysis techniques are by their nature less informative than detailed genome wide analysis methods. However, even so, they can still tell us that methylation is happening, that it is being affected by pollutants and other stressors in a concentration dependent manner and if these factors are affecting methylation randomly across the genome or at particular 5' – CCGG - 3' sites. Because of the specific design and course level of assessment of such methods they may though miss important DNA methylation changes if they occur in small amounts in areas which do not contain a 5' – CCGG - 3' site (Laird 2010).

In this project I will be looking at earthworms, a very important group of species for the terrestrial ecosystem, soil fertility and one that has been found to have adapted to living in very highly polluted sites (Kille et al., 2013). It is also a commonly used animal in ecotoxicological studies and has well established testing methods for toxicology, like mesocosm cultures and tests for sensitivity to pollutants (Heggelund *et al.*, 2014). Earthworms are also subject to a growing number of molecular studies. *E. fetida* and *E. andrei*, two subspecies of earthworms have an explored transcriptome (Lee *et al.* 2005; Gong *et al.* 2010) and *L. rubellus* has a sequenced (unpublished) genome. These species are also the focus of this thesis.

1.4. Research aims

Epigenetic processes are very likely implicated in short term adaptations and possibly even long-term evolutionary change of organisms and are mechanisms involved in responding to stress. Pollution and other toxicants have been shown to influence epigenetic processes and cause effects in offspring of exposed individuals for multiple generations, therefore it is possible, that, if epigenetic influence on evolution is present, pollution is a factor influencing it. Exploring epigenetic changes caused due to toxic exposures is also very important in understanding the impact pollution and pesticides may have on organisms in a longer time scale than that usually explored in ecotoxicology studies. Therefore, in this project I set out to investigate if pollution has an impact on the epigenetic processes of earthworms and whether this could be linked to more long-term adaptations by the organisms.

The following investigations were carried out:

1) A three generations long exposure of the earthworms *Eisenia fetida* and *Eisenia andrei* to arsenic, cadmium and imidacloprid in order to assess phenotypic effects caused by pollution when the animals are exposed continuously for three generations and after removal of the toxicant for one or two generations. This was done in order to test if the long-term exposure has different effects on the organisms compared to a short-term exposure and if any effects are carried onto the generations after exposure.

2) DNA methylation analysis of populations of the earthworms *L. rubellus* adapted to living in a gradient of sites with varying levels of heavy metal pollution and in *L. rubellus* exposed to three different toxicants for a lifetime in a laboratory setting. This was done in order to test if DNA methylation forms a part of the adaptive response to pollution in the wild and a laboratory setting.

3) DNA methylation and gene expression analyses of *L. rubellus* earthworms collected from a site polluted with heavy metals and a control site and transplanted between the two soils in a laboratory setting. This was done in order to a) test the molecular mechanisms behind the success of the extremophile L.rubellus population adapted to living in the heavily polluted soil; b) to test the mechanisms activated by the control population when exposed to pollution; c) to compare the short- and long-term adaptations and d) to establish what role epigenetics plays in these.

Chapter 2. The effects of life-time exposure to environmentally relevant concentrations of arsenic, cadmium and imidacloprid on the growth and reproduction of earthworms from the *E. fetida /E. andrei* complex.

2.1. Introduction

Chemical pollution of the terrestrial environment is a widespread and commonly occurring issue that can have many different adverse effects on soil dwelling organisms (Cortet *et al.*, 1999). Soil is a major sink for pollutants that are released into the terrestrial environment. Pollutants can be added to soils from one-off events such as a nuclear plant meltdown or an industrial accident, repeatedly, as in the case of pesticide applications to agricultural land or continuously, for example, from regularly emitting pollution sources, such as industrial emissions and traffic derived pollution. Many of these pollutants, like heavy metals and some organic chemicals, can be persistent in soils (Micó *et al.*, 2006). Therefore, when pollution events occur, affected terrestrial organisms are often exposed throughout their full developmental period and life-span. This long-term exposure has the potential to cause adverse effects in soil dwelling organisms that may go beyond those observed during the short-term exposures that are commonly used to investigate the biological effects of chemical exposure in laboratory-based toxicity tests.

The potential for the long-term presence of pollutants in soil to adversely affect growth, development and reproduction in earthworms, one of the most functionally important soil taxa, has been demonstrated in a number of studies. Spurgeon and Hopkin (1996) showed a reduction in the rate of growth, delays in sexual maturity and a reduction in rates of cocoon production (linked potentially to reduced rates of sexual maturation) in earthworms (*E. fetida*) chronically exposed to metal polluted soils collected from sites along a contamination gradient from a cadmium/zinc/lead smelting works (Spurgeon and Hopkin, 1996). In a similar experiment, Van Gestel et al. (1993) exposed juvenile *E. andrei* earthworms to cadmium (Cd), copper (Cu) or pentachlorophenol spiked

separately into soils throughout their full developmental period. Cd was found to reduce juvenile growth rate and to slow sexual development, while exposure to Cu resulted in slightly increased growth rate and earlier sexual maturation, the mechanisms of this effect are not understood, they may be due to the effects of copper on bacteria or another mechanism . Pentachlorophenol did not affect the size or maturation rate at the tested concentrations (van Gestel *et al.*, 1991; van Gestel *et al.*, 1993) . These results indicate a range of effects on developmental trajectories of earthworms exposed to toxicants in the field over extended timescales. The full dynamics of such effects could not be captured during standard ecotoxicology experiments with earthworms which generally involve short-term exposure (often <28 days) of mature adults.

Using life-history based modelling, studies have indicated that population growth rate in earthworms is more sensitive to changes in juvenile development (e.g. time to maturity) than to changes of a similar extent to survival rates in exposed adults or indeed to effects on reproduction rate (Jager and Klok, 2010). The strong effect of development times on population growth rate are governed by an increase in generation time when individuals grow slower. Therefore, any chemical exposure that has a strong effect on the rate of growth and development has the potential to have a significant effect on the demography and potentially overall population size of earthworms in polluted soils. This may lead to a reduction in population size and in genetic diversity of the populations, leading to inbreeding depression. Further, slower development may also result in changes in the phenology of earthworm population that can also lead to potential population effects. For example, maturation of the animals at the wrong time of year, or an increase in the time when they are more vulnerable to predation, may lead to reduced population sizes resulting from lower survival of both adults and juvenile (Haddad *et al.*, 2016). In the case of earthworms, they may need to complete their life-cycle within a specific period during which conditions are suitable before entering resting stages, either through aestivation or as cocoons that are tolerant to adverse environmental conditions (e.g. winter frost or summer drought). Any delay to growth rate may prevent them reaching the stages or body size at which such survival strategies can be deployed at the appropriate time. Delayed maturation rate due to environmental exposure to a mix of heavy metal pollutants (copper, mercury and lead) has been observed in *L.rubellus* (Klok *et al.*, 2006), this article also underlined the importance of this in the trophic chain in the ecosystem. Other types of pollution, namely the synthetic chemicals lindane and deltamethrin, have been observed to induce slower growth rates in earthworms during a laboratory exposure (Shi *et al.*, 2007). In cases like that for copper found by Van Gestel et al (1993) when an increase in growth rate is observed, likely due to changes in resource allocation, such as the effect may potentially have a detrimental effect on

populations in the long term, resulting in, for instance, an uncoupling of growth trajectories from potential food supply or a greater likelihood of disease in later life (Singhal and Lucas, 2004; Preston-Martin *et al.*, 1990). Therefore, it is important to look at long term sublethal effects of stressors in species on life-cycle traits that might have an ecological and/or evolutionary impact on exposed populations (Spurgeon and Hopkin, 1996).

A further impact that may result from long term pollutant exposures is that species at different life stages may exhibit differential sensitivity to stress. Hence, effects measured only on adults may not reflect those for other exposed life-stages. For example, Spurgeon and Hopkin found that juveniles exposed to heavily polluted soil, which contained various heavy metals, resulted in increased mortality (Spurgeon and Hopkin, 1996), while adults did not show such an effect at the same exposure concentrations (Spurgeon and Hopkin, 1995). Similarly, in *E. andrei* chronic exposure to cypermethrin indicated a higher sensitivity for effects on juveniles growth rate compared to adult weight change and reproduction in adults has been found (Zhou *et al.*, 2008). Long-term exposures designed to measure effects on juvenile and adult traits are a potentially valuable approach through which to identify such variations in sensitivity.

To understand the effects of long-term chemical exposure experiments studies are needed to trace the effects of persistent pollutants on a range of traits over the full developmental period. Earthworms are valuable for such studies for a number of reasons. They are recognised as sentinel species in ecotoxicological research (van Gestel *et al.*, 1993; Spurgeon *et al.*, 1994; Luo *et al.*, 1999; Heggelund *et al.*, 2014). As highlighted above, there are already a number of studies suggesting effects of long-term exposure on a range of relevant developmental traits (Spurgeon and Hopkin, 1996; Schnug *et al.*, 2013). Earthworms are an important group of species in soil ecosystems, providing a range of ecosystem services related to soil fertility (Edwards and Bohlen, 1996) and soil structural characteristics, as well as being a food source of numerous other species. Earthworm have a longer life-span and development time (e.g. 10 weeks of growth and 10 – 12 weeks for sexual development for *E. fetida* (Spurgeon and Hopkin, 1996) than that of other well-studied invertebrate ecotoxicological models, such as *D. magna* (Brennan *et al.*, 2006) and *C. elegans* (Contreras *et al.*, 2013). However, this length of time is manageable in a laboratory experiment. By working with species such as *E. fetida* and *E. andrei* that have relatively short growth and development periods compared to those of other earthworms, such as *Lumbricus terrestris* (Spurgeon *et al.*, 2004). Therefore, using *E. andrei* and *E. fetida (Eisenia sp*.), there is the potential to explore the effects of extended continuous chemical exposure in a relatively long-lived species in a laboratory setting.

In this experiment, juvenile *Eisenia sp.* earthworms were exposed over their full developmental period to three persistent toxic chemicals: arsenic, cadmium and imidacloprid. These three chemicals are all recognised for the long residence time in soil (Li and Thornton, 2001; Camm *et al.*, 2004; Dankyi *et al.*, 2014). Cd and As, as trace elements, persist in soil and can only be removed by a physical process, such as leaching to groundwater. As an organic chemical, imidacloprid can degrade. However, studies with this substance have shown a half-life of between 990 and 1230 days indicating the potential for continuous exposure in areas subject to annual treatment with this widely used insecticide (Baskaran *et al.*, 1999). This means that the exposure of earthworms to these chemicals can be expected to occur in natural conditions for whole generations. Furthermore, as well as being persistent, all three chemicals have been observed to have a negative impact on *Eisenia sp.* phenotypes (Spurgeon *et al.*, 1994; Luo *et al.*, 1999; Lock and Janssen, 2002). While most of these effects are likely to be detrimental for populations, this is not always the case. For example, continuous exposure to low doses of imidacloprid has been observed to cause hormesis for multiple generations of green peach aphids, resulting in an overall increase in population numbers (Ayyanath *et al.*, 2013). Using these three chemicals, there is an opportunity to explore the lifelong impact of toxicants with different modes of action over longer timescales (Liu and Casida, 1993; Kitchin, 2001; Matovic *et al.*2011).

Natural populations and laboratory culture organism stocks can contain individuals of various lineage and family backgrounds. This genetic diversity may have an effect on the results of toxicity tests. For example, in an experiment with the herbivorous amphipod *P. parmerong* conducted according to a split family design (Green 1975), family background had a significant effect on offspring survival and feeding rates following copper exposure, but no such effect was found in the control treatment (Pease *et al.*, 2010). *Eisenia sp.* laboratory culture stocks have widely been shown to contain a mix of different lineages and these stocks may even suffer from inbreeding depression (Pietermaritzburg *et al.*, 2015). Such genetic variation has the potential to affect earthworm species responses to chemical exposure. Ecotoxicologically relevant phenotypic differences in response to arsenic have been observed between different genetic clades of the earthworms *L. rubellus* (Anderson *et al.*, 2013) and epigenetic response of clades species exposed to arsenic mine spoil have also be shown (Kille *et al.*, 2012). Such observation suggest that it is important to consider the family genetic background of organisms when assessing the effects of pollution.

In this experiment, I set out to test a) the long-term effects of environmentally relevant concentrations of arsenic, cadmium and imidacloprid on earthworms in *Eisenia sp.* and b) to take account of the family background and species background when assessing the effects of the three toxicants. *E. andrei* and *E. fetida* were exposed to arsenic, cadmium and imidacloprid throughout their development. Growth rate, fertility and cocoon viability were measured over the time course. It is expected that the exposure will have an impact on growth and reproduction of the exposed earthworms, effects that may themselves vary according to earthworm genotype.

2.2. Materials and Methods

2.2.1. Selection of test treatments and soil preparation

Separate experiments were conducted to assess the effect of long-term exposure on *Eisenia sp.* for three different chemicals. The chemicals selected represent a non-essential metal cadmium, a non-essential metalloid arsenic and a neonicotinoid insecticide imidacloprid. The soil concentrations selected for testing were chosen based on available toxicity data to:

a) represent environmentally relevant levels of these pollutants in soils polluted by metal mining and processing and from the application of imidacloprid (the most widely used insecticide worldwide) as a spray treatment for pest control. (Camm *et al.*, 2004; Dankyi *et al.*, 2014;Li and Thornton, 2001; Wang *et al.*, 2007);

b) be low enough not to be expected to result in elevated mortality of the exposed earthworms, even over the extended test duration (Lock and Janssen, 2002; Spurgeon *et al.*, 1994; Wang *et al.*, 2015);

c) be in the range anticipated to have a potentially significant effect on reproduction, based on published toxicity test (Lock and Janssen, 2002; Spurgeon *et al.*, 1994; Wang *et al.*, 2015) which, alongside possible growth inhibition, was chosen as a marker for toxicity in this experiment.

For arsenic and cadmium, concentrations chosen were 12 mg/kg and 40 mg/kg respectively ((Lock and Janssen, 2002; Spurgeon *et al.*, 1994). Because the available earthworm toxicity data for imidacloprid is more limited than for cadmium or arsenic, exposures for this chemical were conducted at two concentrations. Thus, 0.1 mg/kg was selected as a concentration that was found in the environment (Dankyi *et al.*, 2014), the 1 mg/kg concentration is known to have a significant effect on cocoon

production (Wang *et al.*, 2015). A further value of testing at two concentrations for this substance was that use of higher concentrations could ensure sustained exposure even if degradation reduced concentration in the later stages of the exposure.

2.2.2. Soil preparation

The soil used for all exposures was a clay loam soil (Broughton Loams, Kettering, UK) with a pH of 7.5 and 6% organic matter content. The test soil was sieved through a 2 mm mesh and then amended with 3% organic material ("GroSure" by Westland, Dungannon, UK) to both further increase soil organic content and also improve the soil structure. To add the test chemical to the soil, a stock solution either cadmium (40 mg/kg dry weight, applied as CdCl2 (Sigma Aldrich, Bournemouth, UK), arsenic (12 mg/kg dry weight, applied as HAsNa2O4.7H2O (Sigma Aldrich, Bournemouth, UK) or imidacloprid (0.1 and 1 mg/kg dry weight (Bayer Crop Science, Cambridge, UK) was prepared in MiliQ water and sufficient volume of the stock solution then added to the test soils to produce the required chemical concentration and soil water content (60% of water holding capacity). Added stock solution was thoroughly mixed into the test soil to ensure the toxicants were evenly mixed. After addition of cadmium and arsenic, the soils were left to stabilise for one week to ensure that the added metal/metalloid obtained a more realistic speciation in the tested soils. The imidacloprid treatments were left overnight to allow the added water to distribute throughout the soil before the earthworms were added.

2.2.3. Earthworm rearing and maintenance in the experiment

2.2.3.1. Earthworm rearing

Throughout the experiment, all earthworm pairs were kept in a 20°C controlled temperature room in a 12 hour light, 12 hour dark cycle. The parent *Eisenia sp*. (P0) generation from which the P0 pairs were taken was initially reared in a bulk culture on a medium comprising by volume of one third natural silt-loam field soil, one third commercial Sphagnum peat and one third composted bark (LBS Horticultural, Colne, UK). To produce the F1 population, individuals from the P0 generation were selected from this stock culture at late juvenile stage, before they had developed clitella. This was done in order to be sure of the paternity of the offspring that would be produced from these worms after pairing, as earthworms are known to retain exchanged sperm within the spermathecal organ

after mating. The earthworms were paired and kept in control or spiked soil held in circular 200 ml plastic pots throughout the exposure period (See Figure 2.1).

The genotypes of the parent worms were unknown at the beginning of the experiment; however, each pair was numbered and the pedigree of offspring tracked and linked to parent pair. The pairs of P0 earthworms were initially incubated in treatment soils to produce cocoons. These cocoons were hatched and the resulting juveniles paired and placed in the control spiked soils, making up the F1 generation. Both individuals in each pair came from the same P0 parents. Similarly sized juveniles were picked from each P0 pair to make the subsequent assessment of growth and development in each of the differently exposed cohort populations more comparable. The paired offspring were spread between different treatment groups to ensure the genetic background would not impact on the effects of the treatments. There was a minimum of 15 replicates in each treatment pair.

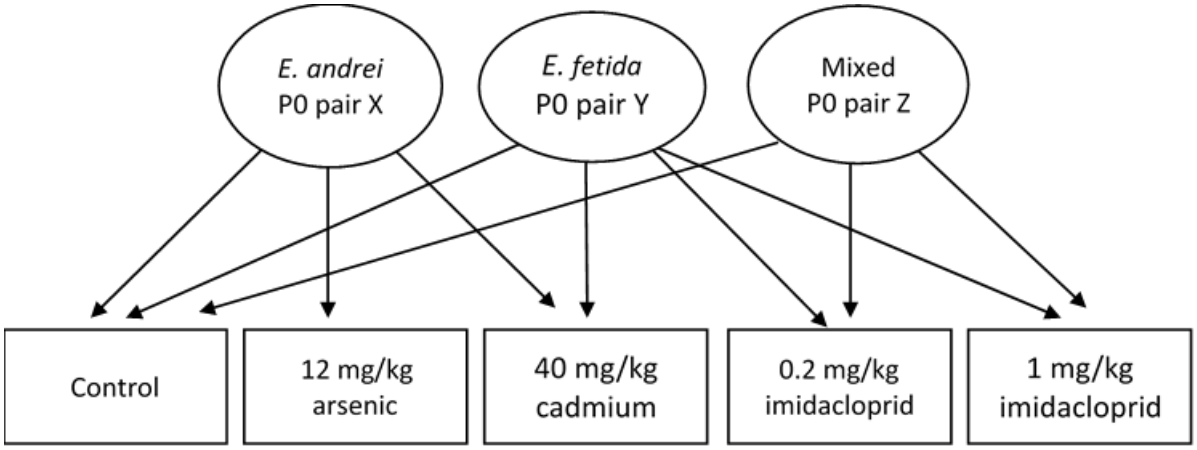


Figure 2.1. Experimental scheme showing the treatment strategy for Eisenia sp. earthworms used in the lifelong exposure study in which 14 P0 pairs were used, 7 of these *E. andrei*, 3 *E. fetida* and 4 were mixed (e.g. containing an individual from each lineage). Exposure of the F1 earthworms was conducted for 62 pairs of *E. andrei*, 34 pairs of *E. fetida* and 33 mixed pair with each worm in the pair coming from the same P0 parents. Arrows represent an earthworm pair from each P0 pair. Most P0 pairs did not produce enough offspring to have their F1 couples in every treatment, however, every P0 pair is represented in the control treatment and individuals from all three lineage backgrounds were represented in each treatment.

2.2.3.2. Feeding

All earthworms were fed 1 g (dry weight) horse manure once a month. The manure was previously collected at a farm in Oxfordshire, UK taken from an animal grazing uncontaminated pasture that was not subject to recent medication. The manure was initially frozen at -20 °C, oven dried and rewetted with miliQ water at a 1:4 weight ratio. The manure was spiked with the same concentration of toxicants as the soil in order to ensure both contact and dietary exposure. It has been reported that a spiked manure ensures higher accumulation in exposed *E. andrei*, which was significant at a dose of 10 mg/kg cadmium, but not at higher concentrations (van Gestel *et al.*,

1993), which implies that manure spiking to provide dietary exposure has an overall impact on total chemical uptake. Further, as avoidance behaviour was a possibility (e.g. see Chapter 4), spiking of manure also ensured that the exposed earthworms would not be able to escape into uncontaminated manure. The food was placed onto the soil surface in each container and spread out to avoid any possible effects of territoriality. This method was deemed sufficient as it has been reported that there is no significant difference in earthworm exposure to pollutants when manure is placed on top or mixed throughout the soil (van Gestel *et al.*, 1991).

2.2.4. Experimental procedure

The F1 *Eisenia* sp. were recovered from the soil every 28 days over a 112 day exposure. At each sample point, the wet weight of both individuals in the pair was measured and developmental stages assessed. When both of the F1 worms had developed into adults, the pair were placed into containers with fresh control or spiked soil for 6 weeks to allow reproduction rates to be quantified. In an exposure to a gradient of polluted soils, Spurgeon and Hopkin (1996) found that *E. fetida* became sexually mature and started cocoon production at different rates depending on exposure conditions. Hence, transfer of the earthworm pair to a fresh soil allowed assessment of cocoon production over a known period allowed rates to be better assessed. In the cases where the earthworms had not matured after 6 months of exposure, reproduction was not recorded for the pair.

For the initial reproduction test, the earthworm pairs were placed in soil spiked at the same concentrations in which they were reared. After 6 weeks, the adults were removed, dissected and snap frozen for further analysis. The number of laid cocoons and hatched juveniles was then counted after wet sieving of the soils. This technique was used rather than other methods for juvenile counting such as heat extraction as it was thought likely to provide less stress for the juveniles, which were due to be used as an F2 cohort for each pair in a subsequence multi-generational experiment (Chapter 3). The cocoons recovered were placed in fresh soil for observation of hatching success.

2.2.5. Growth and reproduction data analysis

Data handling and analysis was performed using Microsoft Excel, R and R studio; graphs were made using Microsoft Excel, Microsoft PowerPoint and R and R Studio. The number of juveniles per cocoon was used in order to find out the number of offspring produced in the initial six week reproduction

test by multiplying the number of hatchlings per cocoon and the number of cocoons produced in six weeks and adding the number of juveniles produced in that time. Therefore, for the fertility statistics, two numbers were used – the overall number of offspring produced in six weeks and the number of juveniles hatched per cocoon (cocoon viability).

The max growth rates and weights were calculated using the gfit package in R, which produced the maximum growth rate from the period of time (one of the 4 week periods) at which the organisms grew the fastest, calculating by how much the earthworms had grown in that time in g/4 weeks. The maximum weight was acquired from the point in time at which the earthworms were the heaviest in each pair.

Shapiro-Wilks test and the model aov command on R were used to test for normality. For assessing statistical difference between treatment Wilcoxon-Mann-Whitney test and the Kruskal-Wallis test were used as the most appropriate tests for the data as it was not normally distributed.

The total numbers of individuals in the growth measurements and pairs in the reproduction tests can be seen below. These numbers refer to the whole experiment which was three generations long, the results from the second and third generation are described in Chapter 3.

	F1			F2			F3		
	E.andrei	*E. fetida*	mixed	*E.andrei*	*E. fetida*	mixed	*E.andrei*	*E. fetida*	mixed
F1:As,F2: As, F3:As	24	12	18	28	16	16	18	8	8
F1:Cd,F2: Cd, F3:Cd	20	10	12	28	10	10	22	22	10
F1: 0.2 mg/kg Im,F2: 0.2 mg/kg Im,F3: 0.2 mg/kg Im	14	12	16	6	10	12	8	8	6
F1: 1 mg/kg Im,F2: 1 mg/kg Im,F3: 1 mg/kg Im	26	8	8	24	6	14	16	4	14
F1:Ctr,F2: Ctr, F3:Ctr	40	24	30	46	14	22	38	4	14
F1:As,F2: Ctr, F3:Ctr	NA	NA	NA	4	12	12	4	20	24
F1:Cd,F2: Ctr, F3:Ctr	NA	NA	NA	26	8	6	46	20	2
F1: 0.2 mg/kg Im,F2:Ctr,F3:Ctr	NA	NA	NA	6	14	14	6	16	12
F1: 1 mg/kg Im,F2:Ctr,F3:Ctr	NA	NA	NA	8	2	8	12	4	22
F1:As,F2:As,F3: Ctr	NA	NA	NA	NA	NA	NA	12	18	6
F1:Cd,F2:Cd, F3:Ctr	NA	NA	NA	NA	NA	NA	28	6	0
F1: 0.2 mg/kg Im,F2:0.2 mg/kg Im, F3:Ctr	NA	NA	NA	NA	NA	NA	16	4	4
F1: 1 mg/kg Im,F2:1 mg/kg Im, F3: Ctr	NA	NA	NA	NA	NA	NA	20	4	14

Table 2.1. The number of individual earthworms belonging to each treatment in growth experiments (any earthworms that died during the experiment or were excluded for other reasons have been omitted).

	F1			F2			F3		
	E.andrei	*E. fetida*	mixed	*E.andrei*	*E. fetida*	mixed	*E.andrei*	*E. fetida*	mixed
F1:As,F2: As, F3:As	7	5	9	10	10	9	10	8	10
F1:Cd,F2: Cd, F3:Cd	10	6	5	11	5	7	14	10	7
F1: 0.2 mg/kg Im,F2: 0.2 mg/kg Im,F3: 0.2 mg/kg Im	6	4	6	2	7	7	2	3	3
F1: 1 mg/kg Im,F2: 1 mg/kg Im,F3: 1 mg/kg Im	10	3	4	10	1	4	6	1	5
F1:Ctr,F2: Ctr, F3:Ctr	27	17	14	18	8	11	18	2	8
F1:As,F2: Ctr, F3:Ctr	NA	NA	NA	1	6	6	2	9	10
F1:Cd,F2: Ctr, F3:Ctr	NA	NA	NA	10	4	3	12	9	4
F1: 0.2 mg/kg Im,F2:Ctr,F3: Ctr	NA	NA	NA	4	5	7	4	7	3
F1: 1 mg/kg Im,F2:Ctr,F3: Ctr	NA	NA	NA	3	1	3	6	2	9
F1:As,F2:As,F3: Ctr	NA	NA	NA	NA	NA	NA	4	8	3
F1:Cd,F2:Cd, F3:Ctr	NA	NA	NA	NA	NA	NA	7	3	6
F1: 0.2 mg/kg Im,F2:0.2 mg/kg Im, F3:Ctr	NA	NA	NA	NA	NA	NA	6	2	2
F1: 1 mg/kg Im,F2:1 mg/kg Im, F3: Ctr	NA	NA	NA	NA	NA	NA	9	1	4

Table 2.2. The number of earthworm pairs belonging to each treatment in reproduction tests (any earthworms that died during the experiment have been excluded)

2.2.6. Earthworm genotyping

2.2.7. Dissections

The earthworms were dissected, separating the clitellum, head and tail samples were for use in additional analyses. The head samples were used for the isolation of DNA for earthworm genotyping and the tail samples were used for chemical analysis of pollutant content, meanwhile, the clitellum was not used for further analysis in the experiments described in this thesis. The samples were then kept at -80 °C until required for the relevant downstream analyses. All equipment was cleaned in 70% ethanol between dissections to ensure that there was no cross contamination between individual specimens.

2.2.8. DNA extraction

To acquire DNA for *Eisenia sp.* genotyping, head samples that had previously been kept frozen at -80 °C were ground up using a sterilised and cleaned pestle and mortar under liquid nitrogen. An aliquot of powdered tissue taken from this ground sample was used for the DNA extractions.
DNA samples were extracted using the Qiagen blood and tissue DNA extraction kit (Qiagen Inc., Crawley, UK) according to the manufacturers' instructions. The DNA isolated from the extracted tissues was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) to provide a quantitative measure of DNA concentrations and their quality was checked using gel electrophoresis. The DNA samples were then kept at -20 °C prior to all downstream analyses.

2.2.9. Gel electrophoresis

Gel electrophoresis was run in order to check the quality of DNA after extractions and DNA after PCR. For preparation of the electrophoresis gels, agarose was dissolved in autoclaved 1X TAE buffer, in a microwave, and cooled to an optimal temperature. SYBR™ Safe DNA Gel stain (Invitrogen, Thermofisher scientific, Gloucester, UK) was added (10 µl/100ml for DNA, 15 µl/100ml for RNA), the solution mixed and poured into the gel mould. After leaving the gel to set for approximately 30 minutes, the DNA samples were mixed with 1 µl electrophoresis loading dye and the samples were loaded onto the gel alongside a DNA ladder (Hyperladder™, Bioline, London, UK). The samples were resolved by electrophoresis in 1X TAE buffer for sufficient time to allow the separation of the

relevant bands needed to allow the assessment of sample integrity. Nucleic acid bands were then visualised in a molecular imager (BioRad, Watford, UK).

2.2.10. Genotyping procedure

In order to use the extracted DNA samples for genotyping they were diluted to equalise their concentrations. For the following polymerase chain reactions (PCR) the following COI barcoding primers were used: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Sigma Aldrich, Bournemouth, UK). After initial denaturation at 5 min at 95 °C the DNA was amplified over 40 cycles of 30 s 95 °C, 30 s 48 °C and 60 s 48 °C. Both the primers and the PCR procedure used was described by (Folmer *et al.*, 1994) and (Spurgeon *et al.* 2016).

The PCR ingredient were mixed according to the following for all reactions:
40.75 µl DNAse/RNAse free Water
5 µl 10X PCR Buffer (Sigma Aldrich, Bournemouth, UK)
1 µl Deoxynucleotide mix (Applied Biosystems, Thermo Fisher Scientific, Birchwood, UK)
1 µl Forward primer (Sigma Aldrich, Bournemouth, UK)
1 µl Reverse primer (Sigma Aldrich, Bournemouth, UK)
0.25 µl Taq DNA polymerase including MgCl (Sigma Aldrich, Bournemouth, UK)
1 µl Template DNA

Following completion of the amplification, a 1 µl sample of PCR products was checked using gel electrophoresis to ensure the amplification of the target 670 base fragment from each sample. All samples that had undergone successful amplification were then purified using ZR DNA Sequencing Clean-up Kit™ (Zymo Research, Cambridge Bioscience, Cambridge, UK) and a PCR reaction conducted using the BigDyeTM Terminator v3.1 Cycle sequencing Kit (ThermoFisher Scientific, Gloucester, UK) according to manufacturer's instruction, followed by a sample clean-up step using BigDye Xterminator Purification kit (ThermoFisher Scientific, Gloucester, UK) used according to manufacturers' instructions. The samples were then sequenced using the Applied Biosystems 3730 DNA Analyzer. Sequencing data was analysed with the programs Sequencher, Geneious and MEGA. Reference sequences were obtained from GenBank for the analysis of genetic relatability.

The reference DNA barcoding sequences were taken from DDBJ/EMBL/GenBank and/or at Barcode of Life. The creation of the phenotypic tree and the distance table was done using MEGA software.

2.2.11. Soil chemistry data measurements

2.2.11.1. Analysis of cadmium and arsenic content in soil

Five replicates of roughly 5 g of soil were used for this analysis. Firstly, the samples were dried for 48 hours at 80°C before being sieved using a 2 mm sieve. The soil metal analysis was carried out as described by Spurgeon et al. (2011). Briefly, following an aqua regia digestion using a microwave digestion, the samples were quantified with a Perkin Elmer Elan DRC II inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer 4300DV, Cambridge, UK). The samples were tested for arsenic and cadmium content. Quality control for this analysis was carried out using standard reference material ISE 192 (International Soil Exchange, The Netherlands.). All quality control recoveries were within the expected norms. Certified values for reference materials were well aligned with data in this analysis, they were within 10% of certified values for both metals.

2.2.11.2. Analysis of imidacloprid content in soil

For this analysis, three replicates of roughly five grams of soil were collected from freshly spiked soils at the beginning of the exposure. In order to carry out the imidacloprid content analysis in soil, liquid chromatography coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hemsptead; UK) were carried out. This process was interfaced using an ion max electrospray ionisation (ESI) and analyte separation was carried out on a Phenomenex Synergi Fusion column (2.5 µm particle size, 50 mm x 2mm I.D., Phenomenex) using the H2O:MeOH mobile phase gradient. The residues were compared to spiked samples (0.1g) labelled with internal imidacloprid standards. The quantification of imidacloprid residues was based on the respective response factors to these internal standards. The measured chromatographic peaks were integrated using an ICIS algorithm, it was also used to generate linear calibration curves based on a 1/X weighting ($R2>0.99$). Limit of detection (LoD = 0.38 ng g-1) and recovery of the internal standards for the analytes were used in order to assess the performance of the method. Certified values for reference materials were well aligned with data in this analysis, they were within 10% of certified values for imidacloprid.

LoD was established as three times the signal to noise ratio with included assessment of the expanded uncertainty of the method. LoQ (limit of quantification) was calculated as the LoD with added calculated expanded uncertainty of the method (LoQ = 0.53 ng g-1).

2.3. Results

2.3.1. Soil physio-chemistry

The background concentrations of cadmium and arsenic in the unspiked control soil were 0.39 ± 0.03 mg/kg and 32.9 ± 2.14 mg/kg (± SD), respectively. These concentrations are in the range typical of the geological background of these elements in uncontaminated soils (Spurgeon *et al.*, 2008). Concentrations of all other measured trace metals were within the range commonly found in uncontaminated soils. No imidacloprid was detected in the control soils.

In the spiked soils, the mean cadmium content was 37.8 ± 1.09 mg/kg and the mean arsenic content was 51.7 ± 3.69 mg/kg mg/kg. The metals added to the soil were intended to result in an increase in concentration of the two metals by 40 mg/kg for cadmium and 12 mg/kg for arsenic. Actual increases were 37.41 and 18.8 mg/kg, respectively, indicating a good agreement between the desired and achieved added concentrations. The two measured concentrations of imidacloprid in exposure soils were 1.18 ± 0.120 mg/kg and 0.226 ± 0.0183 mg/kg. The concentrations measured for the cadmium treatment and the higher tested concentrations of imidacloprid are in good agreement with the planned nominal increase of 40 and 1 mg/kg respectively (within 20% of intended value). However, the measured concentrations for the arsenic exposure soils and the lowest tested concentrations of imidacloprid exceeded the intended increase of 12 mg/kg and 0.1 mg/kg respectively. The slightly higher levels of measured arsenic in soil is within the range of combined errors of the background and measured value. However, given the relatively small variance found for the 0.1 mg/kg imidacloprid soil, this is not the case for this treatment. It is not possible to conclude whether these differences result from a systematic dosing error, spatial variability leading to the collection and analysis of samples with higher than average concentrations or due to other downstream measurement errors. The measurements do, however, indicate that exposure concentrations are 5-10 fold lower in this treatment than at the higher tested concentration. In the remainder of this thesis, therefore, the intended 0.1 mg/kg treatment will be referred to as 0.2 mg/kg in order to more accurately describe the treatment. The arsenic exposure concentrations will, however, continue to be referred to as 12 mg/kg given the overlap of error values with the scale of variation from the intended increase.

2.3.2. Genotyping

Genotyping of the P0 earthworms showed that two lineages were present in the experimental population. Of the 14 pairs successfully genotyped, 6 contained 2 individuals from the *E. andrei*

lineage, 3 had 2 individuals of *E. fetida* and 4 pairs an individual from each lineage. Within the two lineages, three separate clade groups were found (see Figure 2.2). The level of genetic divergence between the clades was more than ten times higher for the *E. fetida* earthworms than for the *E. andrei* (See Table 2.1). This is consistent with previous findings of high genetic diversity between individuals morphologically identified as from the *E. fetida* lineage (Aira *et al.*, 2016). The level of sequence variation observed between some individuals from within the *E. andrei* and *E. fetida* clades (e.g. *E. andrei* Clade 2 and *E. fetida* Clade 2) are of similar size to the differences between either *E. andrei* or *E. fetida* and *E. japonica*, supporting the hypothesis that the two lineages represent separate species (Domínguez *et al.*, 2005a).

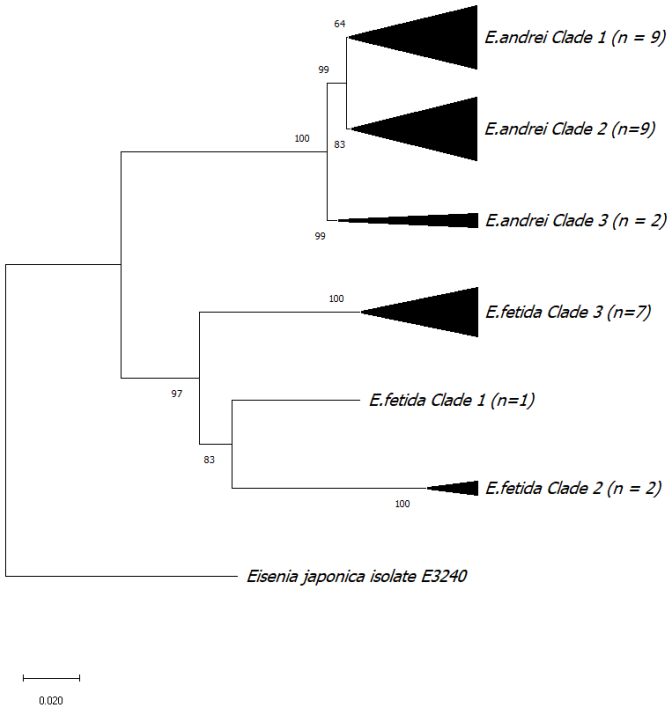


Figure 2.2. Evolutionary relationships between *E. andrei* and *E.fetida*. Phylogenetic relationship between tested individuals inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.44191113 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to branches. Tree is drawn to scale, with branch lengths in the same units as evolutionary distances used to infer phylogenetic relationships. Evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 655 positions in the final dataset. The Clade 1 of each lineage includes the reference sequences for the lineages acquired from GenBank, *Eisenia japonica* was used as the outgroup.

Table 2.3. Estimates of evolutionary divergence over sequence pairs between groups.							
	E. andrei Clade 1	*E. andrei* Clade 2	*E. andrei* Clade 3	*E.fetida* Clade 1	*E.fetida* Clade 2	*E.fetida* Clade 3	*E. japonica* E3240
E. andrei Clade 1	0.000						
E. andrei Clade 2	0.003	0.000					
E. andrei Clade 3	0.012	0.012	0.000				
E.fetida Clade 1	0.166	0.166	0.172	0.000			
E.fetida Clade 2	0.199	0.201	0.199	0.125	0.000		
E.fetida Clade 3	0.164	0.166	0.168	0.121	0.142	0.000	
E. japonica E3240	0.204	0.206	0.194	0.196	0.235	0.218	0.000
The differences are shown as the number of base substitutions per site between groups averaged over all sequence pairs as conducted using the Tamura 3-parameter model with 31 nucleotide sequences with codon positions included were 1st+2nd+3rd+Noncoding. n.b. All positions containing gaps and missing data were eliminated, giving a total of 655 positions in the final dataset.							

2.3.3. Earthworm mortality

All control and exposed groups of earthworms showed low mortality rates despite the extended time-scale of the exposure (total a minimum of 112 days). There was a 100% survival rate among the control, cadmium and arsenic exposed pairs, 96% survival among the 0.2 mg/kg imidacloprid pairs and 97.6% survival rate in the 1 mg/kg imidacloprid exposure pairs. Survival rates between the exposed cohorts did not differ significantly (Kruskal-Wallis $p>0.05$) from control rates. This is consistent with other findings, for example, *E. fetida* showed very high resistance to Cd, with no increase in mortality after 56 days of exposure to 300 mg/kg Cd (Spurgeon *et al.*, 1994).

2.3.4. Growth

The growth measurements were taken every four weeks (28 days) from the start of the experiment. This time period is referred to as a month in the rest of the chapter.

2.3.4.1. Growth statistical analysis

Spurgeon and Hopkin (1996) found that *E. fetida* showed a rapid initial phase of growth in the early stages of development that followed a near linear increase. At this time, as the earthworms entered the start of sexual maturation, weight change slowed until maximum weight was reached. The logistic

model found for weight change in the exposed earthworms in this study accord with the overall pattern of growth. Using the gcFit function in the package grofit in R for the time-series weight data (Kahm *et al.*, 2015), the best fitting growth model to the measured weight data was found to be a logistic function. This model provided the best fit to the observed data for 53% of the curves (75 out of 141) compared to 25% of curves for Richard's model and 20% for the Gompertz model. Fitting of logistic functions to all data-sets was used to derive model parameters identifying the maximum growth rate (g/month) and the maximum weight (g) for each control and exposed earthworm pair (note: individual worms could not be tracked due to their similar size and appearance). The effects of the three chemicals on mean measured size at selected exposure times and parameter values for the logistic model fits for all three exposures given in Table 2.2.

2.3.4.2. Effects of arsenic on growth

Growth of the control and arsenic exposed earthworms could be described by the logistic growth model. After 4 weeks of exposure, the arsenic treated earthworms were significantly heavier than the control individuals ($p<0.01$, $\chi 2 = 7.47$). The difference in size, although significant, was relatively small, with controls being 0.0809 g and arsenic exposed 0.0853 g. Size at later sample times and the logistic model fit parameters for the control and arsenic exposed earthworms did not differ significantly ($p>0.05$) between the groups (See Figure 2.3; Table 2.2).

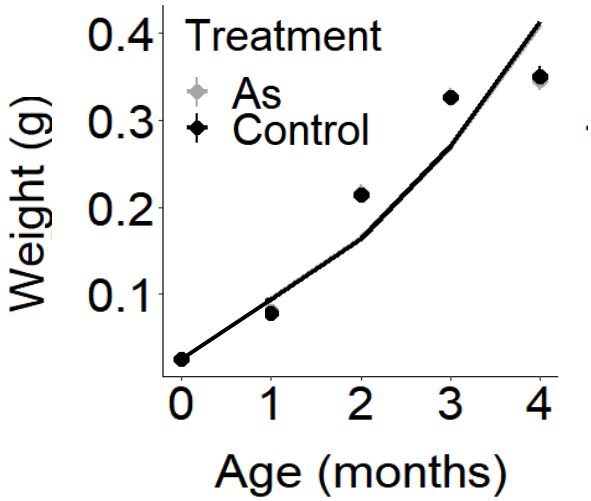


Figure 2.3. Growth of juveniles exposed to control soil and soil spiked with 12 mg/kg arsenic (As). Points show the mean ± standard deviation of measured weight at each measurement time; lines are logistic models fits calculated from the measured body size data. Each month equals an interval of 4 weeks. The control and arsenic treated earthworms showed very similar results, therefore much of the points and lines overlap. Age 0 is the time at which hatched juveniles were exposed to the different soils. Significance values: * $p\leq0.05$; ** $p\leq0.01$;*** $p\leq0.001$.

2.3.4.3. Effects of cadmium on growth

Exposure to 40 mg/kg cadmium significantly reduced earthworm weights compared to controls at 4 (p<0.01, χ2 =7.41), 8 (p<0.001, χ2 = 47.8) and 12 (p<0.001, χ2 = 38.7) weeks. After four weeks of exposure, the average body size was lower in the cadmium exposed earthworms, although this difference was not significant compared to control (p>0.05, χ2 = 1.68), it also reduced the average maximum growth rate reached, with this parameter being affected at a significant level (Figure 2.4; Table 2.2). Model fits indicated a significantly higher final body size in the cadmium exposed population compared to controls, this result is likely not biologically relevant and is a result of the model curve being applied to the data.

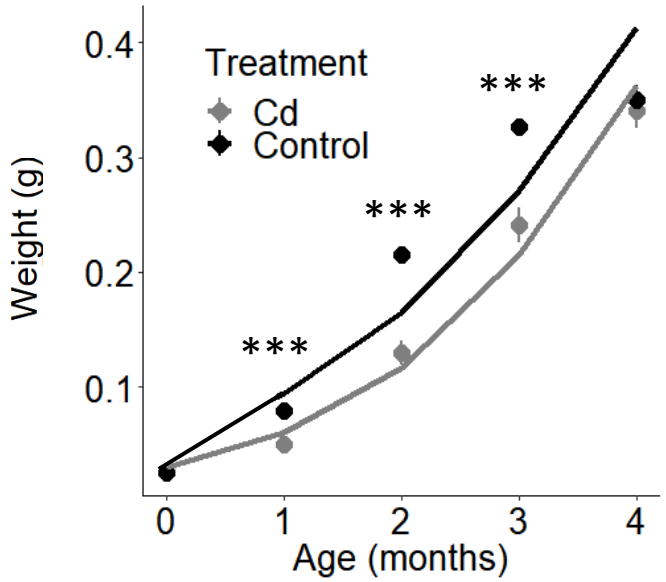


Figure 2.4. Growth of juveniles exposed to control soil and soil spiked with 40 mg/kg cadmium (Cd). Points show the mean ± standard deviation of each measured weight at each measurement time;lines are logistic model fits calculated from the measured body size data. Each month equals an interval of 4 weeks. Age 0 is the time at which hatched juveniles were exposed to the different soils. Significance values: * p≤0.05; **p≤0.01;***p≤0.001.

2.3.4.4. Effects of imidacloprid on growth

Exposure of *Eisenia sp.* to 0.2 mg/kg imidacloprid significantly reduced body size compared to control individuals at 4 (p<0.001, χ2 = 38.33) and 8 (p<0.001, χ2 =45.6) weeks. However, by week 12, the 0.2 mg/kg imidacloprid had "caught-up" and were no longer significantly smaller than the control earthworms. The 1 mg/kg imidacloprid exposure significantly reduced earthworm weight compared to controls and also to the 0.2 mg/kg imidacloprid treatment at 4 (p<0.001, χ2 = 65.9 and p<0.001, χ2 = 22.9, respectively), 8 (p<0.001, χ2 =50.1 and p<0.01, χ2 =6.99) and 12 weeks (p<0.001, χ2 = 27.7 and p<0.001, χ2 =14.9). Neither exposure had a significant effect on body size

after 16 weeks of exposure. Logistic model fits indicated that neither imidacloprid exposure concentration had a significant impact on maximum growth rate (0.2 mg/kg: $\chi 2$ =1.13, p>0.05; (1 mg/kg: $\chi 2$ =2.85, p>0.05) or maximum body size (0.2 mg/kg: $\chi 2$ =0.0295, p>0.05, 1 mg/kg: $\chi 2$ =0.116, p>0.05)(Figure 2.5; Table 2.2).

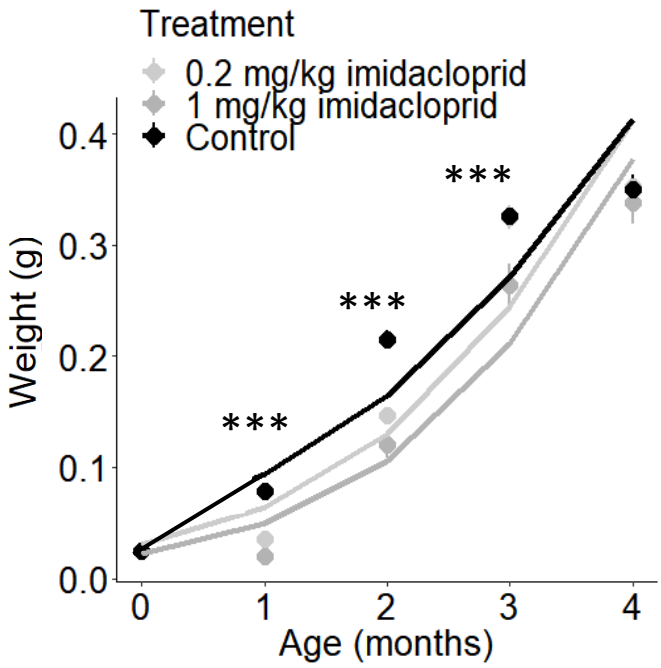


Figure 2.5. Growth of juveniles exposed to control soil and soil spiked with imidacloprid. The points show the mean ± standard deviation of each age group, the lines are the visualisations of a logistic models calculated using the data. Each month equals an interval of 4 weeks. Age 0 refers to the time at which the juveniles were placed in the soil. Significance values : * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$.

Table 2.4. Effects of 40 mg/k cadmium, 12 mg/kg arsenic and 0.2 and 1 mg/kg imidacloprid exposure on *E. andrei* and *E. fetida* during a life time exposure to realistic levels of pollutants.

Soil treatment	% Survival	Average weights (g) at four time points				Max growth rate (g/month)	Max weight (g)
		1st month	2nd month	3nd month	4nd month		
Control	96.49	0.073 ±0.035	0.221 ±0.060	0.329 ±0.068	0.341 ±0.091	0.192 ±0.0659	0.384± 0.0833
12 mg/kg As	93.75	0.085 ±0.037 **a	0.228 ±0.070	0.332 ±0.073	0.338 ±0.080	0.170 ±0.0349	0.381± 0.0744
40 mg/kg Cd	91.42	0.049 ±0.027 **a	0.135 ±0.069 ***a	0.249 ±0.107 ***a	0.340 ±0.103	0.133 ±0.0348 ***a	0.453± 0.122 *a
0.2 mg/kg Imidacloprid	98	0.035 ±0.016 ***a	0.146 ±0.049 ***a	0.322 ±0.065	0.349 ±0.073	0.209 ±0.0712	0.373 ±0.0606
1 mg/kg Imidacloprid	92.30	0.019 ±0.013 ***a ***b	0.122 ±0.052 ***a **b	0.266 ±0.084 ***a ***b	0.336 ±0.078	0.184 ±0.0616	0.394 ±0.0793

Each value (mean ±SD) is calculated from a minimum of 15 replicates with two individuals per replicate. Each month equals an interval of 4 weeks. The statistical significance. * p≤0.05; **p≤0.01;***p≤0.001, a – in comparison to control, b- in comparison to the other concentration of imidacloprid.

2.3.5. Reproduction

The experimental design of the test for reproduction rate effects differed slightly from the tests described in most literature due to the necessity to carry on the exposure of the populations into the two subsequent generations to measure the effect of multi-generational exposure (Chapter 3). To measure effects on reproduction and also to allow the collection of a sufficient number of individuals for the subsequent multigenerational exposure (Chapter 3), the tests with all four exposure treatments were run over an extended 6 week exposure designed to ensure enough offspring would be available for the next generation. The extended duration meant that cocoons hatched in the test system. Therefore, juvenile number rather than the number of laid cocoons (note *Eisenia* sp. can hatch more than one juvenile from each cocoon) was used as the reproduction endpoint. To ensure that the juveniles were not adversely harmed, manual sorting through soil rather than heat extraction was used. It is possible that fewer juveniles were retrieved by soil sorting than via the heat removal method. Further, there is also the potential for hatched juveniles to die during their time spent in the exposed soil. However, given the high observed survival in the test system, such loses are likely to be low and comparable between treatments. Cocoons that did not hatch during the six week exposure period of the reproduction exposure were further incubated in dosed soil for an additional six weeks to allow hatching.

Control earthworms on average produced 0.537±0.561 juveniles per cocoon. This was calculated from all cocoons placed in soil, the number of cocoons that did not hatch at all was not recorded. Presumably, this difference in design is the main reason why this value is lower than values previously reported. For example, Spurgeon and Hopkin reported cocoon viability at 2.01 (range 1-5) worms per cocoon (Spurgeon, Hopkin *et al.* 1994). Differences in results may possibly also be due to different methods of counting or death in early life of the newly hatched juveniles and/or the density and number of worms being kept in an individual container during the experiment. Some of this effect may be due to the fact that mixed lineage pairs were used in the experiment, this is addressed later in the chapter.

2.3.5.1. Effects of arsenic on reproduction

There was a significant increase in the total number of juveniles produced in the 6 week reproduction test ($\chi 2 = 4.23$, $p<0.05$) (Figure 2.6). Average juvenile production per pair was slightly higher in the arsenic exposed *Eisenia sp.* compared to the control exposed earthworms. No impact of arsenic on the number of juveniles emerging per cocoon was observed.

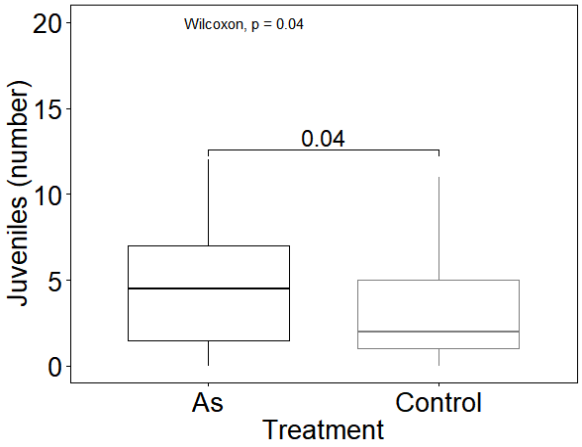


Figure 2.6. Total number of juveniles produced in a 6 week reproduction test by *E. fetida* and *E. andrei* after a lifetime exposure to control of arsenic (12 mg/kg) spiked soil showing the means and distributions of the two sample sets as well as the p value calculated using the Wilcoxon-Mann-Whitney test.

2.3.5.2. Effects of cadmium on reproduction

No significant effect of cadmium was observed on either the total juvenile production ($\chi 2 = 1.19$, $p<0.05$) or the number of juveniles per cocoon ($\chi 2 = 1.09$, $p<0.05$) (Figure 2.7). The absence of an effect of cadmium exposure on reproduction is in contrast to previous findings. For example, Spurgeon et al. (1994) found that earthworm reproduction rate was significantly lower in individuals exposed to 20 mg/kg Cd than in a control soil. The EC50 for effects on cocoon production was calculated as 46.3 mg/kg Cd (Spurgeon, Hopkin *et al.* 1994). Hence findings here suggest a less

detrimental effect of cadmium on reproduction following extended exposure than in the short-term (28 day) toxicity test as conducted by Spurgeon et al. (1994).

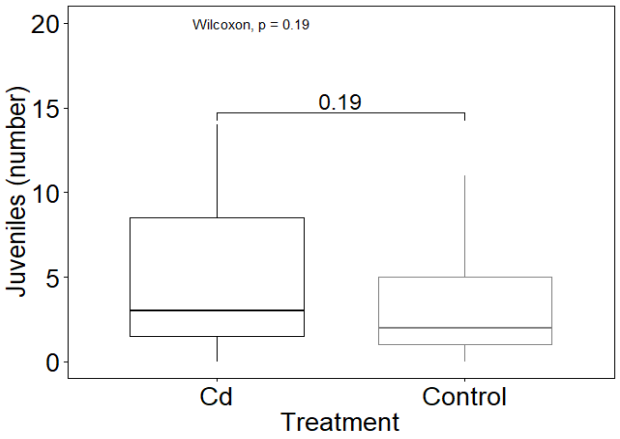


Figure 2.7: The number of juveniles produced in a 6 week reproduction test by *E. fetida* and *E. andrei* after a lifetime exposure to control or cadmium (40 mg/kg) spiked soil showing means and distributions of the two sample sets as well as the p value calculated using the Wilcoxon-Mann-Whitney test.

2.3.5.3. Effects of imidacloprid on reproduction

Exposure to the lower concentration of imidacloprid significantly reduced (22% lower than controls) the number of juveniles produced ($\chi 2 = 7.05$, $p<0.01$) (See Table 2.3; Figure 2.8). In contrast, in the higher imidacloprid exposure, no significant impact on juvenile production per pair compared to controls was found (See Table 2.3; Figure 2.8). The number of juveniles from cocoons was significantly higher at 0.2 mg/kg imidacloprid compared to controls. Hence, the lower overall juvenile production found in this treatment is primarily associated with reduced cocoon production, rather than any effect on hatchability.

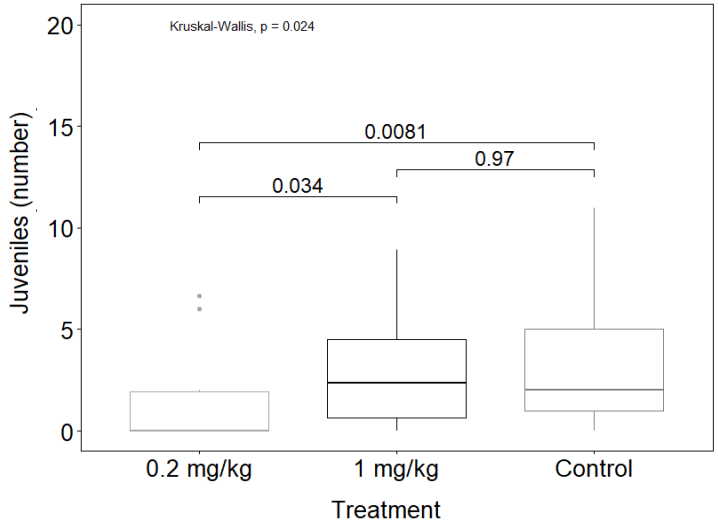


Figure 2.8. The number of juveniles produced in a 6 week fertility test by *E. fetida* and *E. andrei* after a lifetime exposure to either imidacloprid spiked (0.2 mg/kg and 1 mg/kg) or control soil. The reproduction test was carried out within the same soil types. The boxplot shows the means and distributions of the two sample sets as well as the p value calculated using the Kruskal-Wallis test.

Table 2.5 .Effects of As, Cd and imidacloprid at environmentally relevant concentrations on the fertility of *E. andrei* and *E.fetida*

Treatment	Juveniles/worm/week	Cocoon viability (juveniles per cocoon)
Control	0.250±0.240	0.537±0.561
12 $mgkg^{-1}$ As	0.406±0.309*[a]	0.486±0.708
40 $mgkg^{-1}$ Cd	0.382±0.352	0.835±0.881
0.2 $mgkg^{-1}$ Imidacloprid	0.193±0.368**[a]	0.657±0.734***[a]
1 $mgkg^{-1}$ Imidacloprid	0.248±0.236*[b]	0.381±0.486

Mean ±SD for juvenile production and number of juveniles/cocoon based on a minimum of 14 replicated containers with either a pair of earthworms or cocoons. The values for juveniles per week were acquired from a 6 week reproduction test after the individuals had reached sexual maturity, number of juveniles per cocoon calculated following hatching of cocoon on the same soil and used for the exposure for 6 weeks. Significance values: * p≤0.05; **p≤0.01;***p≤0.001, a –comparison to control, b- comparison between the two imidacloprid treatments.

2.3.6. Effects of genetic background on growth and reproduction

2.3.6.1. Phenotypic differences between families.

When looking at all the individuals within a certain family across all the exposures, there were observable differences between the families' reproductive output (from 0.45 to 0.067 offspring per worm per week and from 1.22 to 0.314 juveniles hatched per cocoon) and some differences between weights at the developmental growth stages (e.g. from 0.0973 to 0.222 g at the 8 week growth stage), however, neither of these differences were significant. This information is summarised in Table 2.4. No correlations between the different parameters were observed.

Table 2.6. Comparisons between individual families of *E. fetida*, *E. andrei* and mixed lineage background (one parent *E. andrei*, the other *E. fetida*) pairs exposed to control, 12 mg/kg As, 40 mg/kg Cd, 0.2 mg/kg and 1 mg/kg imidacloprid (four separate exposures).

Family (Line) number	N (F1)	Lineage	Weights (g) at individual growth stages				Offspring per worm per week	Cocoon viability (juveniles/ cocoon)
			1st month	2nd month	3rd month	4th month		
3	12	*E. andrei*	0.0732± 0.0490	0.205± 0.0687	0.304± 0.0796	0.306± 0.0984	0.390± 0.261	0.582± 0.540
11	17	mixed	0.0707± 0.0446	0.187± 0.730	0.291± 0.0822	0.319± 0.0910	0.365± 0.372	0.614± 0.847
25	5	*E. andrei*	0.0341± 0.0213	0.149± 0.677	0.339± 0.113	0.409± 0.105	0.067± 0.042	0.408± 0.426
32	10	*E. fetida*	0.0725± 0.0376	0.191± 0.0437	0.309± 0.0760	0.357± 0.109	0.188± 0.153	0.663± 0.547
35	19	*E. fetida*	0.0540± 0.0355	0.176± 0.0683	0.323± 0.0692	0.378± 0.0905	0.360± 0.278	0.733± 0.824
42	7	*E. andrei*	0.0513± 0.0275	0.193± 0.0652	0.298± 0.0837	0.316± 0.0893	0.333± 0.282	0.576± 0.398
44	3	mixed	0.0499± 0.0185	0.158± 00748	0.303± 0.129	0.358± 0.0825	0.450± 0.357	0.314± 0.335
51	4	mixed	0.0335± 0.0247	0.0973± 0.0443	0.252± 0.105	0.358± 0.125	0.178± 0.211	1.22± 0.975
59	4	*E. andrei*	0.0833± 0.0597	0.177± 0.0702	0.243± 0.0458	0.293± 0.113	0.287± 0.036	0.170± 0.204
65	3	mixed	0.0593± 0.0123	0.186± 0.0430	0.319± 0.0543	0.288± 0.0901	0.111± 0.128	0.650± 0.238
67	6	*E. fetida*	0.0537± 0.0268	0.188± 0.0496	0.324± 0.530	0.372± 0.0539	0.255± 0.254	0.424± 0.602

74	11	mixed	0.0633± 0.0326	0.222± 0.0544	0.373± 0.0508	0.427± 0.0798	0.249± 0.348	0.642± 0.484
80	15	*E. andrei*	0.0447± 0.0295	0.167± 0.0686	0.304± 0.0964	0.368± 0.0976	0.230± 0.326	0.467± 0.553
97	17	*E. andrei*	0.0489± 0.0306	0.188± 0.0830	0.319± 0.0864	0.332± 0.0696	0.226± 0.184	0.444± 0.687
Data is the value for all treatments, n refers to the number of pairs of earthworms from each family. Each month equals an interval of 4 weeks. The values are expressed in mean ± standard deviation, the reproduction values from tests to measure reproduction rate and juveniles / cocoon.								

2.3.6.2. Growth

Mean earthworm weights were not significantly dependent on lineage or family background at any of the sample points (4, 8, 12 and 16 weeks) both within the control group and all worms when weight of individuals from all treatment groups were included in the analysis. While there were no effects at individual timepoints, logistic model fits to weight change data over the full time series did indicate significant differences in the patterns of growth depending on both lineage and family background. Maximum growth rate differed significantly depending on both lineage background and family (Kruskal-Wallis p=0.0007 and 0.0016, respectively). Across all treatments, *E. andrei* pairs showed significantly faster maximum growth rate than both *E. fetida* (p<0.001) and mixed lineage pairs (Kruskal-Wallis p = 0.04). Maximum weight was also significantly dependent on family (Kruskal-Wallis p = 0.015)(Table 2.5). However, when looking at the direct measurements of growth weights, *E. fetida* earthworms were on average slightly heavier at each growth stage, possibly because *E. fetida* grew faster at the beginning before the first measurement, or perhaps in general showed greater, but more measured growth. These observations, however, were not significant. While both genetic background and individual chemical treatments (e.g. Cd 0.2 and 1 mg/kg imidacloprid) had a significant impact on the earthworms, no interactions were found between the two genetic background measurements and chemical treatment effects on either maximum growth rate or maximum weight.

Table 2.7. Lineage background and growth parameters.

	E. andrei	*E. fetida*	Mixed
Maximum growth rate (g/4weeks)	0.205 ± 0.0625***[a], *[b]	0.155 ± 0.0207	0.168 ± 0.0317
Maximum weight (g)	0.397 ± 0.0393	0.383 ± 0.0249	0.403 ± 0.0492

Mean ± standard deviation of logistic model parameters fitted to growth data for earthworm in all experimental treatments. Significance values: * p≤0.05; ** p≤0.01;*** p≤0.001, a – in comparison to *E. fetida*, b- in comparison to mixed background, c– in comparison to the other soil type

2.3.6.3. Reproduction

The number of offspring produced by *E. andrei* during the 6 week fertility test was slightly lower than in either the *E. fetida* or mixed lineage pairs (3.09 versus 3.51 and 3.58 offspring in 6 weeks, respectively). Slightly fewer juveniles per cocoon were produced by *E. andrei* earthworms (0.529 juveniles per cocoon) compared to the *E. fetida* and mixed lineage pairs (0.654 and 0.576 respectively). The differences in these reproduction parameters were, however, not significantly different between the lineage background pairs (*E. andrei, E. fetida* or mixed lineage) ($\chi 2=2.32$, $p>0.05$). These results are not in accordance with the results from a previous study where *E. andrei* were found to produce the same number of cocoons as *E. fetida*, but more juveniles per cocoon (Domínguez *et al.*, 2005a).

The mixed lineage pairs combining an individual *E. fetida* and *E. andrei* were capable of producing fertile offspring. Indeed, the average number of juveniles hatched per cocoon from the mixed pairs was not significantly different from that for either single lineage pair ($\chi 2=0.0354$, $p>0.05$ in comparison to either *E. fetida* or *E. andrei*). The number of pairs of mixed lineage background that did not produce any hatchlings was, however, higher than for either single lineage pair (43.8% versus 33.8% for *E. andrei* and 31.1% for *E. fetida*). As *E. andrei* and *E. fetida* are capable of self-fertilisation, it cannot be established if the two lineages have truly interbred during this experiment. A previous experiment has demonstrated potential cross fertilisation between individual from these two linages (Plytycz *et al.*, 2018). Hence, the possibility remains that the two lineages are not truly reproductively isolated and indeed when paired can reproduce at rates consistent with those for either lineage.

When carrying out a Kruskal-Wallis test on the data of all of the families, no significant effect of family background on the total number of juveniles produced in the reproduction test was found. However, a significant effect of family on the number of juveniles hatching from each cocoon was present. As there were different numbers of individuals from each family in each treatment group, further analysis was carried out to find out if the family background interacts with the treatment effects. Analysis using a generalised linear model indicated no family and treatment interactions indicating that reproductive effects in response to exposure were not determined by family pair.

2.4. Discussion

All three chemicals used in this study (arsenic, cadmium and imidacloprid) have known negative impacts on *Eisenia* earthworms and other terrestrial organisms (Lock and Janssen, 2002; Spurgeon *et al.,* 1994; Wang *et al*., 2015). However, to date, only Cd has been previously investigated for effect on multiple life-cycle traits on earthworms from the *E. fetida/E. andrei* complex throughout their full developmental period. This study addressed this gap in knowledge for arsenic and imidacloprid which are widespread and persistent pollutants. Further, the impacts of genetic background on growth and reproduction both in control soils and also in response to the long-term chemical exposures was assessed in order to better understand how mixed lineage backgrounds common in laboratory stock cultures may respond to toxic exposures and how different levels of genetic diversity (resulting from natural or artificial bottleneck events) may have an impact on the earthworms' phenotypes in response to pollution.

Exposure to all three chemicals resulted in a significant change in one or more of the growth and/or reproduction parameters measured. The effects of exposure were primarily negative. For example, exposure to both cadmium and 1 mg/kg imidacloprid significantly reduced the size of juveniles at the first three measured growth stages compared to control and exposure to both concentrations of imidacloprid significantly reduced overall reproductive output. In some of the exposure cases, positive ("hormesis") effects on some measured traits were found. For example, total reproductive output of the exposure was significantly increased in arsenic exposed worms compared to controls and cocoon viability was also significantly higher in cocoons produced in the 0.2 mg/kg imidacloprid, especially when these cocoons were left to hatch in control soil. This difference may have resulted from higher mortality of freshly hatched juveniles in the imidacloprid treated soil in comparison to untreated soil or possibly some other beneficial impact of control soil. Meanwhile, however, fewer cocoons were hatched as a result of the 0.2 mg/kg imidacloprid exposure, therefore the overall reproductive output was decreased.

For arsenic, exposure throughout the juvenile development period results in an apparently reduced toxicity compared to the results of other exposures of adult *Eisenia* earthworms to this metalloid. For example, Lock and Janssen (2002) found a 21 day EC50 for cocoon production of 10.8 mg/kg for adult *E. fetida*. In contrast, in the current study, the added arsenic concentration of 12 mg/kg used did not result in a negative effect on reproduction. Indeed, juvenile production was significantly higher in this treatment compared to controls. Arsenic exposure also had a small, but significant, positive effect on

average *Eisenia sp.* weight after 4 weeks of exposure. The lower apparent toxicity of arsenic found here may reflect differences in bioavailability between the natural soil used in this study and the artificial soil used by Lock and Janssen, 2002. However, it is also possible that the extended exposure time may allow for the earthworms to acclimatise, so that the negative effects of short-term exposure are alleviated through phenotypically plastic response to arsenic exposure.

The small positive effects of arsenic on reproduction and growth parameters may imply a potentially positive impact of exposure to this concentration on population growth rate and as a result resilience. However, although arsenic exposure does have a positive effect on growth, a larger body may not necessarily lead to a greater likelihood of survival. Predators, particularly larger vertebrate predators, and pathogens can select for larger prey, which can potentially reduce the likelihood of survival in larger individuals (Nylin and Gotthard, 2002). It has been suggested that in non-seasonal environments a faster development is generally beneficial (Nylin and Gotthard, 2002), however, a faster growth rate can result in poorer survival and reduced juvenile viability as well as an increase in susceptibility to bacteria and viruses (Nylin and Gotthard, 2002). These findings of the impact of arsenic need to be further examined in order to establish if similar effects would be observed in wild populations and if other earthworm species may be affected in the same way in response to life-time exposure.

In contrast to arsenic, exposure to the other two toxicants resulted in mostly detrimental effects. Cadmium exposure over the full developmental period resulted in significantly reduced growth compared to controls for the first three months. This was confirmed through a significantly effect of cadmium exposure on growth rate from the fitted logistic models. By the end of the 16 week exposure period, the cadmium exposed group had reached a similar weight to the controls. Observations of an effect of cadmium on growth rate during juvenile exposure are consistent with previously reported results. For example, in an experiment that exposed *E .fetida* to soils polluted with a mixture of metals, including cadmium, zinc, arsenic and lead, Spurgeon and Hopkin (1996) found reduced growth rates leading to an increase in the length of the early linear stage of growth compared to a clean control soil. Within this study, exposure to the metal polluted soil for 5 and 8 weeks resulted in a lower body mass than controls. However, over an extended time period, the metal exposed individuals “caught-up” with the control worms, eventually reaching a similar final body size. Van Gestel et al. (1993) exposed *E. andrei* juveniles to Cd throughout their developmental stage. The exposure reduced the size of juveniles at 4 weeks from the 10 mg/kg concentration onwards, but at 10 and 32 mg/kg body sizes of the exposed earthworms recovered after 6 and 12 weeks, respectively, such that they were similar to

those of control individuals (van Gestel *et al.,* 1993). These results together suggest an effect of cadmium exposure on body during the early stages of growth, but no effect on the capacity of earthworm to achieve a similar final body size over the full developmental period.

Imidacloprid also significantly reduced measured body weights in the early stages of the exposure and fitted logistic growth rate of the exposed *Eisenia sp.,* although, as for cadmium, after 16 weeks of exposure weights of the treated earthworms were similar to those of the controls. Consistent with expectations, exposure to 0.2 mg/kg imidacloprid resulted in a smaller effect on body weights over a more limited time-scale than exposure to the 1 mg/kg. Contrary to expectation, however, exposure to the lower concentration of the insecticide had a significant impact on both reproduction criteria, while the higher concentration did not result in a significant effect on reproductive traits. The non-monotonic nature of the reproductive response to imidacloprid exposure is not easily explained by current ecotoxicological theory. It is possible that this chemical may cause specific changes in energy budget allocation that divert resources to growth above reproduction at the lower tested concentration, but to reproduction above growth in the higher exposure treatment. Energy allocation may also explain the finding that, while 0.2 mg/kg imidacloprid caused an overall significant reduction in juvenile numbers, it had a positive impact on cocoon viability. Therefore, the earthworms allocated their energy budget to fewer, but more viable cocoons (Jager and Klok, 2010). In an experiment that exposed earthworms to a soil polluted with a mixture of metals, Spurgeon and Hopkin (1996) found a significant increase in *E. fetida* cocoon size in the most highly polluted soil. Reproduction rate in this soil was also lower. This suggests that the reduction in cocoon production may lead to an increase in the energy available to invest in each cocoon, leading to fewer, but larger, cocoons (Spurgeon and Hopkin 1996). In the current study, the size of the cocoons was not measured, however, the lower number of cocoons, but better hatchability at 0.2 mg/kg imidacloprid may indicate a greater amount of resource availability to support successful hatching in cocoons from the exposed earthworms.

As a neonicotinoid, imidacloprid is likely to interact with the post synaptic nicotinic receptors in earthworm nervous systems (Liu *et al.*, 2005). Within the *E. fetida* genome, there is an enhanced compliment of nicotinic receptors compared to, for example, insect species such as *Drosophila melanogaster* (S. Short, Pers. Com.). The presence of a range of potential receptor constructs with different sub-unit and structural confirmations in earthworms has the potential to cause complex response patterns following exposure to this insecticide. It has been previously reported to produce complex responses in other invertebrate species, for instance, induction of hormesis in the green peach aphid (Ayyanath *et al.*, 2013) and abnormal foraging behaviour in honey bees (Yang *et al.*, 2008),

which resulted from exposures to low concentrations of imidacloprid. The exact mechanisms of the variety of effects by this chemical are unknown in earthworms. Other species of earthworms are more sensitive than *E. fetida*. For example, reproduction in *Amynthas gracilis* is 10-fold more sensitive to imidacloprid exposure than *E. fetida* (A. Robinson. Pers. Comm; Kreutzweiser et al., 2008), therefore the negative effects seen in this experiment are important as an indication of the toxicity of imidacloprid on other earthworm species at realistic exposure concentrations.

Within ecotoxicology experiments, it may be important to consider the genetic differences between treatment groups. In outbred populations, the genetic variability present within populations may result in an increase in the variance of the response of different individuals following exposure. This may be particularly important when working with *E. andrei* and *E. fetida* earthworms as the lineages found in experimental stocks are often mixed and at times misidentified by the researchers (Aira *et al.*, 2016). It has been suggested previously that some of the reported variety in effects of pollutants on reproduction between different experiments may be due to the genetic variation within culture populations. Further, hybrid populations found in different labs may produce sterile offspring, which can result in affecting the result of the experiment if this hybrid mix is disproportionate between treatment groups (Aira *et al.*, 2016). Therefore, genotyping of individuals used in this experiment was conducted. Both *Eisenia sp*. lineages were found in the stock culture used. Pairs from all four mixed lineage families (i.e. pairs with one *E. fetida* and one *E. andrei*) produced fertile offspring and their overall reproductive output was not significantly reduced compared to the pure *E. andrei* or *E. fetida* lineage pairs. Where a difference between the two lineages was present, mixed lineage pair offspring generally showed a trait response between those of pairs from the two pure lineages, indicating and intermediate phenotype.

Due to the possibility of self-fertilisation, it is not clear if the two *Eisenia sp.* lineages truly interbred during this experiment. More infertile pairs were observed in the mixed lineage group than for pairs comprising of two *E. andrei* or two *E. fetida*. It is possible that the greater occurrence of infertility among the mixed lineage pairs may result from the greater degree of genetic difference, which in some cases prevents successful fertilisation and embryo development. The two lineages have been shown to interbreed and produce sterile offspring in a recent study (Plytycz *et al.*, 2018), therefore, this potential genetic incompatibility between earthworms from within the two lineages is certainly a possibility.

The genetic relatedness of the two Eisenia lineages has been studied extensively. *E. fetida* and *E. andrei*

have been separated as two species by mitochondrial COI DNA barcoding (Aira *et al.*, 2016; Pérez-Losada *et al.*, 2005), by the 28S nuclear marker (Pérez-Losada *et al.*, 2005), by metabolic profiling (Bundy *et al.*, 2002) and by electrophoresis of esterases (Oien and Stenersen, 1984) (n.b. in the latter experiment *E. andrei* is referred to as *E. unicolor*). Mixed pairs of the two species have been found to produce both viable and inviable cocoons. The results vary between studies, with more evidence indicating two, reproductively isolated, species (summarised by (Domínguez *et al.*, 2005a). Aira et al (2016) found that offspring from mixed parents (one *E. andrei* and *E. fetida*) were infertile and retained visual clues of both parental origin – they were dark red with slight yellow intersegmental stripes. In this study, *E. andrei* and *E. fetida* were collected from 28 international laboratories. Only 17 of these provided completely accurate identifications of their stocks. All the individuals originally identified as *E. andrei* were assigned to the same lineage, while only half of the worms originally identified as *E. fetida* were assigned to the that lineage based on the genetic marker analysis.

This experiment of Aira et al. (2016) was conducted using mitochondrial COI as a barcoding gene. Analysis for this marker indicated that *E. fetida* from different laboratories could be separated into two distinct cryptic lineages (representing possible species) that could not distinguish from one another by visual clues. Huang et al also found a noticeable divergence between *E. fetida* individuals from different parts of China using the COI marker (Huang *et al.*, 2007). These observations are consistent with the current study where the different clades of *E. fetida* were found to be a lot less genetically similar than the clades of *E. andrei*. In the current study, some slight differences in the measured phenotypic traits were altered by both the lineage and family background, but no differences in effects of the toxicant exposures due to the genetic background were detected.

These findings point to a complex relationship between genotype and phenotype which may compromise the potential to ascribe the lineages based on the most commonly used morphological phenotype of banded versus non-banded colouration. This underlies the importance of genotyping the stocks used for experiments.

2.5. Conclusion

In this experiment, exposure to 12 mg/kg As soil resulted in an increase in body size at an early stage of development and an increase in the reproductive output of *Eisenia sp.* indicating a hormetic effect of this metalloid. In contrast, Cd and imidacloprid showed detrimental effects on both growth and reproductive parameters. A significant effect of cadmium exposure on the growth and development of earthworms following extended exposure has also been identified in previous work (Spurgeon

and Hopkin (1996)). The two different concentrations of imidacloprid used, 0.2 mg/kg and 1 mg/kg per dry weight soil, showed an interesting difference in effect. The higher concentration had a more detrimental effect on the growth of the earthworms, while the lower concentration had a lesser impact on growth but a greater effect on overall reproductive output (while producing on average more juveniles per cocoon than the other treatments and controls). This may be a result of differences in energy allocation depending on the scale of toxicant exposure. These effects of toxicants were not influenced by genetic background, neither at a lineage nor a family level. However, genetic background did have an overall impact on the growth and reproductive parameters. This suggests it is important to consider the possible effects of genetic background in earthworms within future toxicity experiments. Interestingly, pairs with mixed parents from the *E. andrei* and *E. fetida* lineages produced viable offspring and the reproductive parameters of these were on average similar to single lineage pairs. However, as self-fertilisation is a possibility, this does not conclusively prove that the two lineages were successfully interbreeding in this experiment.

Chapter 3. Accumulative multigenerational and transgenerational effects of cadmium, arsenic and imidacloprid on phenotypes of *E. fetida* and *E. andrei*.

3.1. Introduction

Toxic chemical pollution can have a strong impact on organisms, reducing fitness through effects on mortality, growth and reproduction (Cortet *et al.*, 1999). Chemical pollutants that are released from a continuously emitting pollution source or that are slow to degrade can be present in the environment for long periods of time (Mulligan et al., 2001). As a result of this long-term presence, affected species are often exposed at time scales that may extend beyond the lifetime of any single individual. In some cases, organisms that are exposed for one or more generations can have offspring that later find themselves removed to a clean environment because of chemical degradation, water runoff or migration from hotspots of contamination to less polluted sites within heterogeneously polluted landscapes (Mulligan et al., 2001; Clostre et al., 2014; Sarkar et al., 2001). Given the different potential generational patterns of long-term exposure, multigenerational and transgenerational exposure studies are necessary in order to assess the effects of persistent and long-term pollution on the natural environment.

There is a growing body of research on the effects of pollution on environmentally important species that is specifically designed to assess long-term and transgenerational exposure impacts (Head et al., 2012; Vandegehuchte and Janssen, 2011). Research has shown, for example, that in a two generation exposure, the pesticide chlorantraniliprole causes exposed *Plutella xylostella* (diamondback moth) offspring to have slower development and lower fecundity than the offspring of unexposed individuals (Guo *et al.*, 2013). Prolonged exposure to low levels of traffic related fine particulate matter and environmentally relevant concentrations of cadmium, copper, lead and zinc have all been observed to cause detrimental effects on lifespan, growth and locomotive ability of offspring of exposed *C. elegans*nematodes (Yu et al. 2013, Zhao et al. 2014). *In a study of the springtail Folsomia candida, exposure to imidacloprid at concentrate ions as low as 0.29 mg/kg per dry weight soil resulted in a consistent detrimental effect on survival and reproduction sustained over three generations (van Gestel et al., 2017).* While most of these effects are clearly detrimental to the affected organisms and to

subsequent generations, in some cases the nature of the effects of long-term multi-generational exposure can be more complex. For example, continuous exposure to low doses of imidacloprid has been observed to cause hormesis for multiple generations of *Myzus persicae* (green peach aphids), resulting in an overall increase in population numbers (Ayyanath *et al.*, 2013). In another study, the insect *Spodoptera exigua* showed significantly lower survival after 1 generation of exposure to 44 mg/kg per dry weight food of cadmium (an environmentally relevant concentration for highly metal contaminated sites), but appeared to develop resistance after 33 generations of exposure by which time survival rates in exposed individuals were similar to controls (Kafel et al., 2012).

Earthworms are a crucial species group for soil fertility maintenance (Edwards and Bohlen, 1996), as well as an established sentinel organism in ecotoxicological research (Spurgeon and Hopkin, 1996; Schnug et al., 2013). Earthworm species have comparatively long developmental and generational time when compared to the other invertebrate species commonly used in experiments, such as the nematode *C. elegans* and *Daphnia* sp. The species used in this experiment, *E. andrei* and *E. fetida*, attain sexual maturity in 40 to 60 days, with a full generation time (from a cocoon being hatched to a fully mature adult) being between 63 and 83 days (Venter and Reinecke, 2015) in comparison to 5-10 days long generation time for *Daphnia pulex* (Colbourne *et al.*, 2011) and a 3-4 days long generation time for *C. elegans* (Muschiol *et al.*, 2009), therefore exploring *E. andrei* and *E. fetida* provides an opportunity to observe the mechanisms used by a more long lived species in experiments that are viable in a laboratory setting. However, partly as a result of longer generation time, little is known about the transgenerational and multigenerational impacts of pollution on this group of earthworms. For regulatory purposes, most tests of toxic exposure in earthworms are performed over no longer than a few weeks and do not look at more than one generation at a time (OECD 2014). This does not give a realistic picture of the potential effects pollution may have over a prolonged exposure time. Therefore, I set out to perform an experiment which explored realistic levels of pollution exposure over multiple generations of the commonly used model earthworm, *Eisenia*. I carried out three generation long exposures to arsenic (As) at 12 mg/kg, cadmium (Cd) at 40 mg/kg and imidacloprid at 0.2 and 1 mg/kg, measuring their growth and reproduction in order to assess if the following hypotheses are correct:

- A multigenerational exposure produces an accumulative effect compared to a single generation long exposure;
- The exposure of a parent will cause parental/maternal and transgenerational effects in its offspring;

- Different lineage/family background will have an effect on the degree to which long-term chemical exposure affects measured trait parameters.

3.2. Materials and Methods

Soil preparation, earthworm rearing and growth and reproduction measurements were carried out the same way as described in Chapter 2 section 2.2.

3.2.1. Experimental plan

Briefly, earthworms of the species *E. andrei* and *E. fetida* were exposed to 40 mg/kg cadmium (Cd), 12 mg/kg arsenic (As) and 0.2 and 1 mg/kg imidacloprid as well as grown in an unspiked control soil for one, two and three generations. After the first (F1) generation, half of the produced cocoons from the spiked soils were placed in control soil to produce recovery populations (F1:Exp|F2:Ctr in the F2 generation and F1:Exp|F2:Ctr|F3:Ctr in the F3 generation). The same was done with cocoons from the continuously exposed F2 groups to produce the F1:Exp|F2:Exp|F3:Ctr recovery group in the F3 generation. Throughout the experiment, individual earthworm weight was measured after every 4 weeks of exposure for 16 weeks. Number of offspring produced in a six week reproduction test and number of juveniles produced per each cocoon hatched (as described in section 2.2.4) were measured in the true control (i.e. continuously unexposed for one or multiple generations), exposed (i.e. continuously exposed for one or multiple generations) and recovery (i.e. exposure for one or two generations followed by maintenance in uncontaminated soil for two or one generation, respectively*)*. The overall experimental scheme for the multigenerational exposure indicating the exposure schedule for the different unexposed generations is shown in Figure 3.1.

The total numbers of individuals in each experimental stage can be seen in Tables 2.1 and 2.2

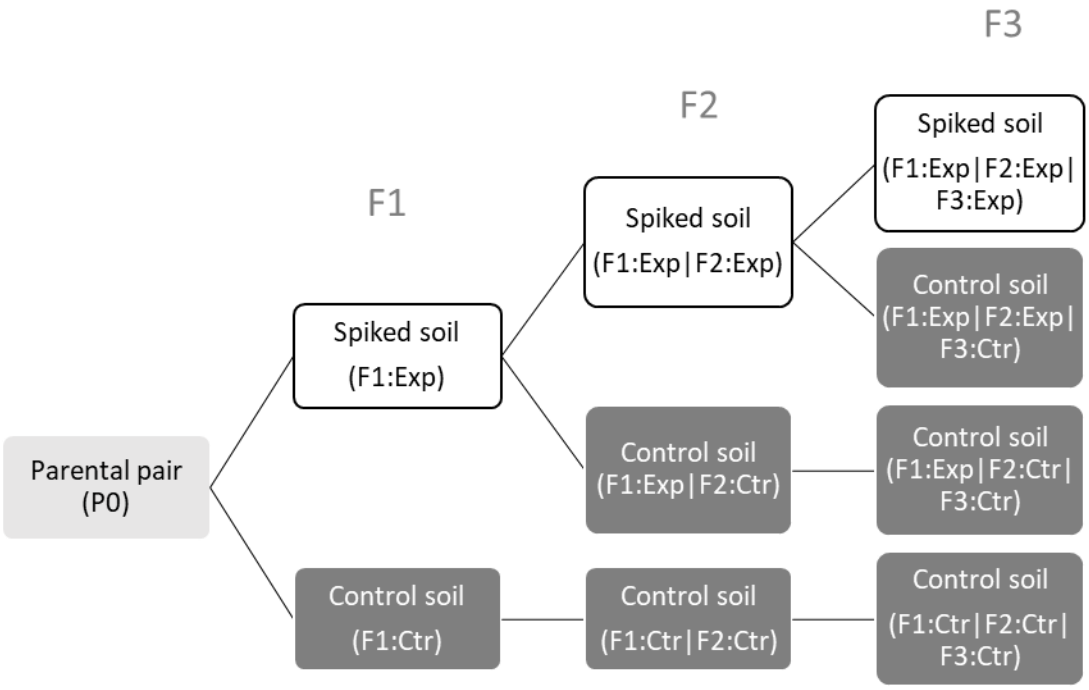


Figure 3.1. Experimental design for the transgenerational experiment on *E. andrei* and *E. fetida* earthworms. Four types of spiked soil were used: 40 mg/kg Cd, 12 mg/kg As and 0.1 and 1 mg/kg imidacloprid, there were 14 parental P0 pairs. The boxes include the soil in which the individuals were placed in and the corresponding exposure group name. Exp – Experimentally spiked soil; Ctr – Control soil.

The details of the results of the exposure experiment for F1 generation are given in detail in Chapter 2. The progeny that were produced from these continuously exposed and unexposed (control) F1 generation individuals for each of the four chemical treatments (arsenic at 12 mg/kg, cadmium at 40 mg/kg and imidacloprid at 0.2 and 1 mg/kg) were used for the F2 generation measurements. Within the F1 generation, individual growth rate was monitored for the first 16 weeks. After exposure, the earthworms were placed in a fresh container of soil for 6 weeks in order to measure reproduction (as described in section 2.2.4). For the F1 control groups, the cocoons produced during the reproduction test were used to establish the F2 control group. From the F1 exposed groups, half of cocoons produced during the reproduction test were placed in the same exposure soil in which the F1 parent were reared, thereby providing the continuous exposed F2 population. The other half of the cocoons produced were transferred to a control soil. The juveniles from the latter population were then raised as the F2 recovery group. After rearing of these F2 groups to adulthood with monthly growth measurements, a second reproduction test was conducted from which the earthworms to produce the tested F3 generations were obtained. For the cohort that were either reared continuously in control soil or had been transferred from the exposed soil to the control soil for the F2 generation, all cocoons were transferred to control soil to give the F3 control and F1:Exp|F2:Ctr|F3:Ctr groups. Cocoons from the F2 exposed groups were then either raised in spiked soil to produce an F3 exposed

population (F1:Exp|F2:Exp|F3:Exp) or placed into control soil to produce the second F3 recovery population (F1:Exp|F2:Exp|F3:Ctr).

Throughout this rearing experiment, offspring from the same P0 pair set-up for the initial study of single generation effects were kept together, thereby producing inbred lines. These lines were named after the original numbering of the P0 pairs. The growth and maturation of the juveniles in each pair were monitored following the same approach as used for the F1 earthworms (Chapter 2 section 2.2).

3.2.2. Cocoon hatching measurements

The cocoons produced in spiked soil from exposed F1 and F2 individuals during the test for effects on reproduction conducted at the end of the growth period were placed in both spiked and control soils and hatching rates measured. The cocoons produced by the F3 exposed individuals were placed only in the appropriate spiked soil. Incubation in control soil was done in order to observe the maternal effects attributable to each chemical for juveniles, which were, thereafter, maintained in an unexposed environment. As juveniles were reared from cocoons that were transferred to unpolluted soils soon after they had been laid, the juveniles for the recovery groups were reared on clean soil (i.e. out of the polluted environment) as soon as feasible. The cocoons from the recovery populations were placed only in control soil.

The cocoons gathered from the six-week reproduction test were placed in freshly made soils supplemented with manure (0.5 g dry weight per cocoon). After six weeks, the juveniles in the soil were counted to obtain cocoon viability data for the full number of cocoons laid. The results of the hatching rates in both the spiked and the control soils are included in this chapter. The experimental scheme of reproduction tests can be seen in Figure 3.2 .

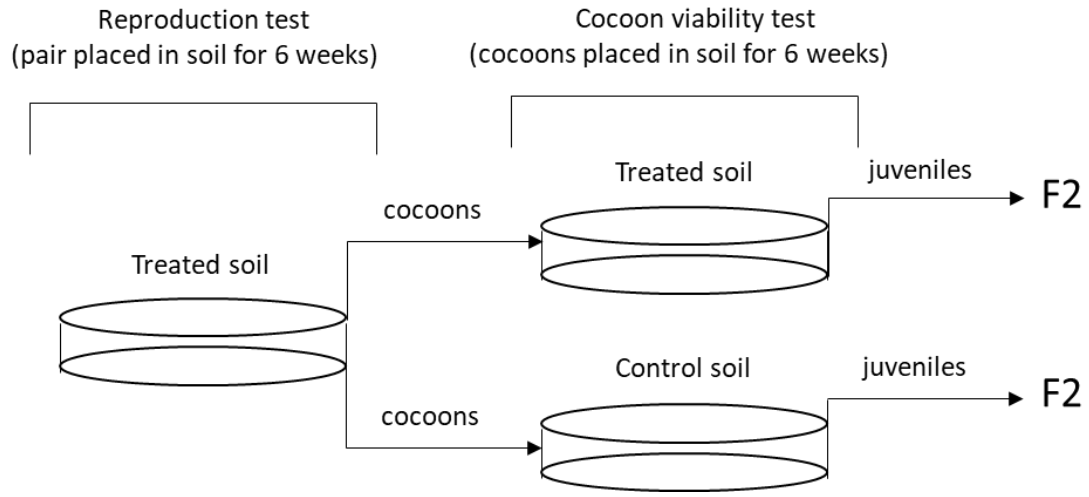


Figure 3.2. Experimental scheme of reproduction tests of the earthworms exposed to either arsenic, cadmium and imidacloprid. A max of 10 cocoons were placed in each cocoon viability test.

3.2.3. Data analysis

Data manipulation, presentation and statistical analysis were performed using Microsoft Office Excel and R statistical software. After testing for normality using the Shapiro-Wilks test it was established that more than half of the tested groups did not fit a normal data distribution. Therefore, Wilcoxon and Kruskal-Wallis tests were used for the establishment of statistically significant differences between the groups (the three mentioned tests are present in the base settings of R). The interactions between the different independent variables were analysed using a generalised additive model which was adjusted to best fit the data analysed (the inverse Gaussian data distribution best fit the reproductive data and the Poisson data distribution best fit the growth data). The lme4 package was used for this analysis (Bates *et al.*, 2014). For the survival comparisons and comparisons of whether a pair was viable the prop.test function in R was used. The gcfit function from the grofit R package (Kahm *et al.*, 2015) was used to establish the growth curve characteristics (maximum weight and maximum growth rate). As in the case of growth in the F1 generation described in Chapter 2. The type of model that best fit the available growth data was a logistic model, therefore, this was used for the comparisons.

3.3. Results

3.3.1. Survival

The survival rates amongst the control population were high at 96% for F1, 98% for F2 and 90% for F3 generation (Figure 3.3).

3.3.1.1. Parental, transgenerational and multigenerational effects of arsenic on mortality

The survival of the F2 generation of *Eisenia sp*. continuously exposed to arsenic was lower than that of the F1 individuals (84% compared to 94%, respectively), however, this difference was not statistically significant ($\chi 2$ = 3.152, p = 0.0759). Survival was significantly lower in the F1:As|F2:As group (84%) compared to the F2 control individuals (F1:Ctr|F2:Ctr) (98%, $\chi 2$ = 5.262, p <0.05). The F1:As|F2:Ctr earthworms also showed a significant reduction in survival rate in comparison to F2 controls (F1:Ctr|F2:Ctr) ($\chi 2$ = 4.682, p <0.05). The F1:As|F2:As and F1:As|F2:Ctr groups were similar with respect to mortality rates, the F1:As|F2:As group had a survival rate of 84% while the F1:As|F2:Ctr groups had a survival rate of 82% . Therefore, it seems likely the common reduction seen in the survival of both F2 populations reared from parents previously exposed to arsenic was established as a result of this parental exposure history. However, by the third generation, this effect of exposure history was not observed, as a 100% survival rate was found. The Survival rate of the two F3 recovery populations was also high at 96% and 97%. The F1:As|F2:As|F3:Ctr group showed no effect of parental arsenic exposure on the survival of subsequent offspring. Hence it seems that the adverse effects of paternal arsenic exposure on the F2 offspring survival represents a transient phenotype that affects the population for one generation after exposure and potentially result in the removal of the most sensitive individuals from the population, such that only more tolerant earthworms remained into the F3 generation. This paternal impact possibly occurs during the development of the F1 individuals, affecting gonad development which then results in more sensitive offspring.

3.3.1.2. Parental, transgenerational and multigenerational effects of cadmium on mortality

The survival amongst the Cd continuously exposed earthworms and the Cd recovery populations remained high throughout all of the three generations. The values ranged from 91% survival in the F1 cadmium exposed group to 100% survival in the F3 continuously exposed and the F1:Cd|F2:Cd|F3:Ctr groups (See Figure 3.3). No consistent or significant treatment or generation related effects of Cd exposure on survival rates were found.

3.3.1.3. Parental, transgenerational and multigenerational effects of imidacloprid on mortality

The survival rates in the *Eisenia sp.* raised in 0.2 mg/kg (per dry weight soil) imidacloprid exposure also remained high throughout the experiment. The continuous exposure survival rates ranged from 98% in F1 and 89% in F3, while within the recovery populations the lowest survival rate was 94% in the F1:Imd|F2:Imd|F3:Ctr and the highest was 100% in the F1:Imd|F2:Ctr|F3:Ctr group.

The 1 mg/kg imidacloprid exposure resulted in a significantly lower mortality in the F3 continuously exposed group. At 70% survival rate for the population, it was the lowest observed within any treatment generation group across all of the three chemicals tested ($\chi 2 = 4.49$, $p < 0.05$ compared to F3 controls). No parental or transgenerational effects of imidacloprid on earthworm survival were observed through observation of significant effects within any of the recovery generations. This indicates that parental exposure to imidacloprid at this concentration does not have an impact on earthworm survival, but perhaps a continuous exposure results in sensitising the organisms to the chemical's detrimental effects on survival in exposures that extend to multiple generations.

3.3.1.4. Effects of genetic background on mortality

Limited differences in survival between the control earthworms belonging to different lineage backgrounds within either of the generations were found. The survival rates within the F1 controls varied from 96% for *E. fetida* to 98% for *E. andrei* to 100% for the group from a mixed background. All three groups from the different lineage backgrounds within the F2 generation, as well as all mixed lineage generation groups within the control treatment showed 100% survival. When comparing different generation groups within lineages, the F3 control *E. andrei* individuals showed a significantly lower level of survival compared to F2 individuals (86% compared to 100%, $\chi 2 = 4.70$, $p < 0.05$). Survival in the F3 population, however, at 86% was still high, hence, while significant, the effects size on survival was comparatively small and did not significantly differ from mixed background individuals ($\chi 2 = 0.411$, $p > 0.05$). The F1 and F2 *E. fetida* earthworms showed similar levels of survival (96% and 100%, respectively). The survival rate of F3 control *E. fetida* was only 67%. However, the number of individuals in this group was too small to allow a reliable comparison with survival for either of the two other tested generations.

Survival rates amongst the families in control treatments also did not show any obvious trends. Within the F1 generation, 11 out of 13 families had a 100% survival rate, with the lowest rate being 75% for Family 25 (*E. andrei*). However, this family only had 4 individuals in this generation, therefore, n was too small to allow robust comparison and differences can be accounted for beyond an idiosyncratic effect. Similar results were found for the other two generations, where the individuals family size

prohibited the establishment of statistical significance. It is possible that with larger sample sizes inbred families may show significantly different survival rates, but it was not possible to elucidate this during the current experiment. Similarly, due to the small representation of each family within each of the treatment groups and the low mortality rates overall, no significant effect of family background on survival differences between treatment groups could be established.

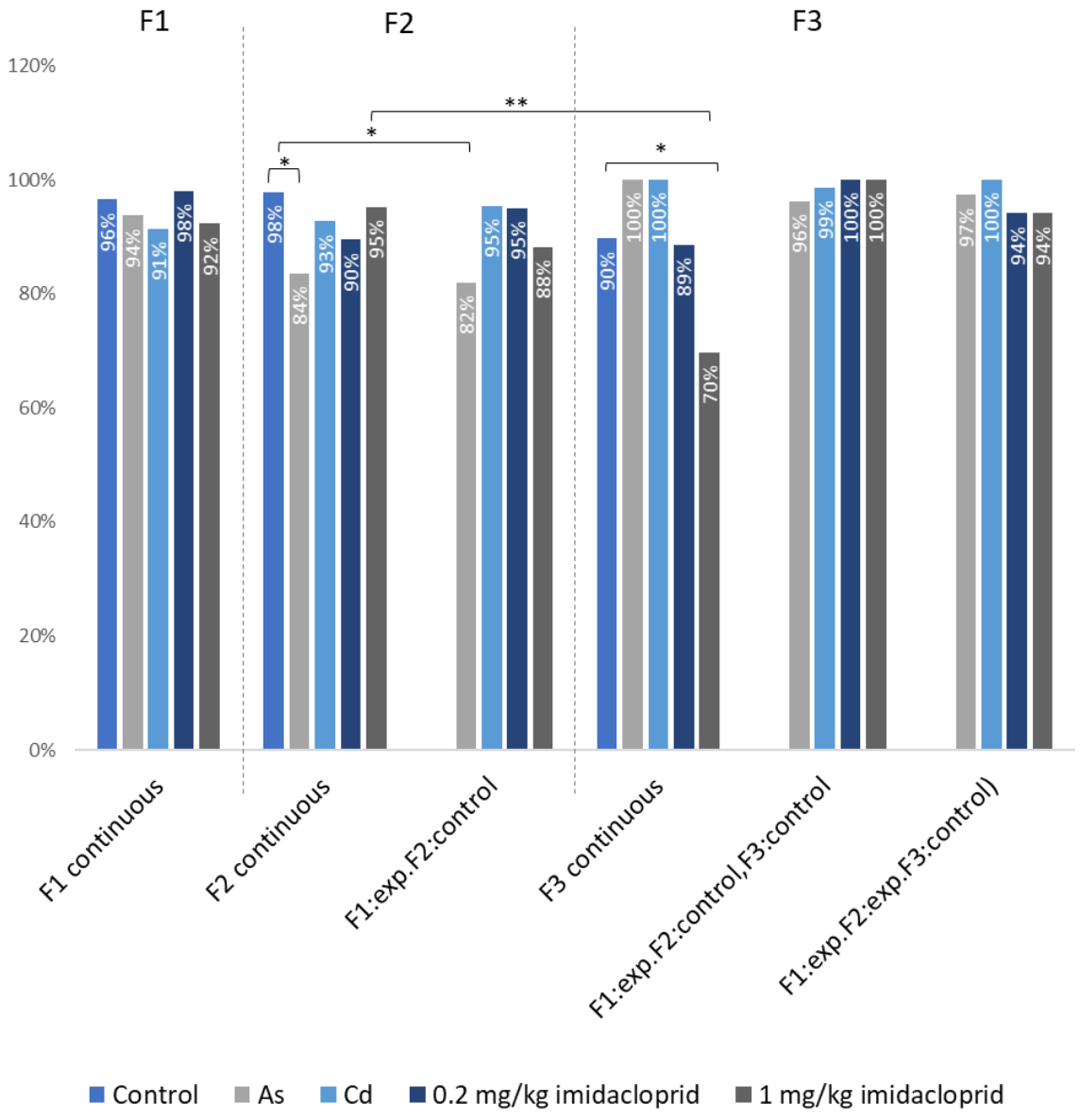


Figure 3.3 .Survival (percentage) of the exposed *E. andrei* and *E.fetida* individuals over three successive generations incubated in unspiked control soil (mid blue) and following exposures to 12 mg/kg (per dry weight soil) arsenic (As)(light grey), 40 mg/kg cadmium (Cd) (light blue) and 0.2 mg/kg (dark blue) and 1 mg/kg (dark grey) imidacloprid in continuous exposure. After the F1 and F2 generations half of the produced cocoons from exposed individuals were placed into control soil for recovery groups and their reproduction parameters were measured (these were F1:exp|F2:Ctr, F1:Exp|F2:Ctr|F3:Ctr and F1:Exp|F2:Exp|F3:Ctr). The statistical significance values: * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$.

3.3.2. Growth

Growth parameters of all earthworms reared in the control soil showed no significant differences between the tested generations ($\chi 2 < 3.65$, $p > 0.05$).

3.3.2.1. Parental, transgenerational and multigenerational effects of arsenic on growth

In the F1 generation, earthworms exposed to 12 mg/kg arsenic were significantly heavier than the control group at 4 weeks of age (Table 2.2). Thereafter, however, The F1 control and F1 As exposed populations showed similar growth, with the weights of the arsenic exposed *Eisenia* not significantly different ($\chi 2 < 0.87$, $p > 0.05$) from the control population at the other growth measurement points. During the F2 generation, the earthworms continuously exposed to arsenic were smaller than the individuals kept continuously on control soil from 8 weeks onwards. The As exposed individuals also showed a significantly lower maximum growth rate compared to the control population (Table 3.1). Comparing across generations, the F1:As|F2:As growth weights measured after 8 and 12 weeks were significantly lower than weights at the same measurement times in the F1 exposed generation (8 weeks: $\chi 2 = 7.24$, $p < 0.01$ and 12 weeks: $\chi 2 = 12.4$, $p < 0.001$). This suggests an accumulative effect on growth of arsenic exposure for individuals experiencing multigenerational exposure. The trend for increased effects in individuals exposed over multiple generations, however, did not continue when successively reared individuals were further exposed into the F3 generation. Conversely, both the continuously exposed (F1:As|F2:As|F3:As) and one of the recovery treatment populations (F1:Exp|F2:Ctr|F3:Ctr) were significantly heavier than the F3 Control group at 16 weeks of age (Table 3.1). The F2 recovery group (F1:As|F2:Ctr) showed no impacts, in terms of growth, of the exposure in the previous generation and had similar measurements to F2 control *Eisenia sp.,* as did the F1:As|F2:As|F3:Ctr group. Therefore, no maternal effects could be seen for this parameter either after one or two generations of exposure to 12 mg/kg As.

Table 3.1. Effects of arsenic on *E. andrei* and *E.fetida* F2 and F3 earthworm growth.							
Soil treatment	Average weights (g) at five growth stages					Max growth rate (g/month)	Max weight (g)
	0 week	4 weeks	8 weeks	12 weeks	16 weeks		
F1 Ctr| F2 Ctr	0.0247± 0.0270	0.0803± 0.0668	0.202± 0.109	0.303± 0.113	0.339± 0.116	0.197± 0.0954	0.476± 0.599
F1:As| F2:As	0.0342± 0.0338	0.0928± 0.0662	0.187± 0.0918	0.258± 0.102 **a	0.308± 0.113	0.112± 0.0445 ***a	0.364± 0.134
F1:As| F2:Ctr	0.0304± 0.0261	0.0846± 0.0517	0.214± 0.0867	0.290± 0.106	0.342± 0.0805	0.149± 0.0349 **b	0.459± 0.145 **b
F1 Ctr| F2:Ctr| F3:Ctr	0.026± 0.0213	0.0782± 0.0475	0.217± 0.0933	0.305± 0.113	0.317± 0.127	0.172± 0.0686	0.345± 0.0855
F1:As, F2:As, F3:As	0.0308± 0.0312	0.0829± 0.0512	0.197± 0.0877	0.292± 0.106	0.358± 0.118 *a	0.134± 0.033	0.391± 0.0801
F1:As| F2:Ctr| F3:Ctr	0.0278± 0.0331	0.0726± 0.0312	0.202± 0.0538	0.295± 0.0807	0.365± 0.105 *a	0.149± 0.0453	0.439± 0.201 *a
F1:As| F2:As| F3:Ctr	0.0178± 0.0138	0.0709± 0.0420	0.192± 0.0727	0.268± 0.101	0.324± 0.119	0.141± 0.0302	0.355± 0.105
Each value (mean ±SD) is calculated from a minimum of 15 replicates with two individuals per replicate. The statistical significance values. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$, a – in comparison to control, b- in comparison to continuously exposed individuals.							

3.3.2.2. Parental, transgenerational and multigenerational effects of cadmium on growth

The F1 cadmium exposed earthworms had significantly reduced growth indicated by lower weights compared to control earthworms at the growth stages of 4, 8 and 12 weeks and a lower maximum growth rate as determined from fitted logistic growth models (section 2.3.4.3). For The F2 continuously exposed group (F1:Cd|F2:Cd), there was no significant effect ($\chi 2$ < 3.44, p >0.05) of cadmium exposure on growth rate when compared to the F2 control (F1:Ctr|F2:Ctr) population (Table 3.2). This suggests that, contrary to our initial first hypothesis, the effects of exposure to Cd over multiple generations does not result in an increase in the severity of effect, but rather that effects are reduced suggesting the development of some form of adaptive tolerance. This trend for a reduced effect of Cd exposure compared to control earthworms was also seen when multi-generational exposure was extended to F3 (F1:Cd|F2:Cd|F3:Cd). For these exposed earthworms, growth was not adversely affected by Cd. Instead, the F3 continuously exposed individuals growth weights were significantly higher compared to those found for the F1:Cd populations at 4, 8 and 12 weeks ($\chi 2$ = 6.48, p <0.05, $\chi 2$ = 11.69, p <0.001 and $\chi 2$ = 5.76, p <0.05, respectively) indicating a significant positive development of phenotypic tolerance over the three generation exposure.

The F1:Cd|F2:Ctr recovery population was not significantly different to the F2 controls at any stage of growth (Table 3.2). However, at 8 and 12 weeks they were significantly larger than the F1:Cd|F2:Cd group (8 weeks: $\chi 2$ = 7.42, p <0.01, 12 weeks: $\chi 2$ = 4.67 , p <0.05) (although this was not the case for the comparison between the F1:Cd|F2:Cd and the F2 control (F1:Ctr|F2:Ctr) group (Table 3.2). This suggest a mild maternal effect causing an increase in growth due to parental exposure. Support for the presence of a maternal effect was strengthened by the fact that the F1:Cd|F2:Cd|F3:Ctr group was significantly larger than F3 controls (F1:Ctr|F2:Ctr|F3:Ctr) at the first three growth stages (0 weeks:$\chi 2$ = 3.99, p <0.05, 4 weeks: $\chi 2$ = 9.1, p <0.01, 8 weeks: $\chi 2$ = 6.85 , p <0.01) (Table 3.2). This maternal effect may perhaps have something to do with a disrupted energy budget allocation that may have arisen due to a stress response in the exposed F2 parents. This effect was not carried over in a transgenerational manner, however, as the F1:Cd|F2:Ctr|F3:Ctr group showed similar growth ($\chi 2$ < 1.88, p >0.05 in all cases) to the F3 controls in all growth stages apart from 16 weeks.

Table 3.2. Effects of cadmium on *E. andrei* and *E. fetida* F2 and F3 earthworm growth.							
Soil treatment	Average weights (g) at five growth stages					Max growth rate (g/4 weeks)	Max weight (g)
	0 week	4 weeks	8 weeks	12 weeks	16 weeks		
F1:Ctr| F2:Ctr	0.0247± 0.0270	0.0803± 0.0668	0.202± 0.109	0.303± 0.113	0.339± 0.116	0.197± 0.0954	0.476± 0.599
F1:Cd| F2:Cd	0.0268± 0.0288	0.0685± 0.0486	0.148± 0.0966	0.272± 0.109	0.319± 0.108	0.167± 0.0647	0.383± 0.124
F1:Cd| F2:Ctr	0.0306± 0.0363	0.103± 0.0734	0.242± 0.138 **b	0.344± 0.150 *b	0.367± 0.128	0.173± 0.0495	0.441± 0.128
F1:Ctr| F2:Ctr| F3:Ctr	0.0267± 0.0213	0.0782± 0.0475	0.217± 0.0933	0.305± 0.113	0.317± 0.127	0.172± 0.0686	0.345± 0.0855
F1:Cd| F2:Cd| F3:Cd	0.0305± 0.368	0.0799± 0.0566	0.199± 0.106	0.319± 0.134	0.374± 0.144 **a	0.148± 0.0394	0.417± 0.116 *a
F1:Cd| F2:Ctr| F3:Ctr	0.0247± 0.0233	0.0799± 0.0534	0.232± 0.0932	0.322± 0.122	0.362± 0.131	0.175± 0.0482	0.393± 0.0943 *a
F1:Cd| F2:Cd| F3:Ctr	0.0453± 0.0348 *a *c	0.132± 0.0629 **a **b **c	0.279± 0.0904 **a **b *c	0.325± 0.0960	0.339± 0.110	0.182± 0.0590	0.354± 0.0854
Each value (mean ±SD) is calculated from a minimum of 15 replicates with two individuals per replicate. The statistical significance. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$, a – in comparison to same generation control, b- in comparison to same generation continuously exposed individuals, c – in comparison to the F1:Exp|F2:Ctr|F3:Ctr group.							

3.3.2.3. Parental, transgenerational and multigenerational effects of imidacloprid on growth

Continuous 0.2 mg/kg imidacloprid exposure caused a significant reduction in growth in the F1:Imd$^{0.2}$ population (see Chapter 2; Table 2.2). The F2 continuously exposed population (F1:Imd$^{0.2}$|F2:Imd$^{0.2}$) was smaller than the F2 control group at all growth stages, although this difference was not statistically significant ($\chi 2$ < 1.99, p >0.05 in all cases). The F1:Imd$^{0.2}$|F2:Imd$^{0.2}$|F3:Imd$^{0.2}$ population did show a significant reduction in weights compared to the F3 control group (F1:Ctr|F2:Ctr|F3:Ctr) at 4, 8 and 12 weeks ($\chi 2$ = 6.74, p <0.01, $\chi 2$ = 17.1, p <0.001, $\chi 2$ =15.3 , p <0.001, respectively) (Table 3.4). This shows a continuous detrimental impact of this concentration of imidacloprid on the growth of *Eisenia sp.* earthworms.

Continuous exposure to 1 mg/kg imidacloprid caused a significant reduction in growth weights compared to the appropriate controls during both F1 and F2 generations (Table 2.2; Table 3.3). However, after an exposure for three generations, these effects were partially alleviated. Thus, the F1:Imd^{1}|F2:Imd^{1}|F3:Imd^{1} group was only significantly smaller than the F3 controls at 8 weeks ($\chi 2$ = 11.33 , p <0.001), while the F2 continuous exposures (F1:Imd^{1}|F2:Imd^{1}) resulted in reduced growth weights in all measurement time except for the final 16 week time point (0 weeks: $\chi 2$ = 5.97, p < 0.05, 4 weeks: $\chi 2$ = 14.8 , p <0.001, 8 weeks: $\chi 2$ = 8.56 , p < 0.01, 12 weeks: $\chi 2$ = 11.9, p <0.001).

The 0.2 mg/kg imidacloprid F2 recovery population was significantly heavier at 4, 8 and 12 weeks than either the continuously exposed or control groups ($\chi 2$ = 7.84, p <0.01, $\chi 2$ =7.97, p <0.01, $\chi 2$ = 7.57, p <0.01, for the three time points respectively compared to control). When continued onto the F3 generation (in F1:Imd$^{0.2}$ |F2:Ctr|F3:Ctr group), this effect was alleviated and the group showed similar growth patterns and weights to the F3 control population, therefore no transgenerational effects were observed. The F1:Imd$^{0.2}$ |F2:Imd$^{0.2}$ |F3:Ctr population mostly showed a similar pattern of growth to the F3 controls, but were significantly heavier than controls at the age of 16 weeks ($\chi 2$ = 4.4, p <0.05)(Table 3.3). Therefore, this type of maternally induced increase in size was more prominent after a single generation of exposure.

The F2 recovery population from the 1 mg/kg exposure were of similar sizes to F2 controls (Figure 3.3). This trend continued into the F3 generation, both the F1:Imd^{1}|F2:Ctr|F3:Ctr and the F1:Imd^{1}|F2:Imd^{1}|F3:Ctr groups showed similar growth weights to F3 controls. Therefore, no maternal or transgenerational effects on this parameter were observed for this exposure (Table 3.3; Table 3.4).

Table 3.3. Effects of imidacloprid on *E. andrei* and *E. fetida* F2 earthworm growth.							
Soil treatment (mg/kg)	Average weights (g) at five growth stages					Max growth rate (g/month)	Max weight (g)
	0 week	4 weeks	8 weeks	12 weeks	16 weeks		
F1:Ctr| F2:Ctr	0.0247± 0.0270	0.0803± 0.0668	0.202± 0.109	0.303± 0.113	0.339± 0.116	0.197± 0.0954	0.476± 0.599
F1:Imd$^{0.2}$| F2:Imd$^{0.2}$	0.0204± 0.0319	0.0687± 0.0820	0.158± 0.102	0.291± 0.109	0.324± 0.0958	0.149± 0.0517	0.339± 0.112
F1:Imd^{1}| F2:Imd^{1}	0.0104± 0.00991 **a	0.0306± 0.0254 ***a	0.120± 0.0702 ***a	0.225± 0.102 ***a	0.323± 0.101	0.156± 0.0464	0.384± 0.101
F1:Imd$^{0.2}$| F2:Ctr	0.0313± 0.0227 **b	0.108± 0.0523 **a **b	0.267± 0.0844 ***a ***b	0.345± 0.0748 **a **b	0.348± 0.0915 *b	0.179± 0.0373 *b	0.364± 0.0378
F1:Imd^{1}| F2:Ctr	0.0188± 0.0184	0.0767± 0.0461 ***b	0.217± 0.0953 **b	0.319± 0.120 *b	0.349± 0.0570	0.185± 0.0656	0.506± 0.267
Each value (mean ±SD) is calculated from a minimum of 15 replicates with two individuals per replicate. The statistical significance. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$, a – in comparison to control, b- in comparison to respective continuously exposed individuals.							

Table 3.4. Effects of imidacloprid on *E. andrei* and *E. fetida* F3 earthworm growth.							
Soil treatment (mg/kg)	Average weights (g) at five growth stages					Max growth rate (g/month)	Max weight (g)
	0 week	4 weeks	8 weeks	12 weeks	16 weeks		
F1:Ctr| F2:Ctr| F3:Ctr	0.0267± 0.0213	0.0782± 0.0475	0.217± 0.0933	0.305± 0.113	0.317± 0.127	0.172± 0.0686	0.345± 0.0855
F1:Imd$^{0.2}$| F2:Imd$^{0.2}$| F3:Imd$^{0.2}$	0.0240± 0.0237	0.0529± 0.0426 **a	0.114± 0.0795 ***a	0.193± 0.114 ***a	0.256± 0.122	0.117± 0.0581 *a	0.334± 0.135
F1:Imd^{1}| F2:Imd^{1}| F3:Imd^{1}	0.0244± 0.0184	0.0636± 0.0412	0.147± 0.0772 ***a	0.261± 0.121	0.295± 0.116	0.147± 0.128 *a	0.354± 0.134
F1:Imd$^{0.2}$| F2:Ctr| F3:Ctr	0.0307± 0.0273	0.0934± 0.0549 **b	0.217± 0.0659 ***b	0.318± 0.0916 ***b	0.360± 0.0932 ***b	0.149± 0.0472	0.392± 0.0802
F1:Imd^{1}| F2:Ctr| F3:Ctr	0.0173± 0.0162	0.0704± 0.0379	0.206± 0.0936 **b	0.333± 0.118 *b	0.371± 0.122 **a **b	0.182± 0.0603 **b	0.416± 0.0664 **a *b
F1:Imd$^{0.2}$| F2:Imd$^{0.2}$| F3:Ctr	0.0284± 0.0214	0.102± 0.0591 **b	0.274± 0.0967 ***b	0.333± 0.108 ***b	0.354± 0.112 *a **b	0.19± 0.0607 **b	0.377± 0.0842
F1:Imd^{1}| F2:Imd^{1}| F3:Ctr	0.0218± 0.0190	0.0719± 0.0503	0.189± 0.0937	0.313± 0.116	0.358± 0.142	0.147± 0.0423	0.398± 0.0891 *a
Each value (mean ±SD) is calculated from a minimum of 15 replicates with two individuals per replicate. The statistical significance. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$, a – in comparison to control, b- in comparison to continuously exposed individuals.							

3.3.2.4. Effects of genetic background on growth

Lineages had no significant effect on the growth patterns on time point weights for either F1, F2 or F3 exposed earthworms when comparing the control individuals within the same generation ($\chi 2$ <1.54, p >0.05 in all cases). The family background did have an impact on the F2 control earthworms at 4 and 8 weeks of age, it also impacted the *Eisenia sp.* F3 control worms at 0, 12 and 16 weeks of age.

When testing for interactions between the effects of the toxic treatments and the genetic background (lineage and family) on growth parameters, no significant interactions could be found (*t* value <1.1, *p* >0.05 in all cases). Therefore, contrary to our starting hypothesise, there was no evidence of different affected associated with toxicants exposure relating to either lineage or family genetic background.

3.3.3. Reproduction

Within the control group, the F2 population produced a significantly higher number of juveniles during the 6 week reproduction test than either the F1 controls or the F3 controls ($\chi 2$ = 4.32, p <0.05 and $\chi 2$ = 4.06, p <0.05, respectively) (Figure 3.4). The higher rates of juvenile production found in the second-generation control population was mainly attributable to a higher number of cocoons being laid during the test, rather than a greater cocoon viability or number of juveniles hatched per cocoon, which were closely comparable in the F2 and F1 generations (Figure 3.5). Interestingly, while the percentage of viable pairs reduced with each successive generation, from 79% in the F1, to 47% in the F3 and the cocoon viability in the F3 generation was also reduced compared to the F1s, the average number of offspring produced per individual was actually slightly higher in the F3 compared to F1 generation (3.00 for F1 and 3.49 for F3). Thus, the F3 pairs that did produce offspring on average produced more juveniles per pair, primarily through high cocoon production, rather than increased viability. The declines in fecundity among pairs suggests a possible effect of inbreeding on reproductive phenotypes including potential incompatibility in some lines, but also a greater reproductive capacity for others that warrants further investigation.

3.3.3.1. Parental, transgenerational and multigenerational effects of arsenic on reproduction

The F1:As earthworms produced significantly more offspring during the 6 week reproduction test than the F1:control group ($\chi 2$ = 4.23 , p <0.05). In contrast, for the populations continuously exposed for two and three generations (F1:As|F2:As and F1:As|F2:As|F3:As), offspring production was not significantly different compared to the generationally matched control populations (See Figure 3.4).

The F1:As|F2:Ctr recovery group produced significantly more juveniles than the F1:As|F2:As individuals, but not the F2 controls ($\chi 2$ =6.65 , p <0.01 and $\chi 2$ =1.73 , p >0.05). In the next generation, offspring production of the this same recovery group (F1:As|F2:Ctr|F3:Ctr) was significantly smaller than for the F1:As|F2:Ctr recovery group ($\chi 2$ = 10.5, p <0.01), but not significantly different to either F3 controls or continuously exposed earthworms ($\chi 2$ = 0.314, p >0.05 and $\chi 2$ = 1.93 , p >0.05). This indicates a possible transient maternal effect of induced reproduction in worms allowed to recover from exposure for an unexposed generation. Offspring production in the F1:As|F2:As|F3:Ctr group

was also not significantly different ($\chi 2$ =1.1, p >0.05) from the F3 control population. Therefore, the maternal effects were only present after a single generation of exposure.

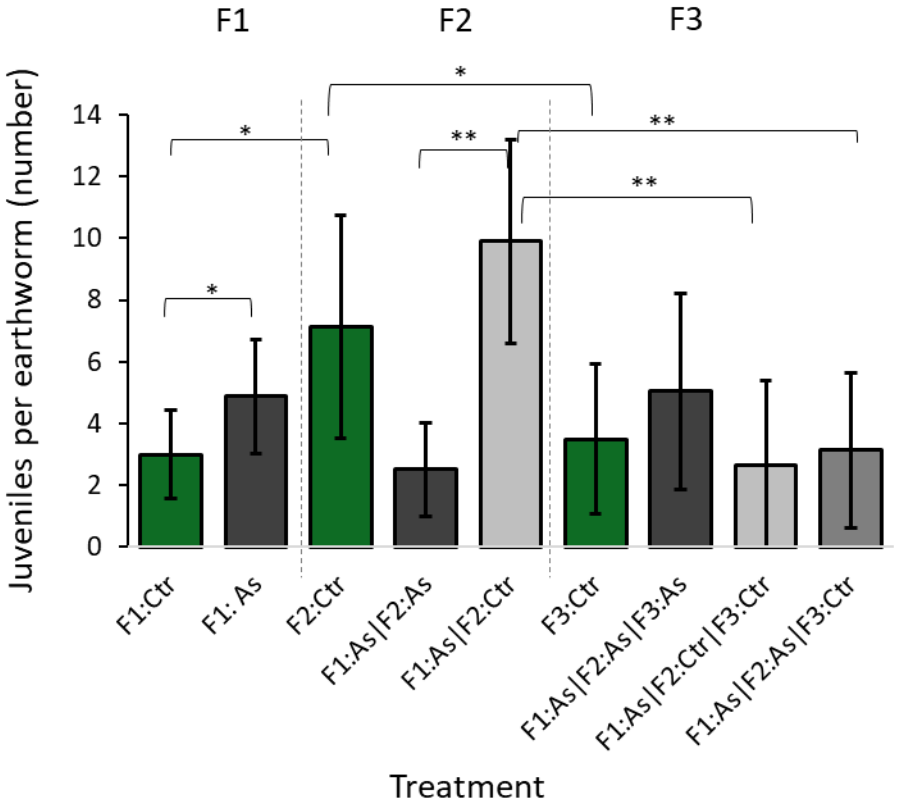


Figure 3.4. Average number (mean and standard deviation) of juveniles per earthworm produced in 6 week reproduction tests across three generations of exposures of 12 mg/kg (per dry weight soil) arsenic (As). After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil for later rearing (F1:As|F2:Ctr, F1:As|F2:Ctr|F3:Ctr and F1:As|F2:As|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels . * p≤0.05; **p≤0.01;***p≤0.001.

Cocoon viability was significantly increased in the F1:As|F2:As|F3:As group in comparison to the F3 controls ($\chi 2$ = 6.01, p <0.05) and the F1:As population ($\chi 2$ = 4.405 , p <0.05), but not the F1:As|F2:As ($\chi 2$ = 1.52, p >0.05). Similarly to control individuals, the proportion of viable pairs reduced with each generation. The F1:As|F2:Ctr group contained more viable pairs than the F1:As|F2:As population which likely provided an explanation for the difference in average juvenile production between the two groups (Figure 3.5).

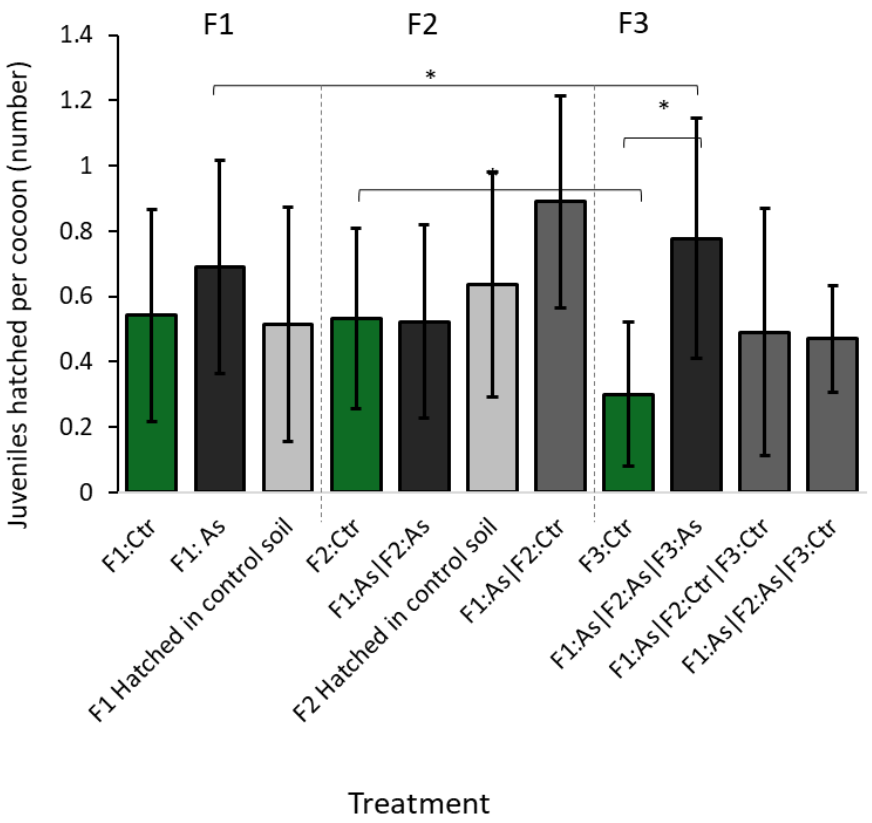


Figure 3.5. Average number of juveniles produced per all cocoons hatched in 6 week reproduction tests across three generations of exposures of 12 mg/kg (per dry weight soil) arsenic (As),. After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil for later rearing (F1:As|F2:Ctr F1:As|F2:Ctr|F3:Ctr and F1:As|F2:As|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

3.3.3.2. Parental, transgenerational and multigenerational effects of cadmium on reproduction

Reproduction in the cadmium exposed F1:Cd *Eisenia sp.* was not significantly different ($\chi 2$ = 1.63, p >0.05) compared to the F1:Ctr population. Offspring numbers in the F1:Cd|F2:Cd exposed population did not differ significantly from the F1:Cd population ($\chi 2$ = 0.02, p >0.05). However, the number of offspring produced by the F1:Cd|F2:Cd|F3:Cd group was significantly smaller compared to both the F1:Cd and F1:Cd|F2:Cd groups ($\chi 2$ = 3.99 , p <0.05 and $\chi 2$ =4.01 , p <0.05, respectively). Reproduction in the F1:Cd|F2:Cd|F3:Cd, however, was not significantly different from the F3 control group ($\chi 2$ = 1.104, p >0.05). This suggests the possible presence of a multi-generational effect on reproduction resulting from rearing within the experiment. However, the fact this was similar in both the continuous control and exposed populations suggests that, counter to our first hypothesis, this is not a result of chemical exposure.

The F1:Cd|F2:Ctr group produced a significantly greater number of offspring than the F1:Cd|F2:Cd ($\chi 2$ = 5.02, p <0.05), F1:Cd|F2:Ctr|F3:Ctr ($\chi 2$ = 11.104, p <0.001) and F1:Cd|F2:Cd|F3:Ctr ($\chi 2$ = 16.86, p <0.001) groups. Reproduction in this group was, however, similar to the F2 controls (see Figure 3.6) ($\chi 2$ = 1.73, p >0.05). This observation indicates a recovery of reproduction following exposure and that there are no effects of maternal exposure among the subsequently unexposed offspring.
The number of juveniles hatched per cocoon was not significantly different between any of the cadmium exposure or recovery groups (Figure 3.7).

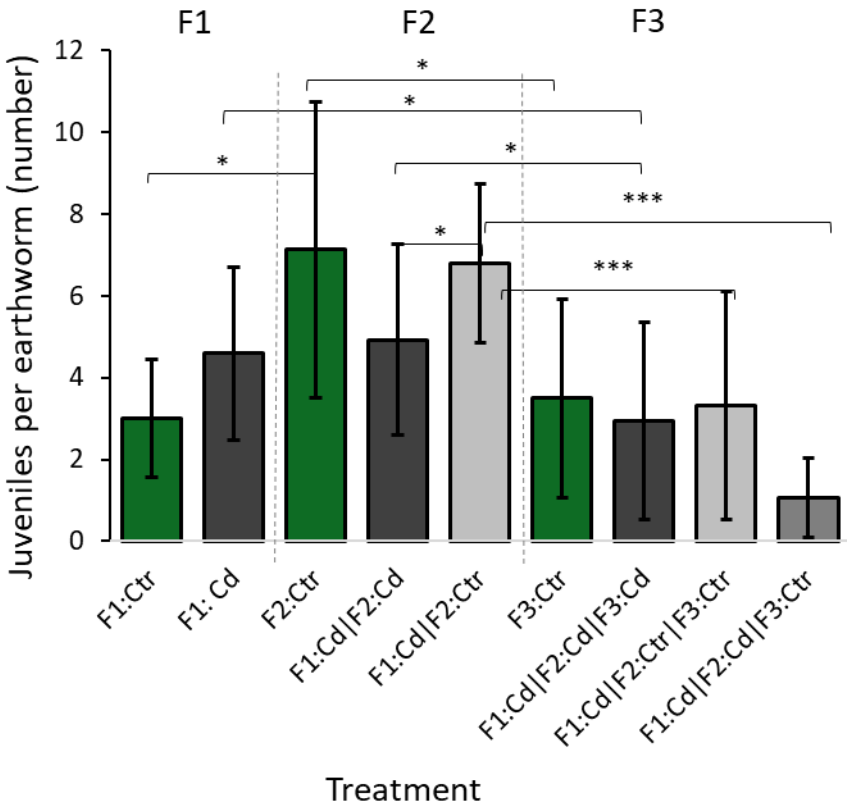


Figure 3.6. Average number (mean and standard deviation) of juveniles produced in 6 week reproduction tests across three generations of exposures of 40 mg/kg (per dry weight soil) cadmium (Cd). After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil for later rearing (F1:Cd|F2:Ctr F1:Cd|F2:Ctr|F3:Ctr and F1:Cd|F2:Cd|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

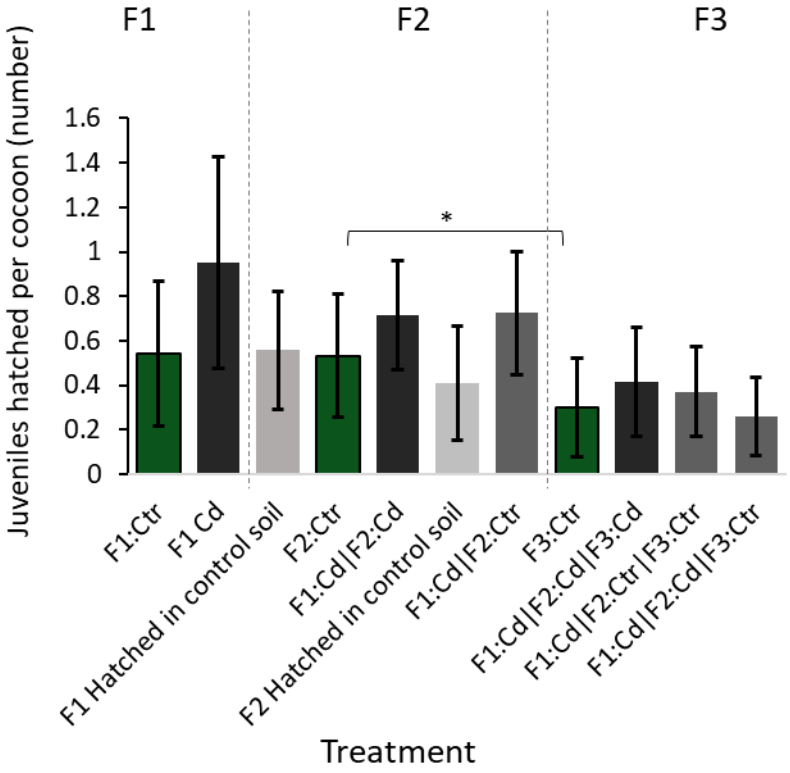


Figure 3.7. Average number of juveniles produced per all cocoons hatched in 6 week reproduction tests across three generations of exposures of 40 mg/kg cadmium (Cd). After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil for later rearing (F1:Cd|F2:Ctr F1:Cd|F2:Ctr|F3:Ctr and F1:Cd|F2:Cd|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

3.3.3.3. Parental, transgenerational and multigenerational effects of imidacloprid on reproduction

Exposure to 0.2 mg/kg imidacloprid resulted in fewer offspring being produced during the 6 week reproduction test than the relevant generational controls for both the F1 and F2 generations ($\chi 2$ = 7.05, p <0.01 and $\chi 2$ = 10.43 , p <0.01, respectively) (Figure 3.8). The proportions of viable pairs in the 0.2 mg/kg treatment were also lower than for the controls in the F1 and F2 generations (F1:50% for 0.2 mg/kg imidacloprid versus 79% for controls; F2: 45% for 0.2 mg/kg versus 71% for controls). This effect was significant for the F1 $\chi 2$ = 4.07, p <0.05), but not F2 generation ($\chi 2$ = 2.88, p >0.05). As in the case of exposures to arsenic and cadmium, the F1:Imd$^{0.2}$|F2:Ctr population had a higher proportion of viable pairs (83%) and produced a significantly higher number of juveniles than either F1:Imd$^{0.2}$|F2:Imd$^{0.2}$ ($\chi 2$ = 8,48, p <0.01) or either F1:Imd$^{0.2}$|F2:Ctr|F3:Ctr (20% viable pairs, $\chi 2$ = 10.1, p <0.01). Cocoon viability for the 0.2 mg/kg F1:Imd$^{0.2}$ populations was higher than for the F1:Ctr, especially when placed in control soil ($\chi 2$ = 15.5, p <0.001 in 0.2 mg/kg imidacloprid soil; $\chi 2$ = 16.4 , p

<0.001 in control soil). This was different for the F2 generation during which the F1:Imd$^{0.2}$|F2:Imd$^{0.2}$ population produced significantly fewer hatchlings than controls ($\chi 2$ = 5.23, p <0.05). In the F3 generation this number was smaller than control, but this effect was not significant (see Figure 3.9).

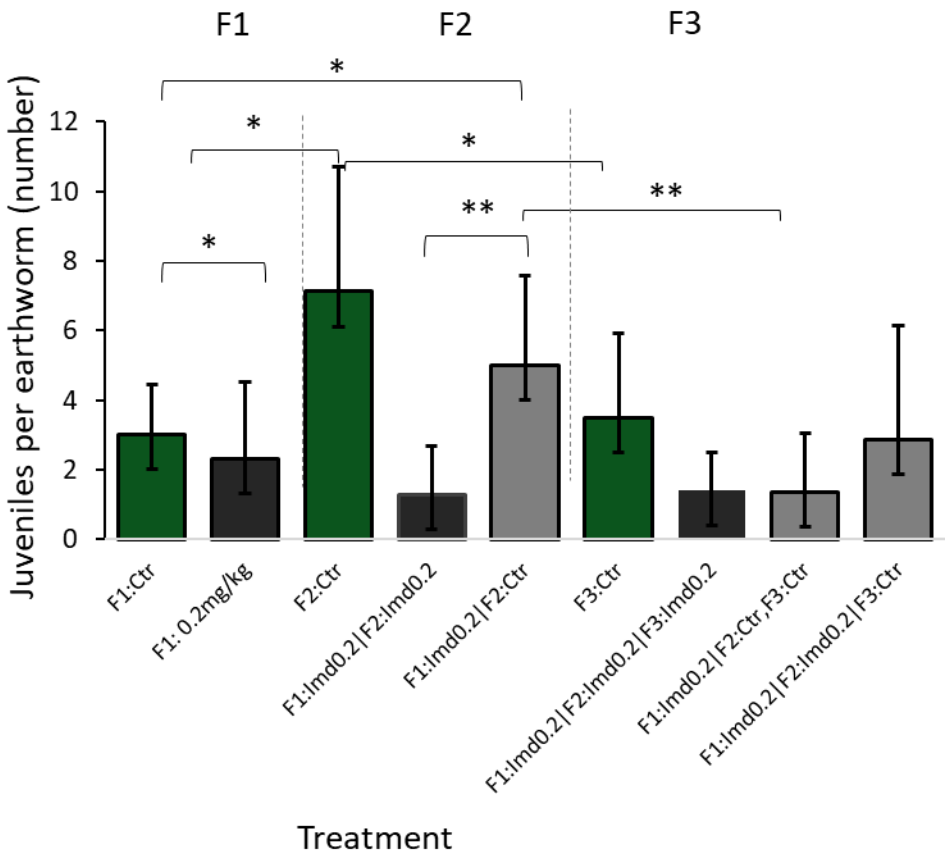


Figure 3.8 .Average number (mean and standard deviation) of juveniles produced in 6 week reproduction tests across three generations of exposures of 0.2 mg/kg (per dry weight soil). After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil. The juveniles grown from these cocoons were then raised in control soil for F2 and F3 generations and their reproduction parameters were measured (F1:Imd$^{0.2}$|F2:Ctr; F1:Imd$^{0.2}$|F2:Ctr|F3:Ctr and F1:Imd$^{0.2}$|F2:Imd$^{0.2}$|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001..

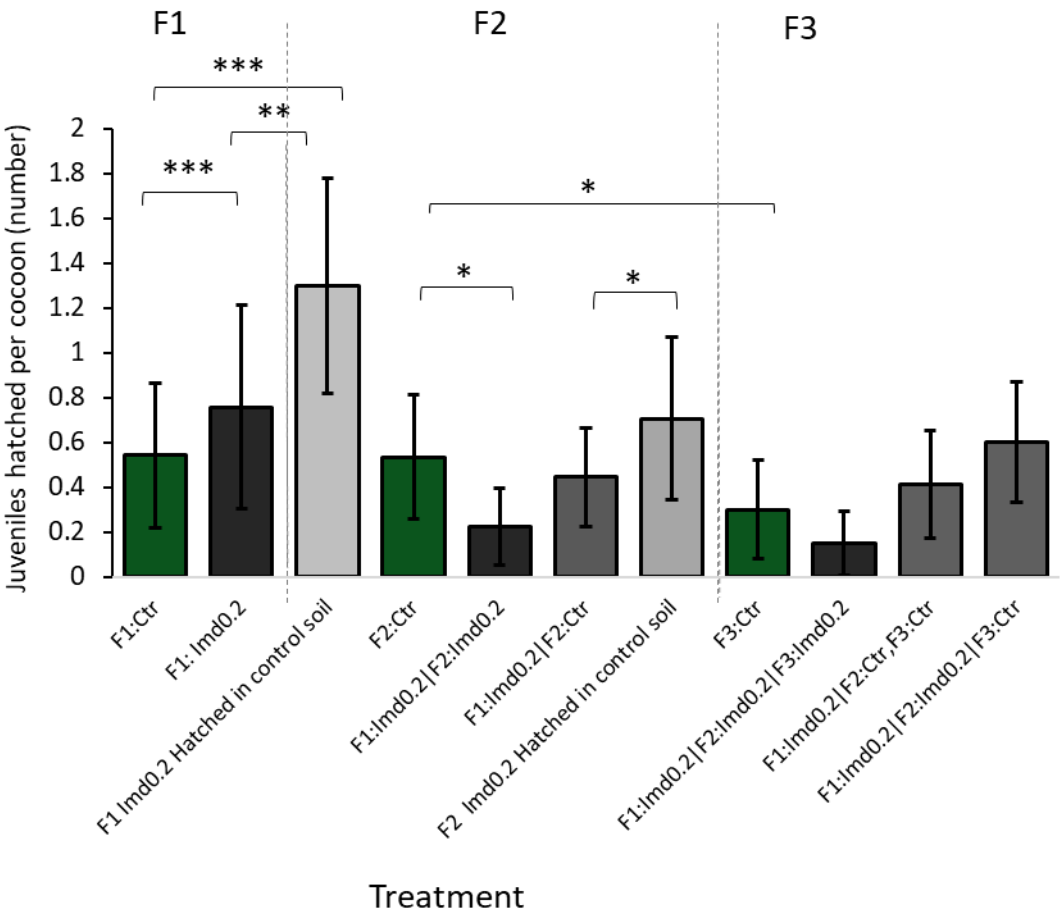


Figure 3.9. Average number of juveniles produced per all cocoons hatched in 6 week reproduction tests across three generations of exposures of 1 mg/kg (per dry weight soil) and 0.2 mg/kg imidacloprid. After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil. The juveniles grown from these cocoons were then raised in control soil for F2 and F3 generations and their reproduction parameters were measured (F1:Imd$^{0.2}$|F2:Ctr, F1:Imd$^{0.2}$|F2:Ctr|F3:Ctr and F1:Imd$^{0.2}$|F2:Imd$^{0.2}$|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

Continuous exposure to 1 mg/kg imidacloprid did not result in a significant effect on juvenile production compared to the generationally matched controls in any generations (F1:$\chi 2$ = 0.011, p >0.05 ;F2:$\chi 2$ = 0.046, p >0.05 ;F3:$\chi 2$ = 0.253, p >0.05) (Figure 3.10). Unlike for the other three exposures, the F1:Imd^{1} |F2:Ctr population did not produce a significantly larger average number of juveniles compared to the F1:Imd^{1}|F2:Imd^{1} group ($\chi 2$ = 1.69, p >0.05), even though the average number of juveniles produced by the F1:Imd^{1} |F2:Ctr population was the largest in any treatments of generational cohort across the whole experiment. The number of hatchlings produced per cocoon was not significantly affected by continuous 1 mg/kg imidacloprid exposure at any point (Figure 3.11). This shows an interesting difference in the response to long-term imidacloprid exposure, with a greater

number and magnitude of significant effects seen on reproduction at the lower tested concentration of 0.2 mg/kg compared to the higher exposure concentrations of 1 mg/kg.

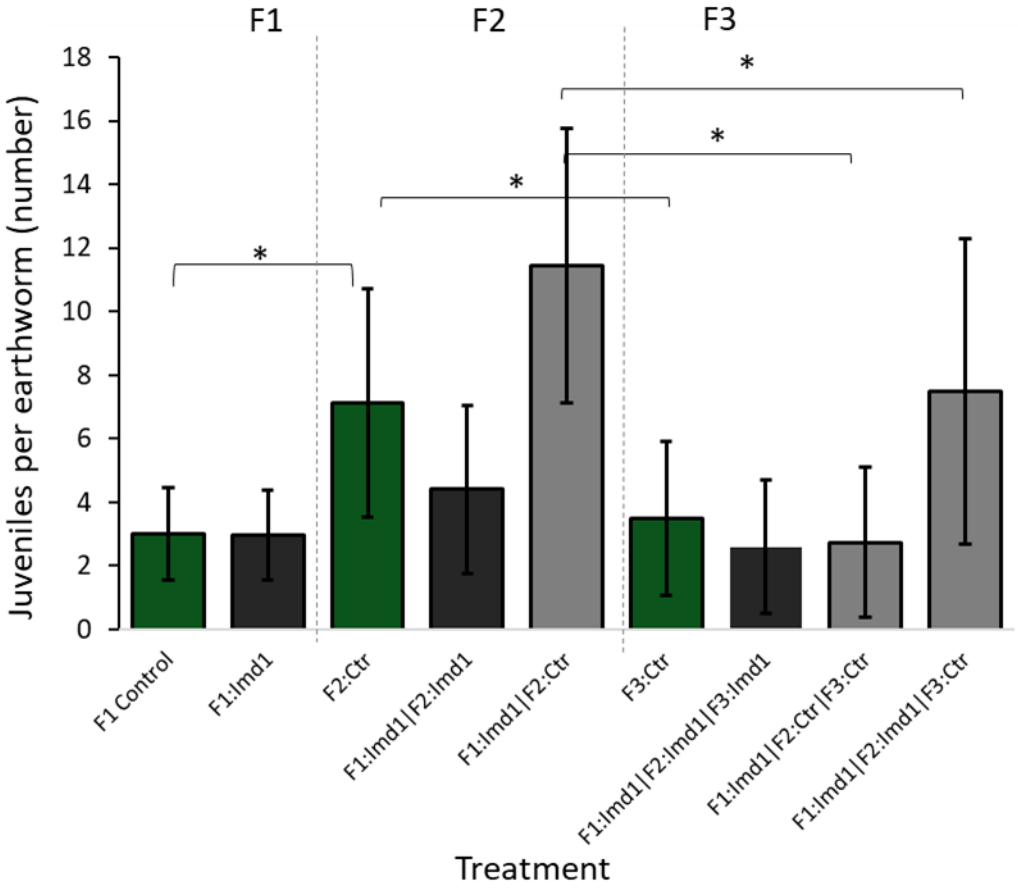


Figure 3.10. Average number (mean and standard deviation) of juveniles produced in 6 week reproduction tests across three generations of exposures of 1 mg/kg (per dry weight soil) imidacloprid. After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil. The juveniles grown from these cocoons were then raised in control soil for F2 and F3 generations and their reproduction parameters were measured (F1:Imd^{1}|F2:Ctr; F1: Imd^{1}|F2:Ctr|F3:Ctr and F1:Imd^{1}|F2:Imd^{1}|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

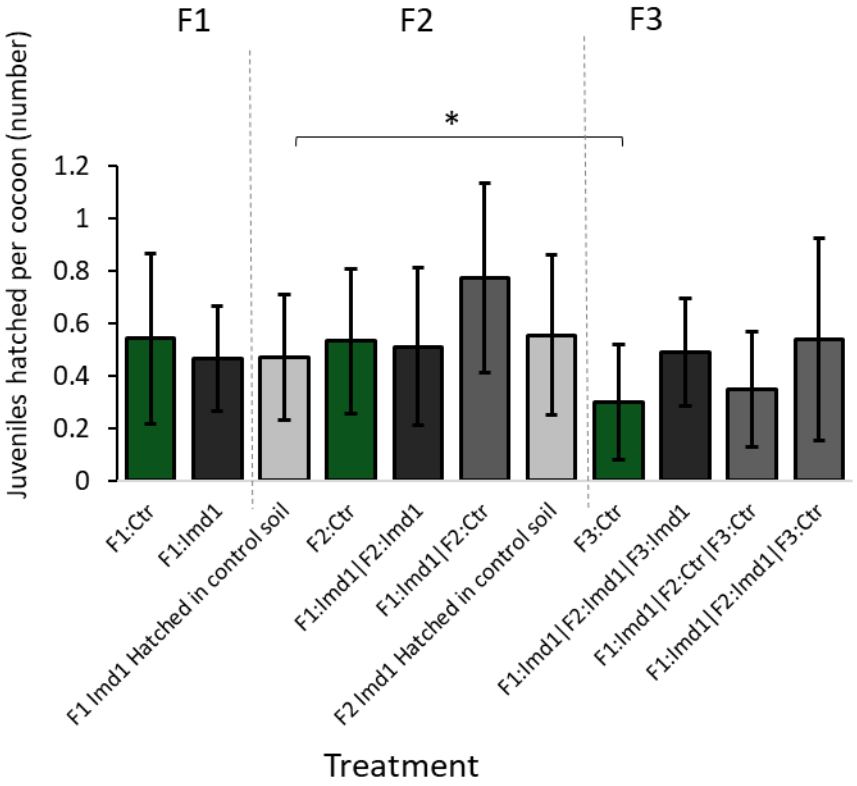


Figure 3.11. Average number of juveniles produced per all cocoons hatched in 6 week reproduction tests across three generations of exposures of 0.2 mg/kg (per dry weight soil) and 1 mg/kg imidacloprid. After the F1 and F2 reproduction tests half of the cocoons from exposed treatments were placed in the same soil as their parents, the other half hatched in control soil. The juveniles grown from these cocoons were then raised in control soil for F2 and F3 generations and their reproduction parameters were measured (F1:Imd^1|F2:Ctr, F1: Imd^1|F2:Ctr|F3:Ctr and F1: Imd^1|F2: Imd^1|F3:Ctr). Green bars are control treatment, dark grey bars continuously exposed, light grey recovery. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

3.3.3.4. Effects of genetic background on reproduction

Species lineage background had no significant impact on any of the reproductive parameters assessed within any one generation (Kruskal-Wallis F1: $\chi 2$ =2.34, p>0.05 , F2: $\chi 2$ =3.063, p>0.05, F3: $\chi 2$ =3.062, p>0.05). When comparing reproductive parameters between generations of the same lineage, however, significant differences were found. The number of offspring produced by *E. andrei* earthworms was significantly larger in the F2 generation than in either F1 ($\chi 2$ =7.16, p <0.01) or F3 ($\chi 2$ =15.89, p <0.001) and was also significantly higher in the F3 generation compared to F1 ($\chi 2$ =5.59, p<0.05) (see Figure 3.12). The percentage of *E. andrei* pairs that produced offspring decreased successively with each generation from 89% in F1 to 72% in F2 to 56% in F3, with the F3 *E. andrei* generation having a significantly lower proportion of viable pairs compared to the F1 ($\chi 2$ = 4.82 , p

<0.05) (See Figure 3.13). The number of hatchlings per cocoon was significantly higher between the F2 and F3 *E. andrei* groups ($\chi 2$ =7.99, p <0.01).

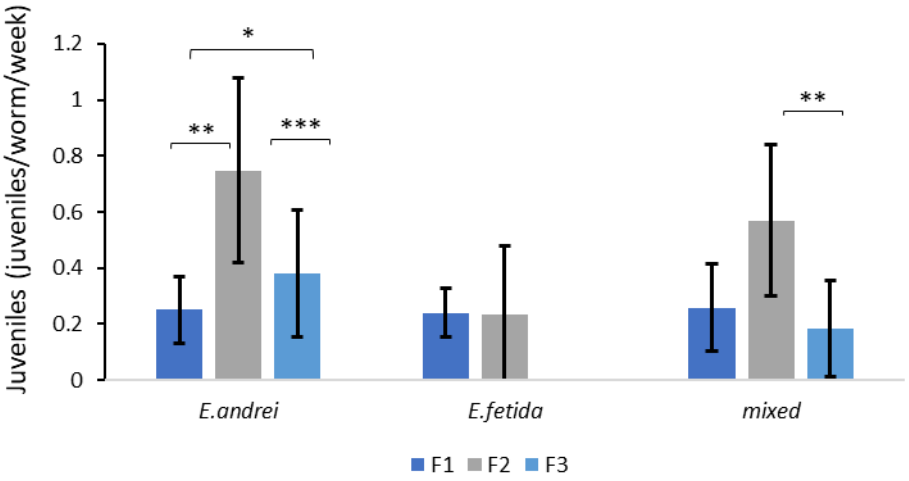


Figure 3.12. .Average number (mean and standard deviation) of juveniles produced in 6 week reproduction tests across three generations of control exposure of *E. andrei*, *E. fetida* and earthworms whose P0 parents were from both *E. andrei* and *E. fetida* lineages. Square brackets indicate statistical significance between treatments at levels: * p≤0.05; **p≤0.01;***p≤0.001.

The *E. fetida* lineage earthworms did not show the same pattern of generation variation in reproduction as for *E. andrei*, producing similar numbers of offspring in the F2 generation compared to F1 earthworms (See Figure 3.12.). A large proportion of the *E. fetida* worms of the F2 generation did not produce offspring. As a result, there were not enough F3 control *E .fetida* pairs to perform a reliable statistical comparison for this generation. Similar to the *E. andrei* lineage worms, the mixed lineage background earthworms also produced the highest number of offspring in the F2 generation. This increase was significant compared to the F3 generation ($\chi 2$ =7.93, p <0.01), but not to the F1s.

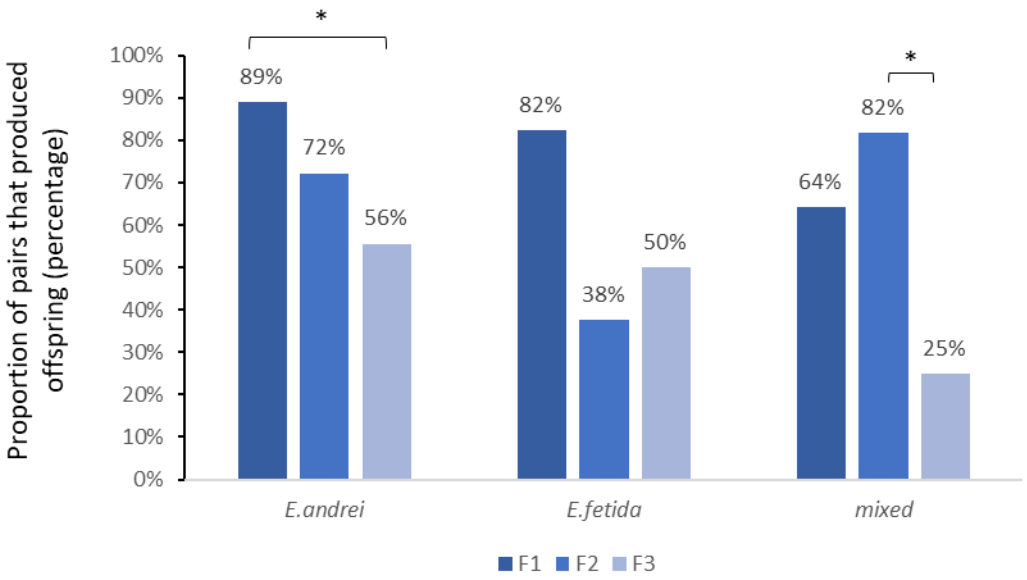


Figure 3.13.. Proportions of viable pairs within the control groups across three generations of the experiment from different lineage backgrounds, *E. andrei*, *E. fetida* and mixed, whose P0 parents were from both lineages.

The *E. andrei*, *E. fetida* and mixed lineage backgrounds had no impact on the effects of the various chemical exposures on the number of offspring produced. Therefore, as in the case of growth parameters, the genetic background of the individuals did not alter how they responded to the toxic exposures. In this respect for their response to the toxicant tested, the two lineages can be considered to be effectively synonymous.

3.4. Discussion

The results reported in this chapter show a variety of effects caused by the three toxic chemicals used in the reported experiment, arsenic, cadmium and imidacloprid. Due to the experimental design of this exposure, the results also provide us with an insight into how inbreeding may impact on earthworm phenotypes as well as earthworm adaptation to different laboratory conditions after multiple generations in moderately polluted environments. For this purpose, the differences between control groups from different generations were assessed. Concentrations of chemicals selected for study are well within the range that are found at sites polluted with trace metals by mining and metal processing (Camm et al., 2004; Li and Thornton, 2001) and for imidacloprid would be expected in surface soil layers after foliar application (Dankyi *et al.*, 2014).

When comparing the individuals within the control treatment between different generations, no significant effects on growth or survival could be found. However, a difference between generations could be seen for reproduction, the F2 generation producing significantly more offspring than for either the F1 or F3. This effect may be due to the acclimatisation of the earthworms adapting to test setting, including the clay loam soil used and the ambient experimental conditions. The percentage of viable pairs reduced with each generation in all of the experiments run. This suggests a generational effect potentially related to inbreeding depression. However, the impact of this reduction in pair viability on overall offspring numbers was mitigated due to F2 and F3 generations producing more offspring per pair than the F1 control group. Within a wider context of earthworm ecotoxicology, this inbreeding effect may result in a reduced genetic diversity of *Eisenia sp.* stocks kept in laboratories, although, as a larger selection of potential mates is available in these compared to the current experiment, this effect may be avoided in mass reared populations. These results show that earthworm inbreeding, at least at a time span of three generations, does not have a severe detrimental impact on overall earthworm fitness. The effects on pair viability do, however, suggest that inbreeding over further generations could have significant fitness impacts in pair viability drop to a level beyond which greater successful pair performance would not make up for losses due to reduced pair viability. In a study on *E. andrei* by Velando et al. (2006) it was found that inbreeding (mating with siblings) and outbreeding (mating with individuals from a different, geographically isolated, population) reduced cocoon production in the earthworms compared to outbreeding within the same population (Velando et al., 2006). In the current study, there were no comparisons between inbred pairs and pairs outbred within the same population, therefore the findings are not directly comparable, however, my results show that there is a possible alleviation of the effects caused by two generations of inbreeding on reproduction.

The exposure of the earthworms to the 12 mg/kg (per dry weight soil) arsenic treatment had a detrimental effect on the survival of the *Eisenia sp.* earthworms in the F1:As|F2:As and F1:As|F2:Ctr groups, but not in the other studied generations. The effect of arsenic exposure on F1:As|F2:Ctr indicates that a maternal effect was established in the F1 generation by arsenic exposure, perhaps during gonad development (Heard and Martienssen, 2014), that impacted the fitness of the F2 individuals through effects on survival probability. A possible reason for why the mortality of the F3 individuals is not as affected as that of the F1:As|F2:As and F1:As|F2:Ctr may be that the greater mortality seen in the F2 generation results in the elimination of the more sensitive individuals from the reproductive cohort that produce the F3s. The F1:As|F2:As group was the only population amongst the arsenic continuously or previously affected groups to show significantly reduced growth compared to the relevant generational controls. Unlike in the case of effects on mortality, however, this effect was not established during the F1 generation exposure as the F1:As|F2:Ctr group's growth was not affected.

The single generation exposure to 12 mg/kg arsenic resulted in a significant increase in the number of offspring produced. This hormetic effect may be linked to a possible trade off in energy budget allocation among the F2 populations, whereby arsenic exposure results in a greater allocation of resources to reproduction at the expense of maintenance costs which results in a higher rate of mortality for the next generation of earthworms (Jager and Klok, 2010). This may also have resulted in higher sensitivity to the arsenic exposure as shown by the F1:As|F2:As group's growth weight differences compare to F1:Ctr|F2:Ctr.

The exposure of the *Eisenia sp.* earthworms to 40 mg/kg cadmium had no significant effect on survival or reproduction for any exposed generations. This is consistent with a previous finding where the same concentration of cadmium did not have a detrimental impact on *E. fetida* juvenile survival rates over an extended exposure time (Žaltauskaite and Sodiene, 2014). Continuous Cd exposure had a significant impact on the growth of exposed F1s compared to generational controls. However, growth was not significantly different for the continuously Cd exposed F2 and F3 generations compared to their relevant control populations. The mitigation of the effect of Cd exposure on growth in the later exposed generations suggests the development of an adaptive phenotype over the time course of the study. Adaptation to Cd by *Eisenia sp.* earthworms has been reported before by Voua Otomo and Reinecke, 2010. The current study supports this possibility even for the relatively low concentration tested. The mechanisms by which this adaptation occurs,

whether through the selection of tolerant genotypes or generational acclimatization mediated by epigenetic change, warrants further investigation. No maternal or transgenerational effects were found as a result of the cadmium exposure.

The two imidacloprid concentrations used resulted in different effects on the exposed *Eisenia sp*. The continuous exposure to both 0.2 mg/kg and 1 mg/kg concentrations resulted in significantly reduced growth for at least two generations each, namely for the F1 and F3 for 0.2 mg/kg (growth was also reduced for the F2 generation of this exposure, but it was not significantly different compared to F2 controls); F1,F2 and, to a smaller degree, the F3 generation for the 1 mg/kg treatment. Only the higher concentration resulted in significantly reduced survival and this effect was seen only in the F3 generation, showing a sensitizing effect caused by the chemical or perhaps an accumulative toxicant effect resulting from the multigenerational exposure (Schultz *et al.*, 2016). The 0.2 mg/kg exposure resulted in a lower reproductive output for the first two generations, but not for the F3 generation, showing a possible adaptive effect (Vedamanikam and Shazilli, 2008).

The F1:Imd$^{0.2}$ |F2:Ctr group showed a significantly higher hatching rate compared to the matched controls. When this group hatched after the F1 reproduction test, significantly more juveniles per cocoon could be observed in this group than in the control treatment. Indeed, this population arose from the most productive cocoons that were reared over the whole experiment. A hormetic maternal effect of low doses of imidacloprid has been previously reported in green peach aphids (Ayyanath *et al.*, 2013). The current experiment shows a similar effect in *Eisenia sp,* at least in terms of cocoon hatching. This implication of such hormetic effects for a wider range of life-cycle traits warrants further investigation, as it has been shown that hormesis for traits relating to exposure can result in impacts on other traits through trade-offs (McClure et al., 2014; Vilca Mallqui et al., 2014), such as the overall lower number of juveniles produced as a result of the 0.2 mg/kg imidacloprid exposure.

Throughout the experiment, some common observations could be seen between different treatment groups. One was the size of the difference for the results of the reproduction tests between the F2 recovery populations (F1:As|F2:Ctr, F1:Cd|F2:Ctr, F1:Imd$^{0.2}$ |F2:Ctr) and the F2 continuously exposed groups (F1:As|F2:As, F1:Cd|F2:Cd, F1: Imd$^{0.2}$|F2: Imd$^{0.2}$). The F1:Exp|F2:Ctr groups for the arsenic, cadmium and 0.2 mg/kg imidacloprid exposures all produced significantly more offspring compared to the relevant F1:exp.F2:exp groups. The F1:As|F2:Ctr and F1: Imd^{1} |F2:Ctr groups also produced noticeably more offspring than controls although neither the recovery nor the continuously exposed groups were significantly different to the F2 controls. This implies a common fast recovery and

possible mild hormetic effect for offspring of individuals exposed to different types of chemicals over sustained exposure (Mallqui *et al.*, 2014).

The lineage background had no significant effect on any of the growth parameters. Further, lineage also had no significant effects on the reproduction of the *Eisenia sp.* observed in this experiment. Therefore, at least in terms of lineage background, the hypothesis that the genetic background will have an impact on the phenotypes of earthworms was rejected. This implies that the findings reported in exposure studies of *E. andrei* and *E. fetida* may show comparable sensitivity meaning that they can be used synonymously, although other publications imply this may not be the case and that the two separate into two distinct species with different morphologies (Domínguez *et al.*, 2005b). The progeny of pairs which comprised of earthworms from both lineages showed no clear signs of outbreeding depression. However, for these earthworms the proportion of viable pairs in the F3 generation was three times lower at 25% compared to the F2 generation of the mixed pairs, and twice as low as the F3 *E. fetida* and *E. andrei*. This difference was not statistically significantly smaller, due to the small number of pairs in the F3. It is, however, noteworthy for the magnitude of the effect size and, hence, warrants further investigation. Therefore, while there is an apparent interbreeding between the two *Eisenia* lineages throughout this experiment, this finding indicates that the previously reported reproductive isolation between the lineages (Domínguez *et al.*, 2005b) might become more apparent after multiple generations. Self-fertilisation is a possibility, therefore, if we imagine that the mixed lineage offspring all resulted from self-fertilisation, this finding may also reflect inbreeding depression.

3.5. Conclusion

Multigenerational exposure to the 1 mg/kg imidacloprid treatment resulted in a long-term accumulative toxicity in multi-generationally exposed earthworm populations. However, as the concentrations used for the As and Cd exposures (12 mg/kg and 40 mg/kg per dry weight soil, respectively) provide only a relative low level of chronic exposures (as visible due to the small impacts of these exposures on the earthworms in this experiment), it is possible that these toxicants may produce similar accumulative effects at higher concentrations. Rather than accumulative toxicity, an adaptive effect, where the exposed earthworms are detrimentally affected for a generation or two and then recover, was observed for these lower concentrations. This was observed for growth weights as affected by Cd and 1 mg/kg imidacloprid, as well as for reproduction of the *Eisenia sp.* exposed to 0.2 mg/kg imidacloprid. Therefore, the hypothesis that a long-term toxic exposure results in sensitizing the species was only shown in the case of 1 mg/kg imidacloprid. While no transgenerational effects were recorded in this experiment, multiple maternal effects were observed. For example, a

detrimental effect resulting from internal exposure was observed in the offspring of the F1:As population and both F1:As|F2:As and F1:As|F2:Ctr groups showed higher mortality compared to controls. Meanwhile, a hormetic effect in the F1:Imd$^{0.2}$|F2:Ctr group was observed for both the number of juveniles hatching and the growth weights of this population. Therefore, the second hypothesis proposed at the beginning of this study (that toxic exposures will result in transgenerational and/or maternal effects) was shown to be correct.

The genetic background of the *Eisenia* earthworms did not have an impact on the toxic exposures on any of the measured parameters. *E. andrei* and *E. fetida* were shown to interbreed for multiple generations (although, as self-copulation is a possibility in this experiment, this is not a definite observation) and inbreeding did not appear to have a detrimental impact on the earthworms' fitness, although some inbreeding or outbreeding depression was observed in offspring from mixed *E. andrei* and *E. fetida* backgrounds. Therefore, the hypothesis that the genetic background will have an impact on the way the measured parameters are affected by toxic exposures was not shown to be the case in this experiment.

Chapter 4. Impacts of life-time exposure of arsenic, cadmium and fluoranthene and of long-term mixed metal exposure on the earthworms' *L. rubellus* global DNA methylation as detected by msAFLP

4.1. Introduction

Tests to measure the toxicity of chemicals are generally performed over short time periods lasting from hours to weeks (OECD 2014). This does not reflect the nature of real life toxic exposures, which can last from months to decades, resulting in organisms being exposed for full lifetimes or for multiple generations depending on aspects such as the half-life of the chemical and the life-span, behaviour and generation time of the organism (Mulligan *et al.* 2001.; Head *et al.* 2012; Vandegehuchte and Janssen, 2011). Species' responses to long-term presence of pollution in the environment can vary greatly. Increased exposure times can lead to greater effects due to the accumulation of the chemical or progression of the accrual of damage; alternatively, species may show phenotypic adaptation to the chemical due to the development of stress hardening traits (Kafel *et al.*, 2012). Therefore, it is important to explore the long-term impacts of pollution on affected organisms to understand the effects of toxic exposure in relevant field cases. Such studies may also help elucidate how measurements of toxicity made in typical short-term experiments relate to effects for typical long-term exposures relevant to field cases of pollution.

Alongside phenotypic effects on population relevant life-cycle traits, such as growth and mortality, long-term exposure to chemicals may also have impacts on organisms at a molecular level. For instance, exposure to chemicals has been shown to result in changes in chromatin structure, detected via DNA methylation (Takiguchi *et al.*, 2003) . These may be indicative of other long term changes at the biochemical and gene expression levels (Boekelheide *et al.*, 2012). Further, changes at a molecular level may also reveal the adaptive mechanisms utilised by exposed organisms to survive under conditions of chronic stress (Boyko *et al.*, 2010). As a result of such observations, there has been a growing interest in environmental epigenetic effects of chemical exposure and their consequences for individuals and populations exposed in the long-term (Head *et al.*, 2012). The quantitative analysis of epigenetic molecular biomarkers may be particularly useful in attempts to

understand impacts of pollution on exposed organisms. Such analyses may characterise the current and also the past exposure histories of local populations, as such molecular markers may show the imprint of the toxic stress exposure experienced by the organism throughout the full life-cycle (Cardenas et al., 2017; Hew et al., 2015).

Exploring molecular markers after both laboratory and wild exposures can provide different insights into the toxicity of chemicals. In a laboratory setting, the doses and manner of organism exposures can be tightly controlled. Such exposures provide a route to elucidating the very particular effects caused by individual chemicals across a range of sub-lethal concentrations under chronic exposure scenarios. Alternatively, studies to assess the effects of chemical pollution exposures in the field provide a realistic picture of the way organisms are affected and, therefore, are more informative of the actual effects and mechanisms associated with real world exposures. Exploring molecular markers from specimens collected at polluted sites in the field may also provide an opportunity to better understand the impacts of pollution for much longer time periods than can be feasibly explored in the laboratory. DNA methylation is commonly the marker of choice for understanding of molecular epigenetic effects due to its importance in normal cell functioning (Saint-Carlier and Riviere, 2015) and the availability of techniques for the exploration of this mark (Kurdyukov and Bullock, 2016; *Alonso et al.* 2018).

L. rubellus is a species of earthworms that has been comparatively widely explored in field and laboratory settings designed to understand the short- and long-term impacts of chemical exposures. Cadmium (Cd) has been found to have an impact on *L. rubellus* growth and Metallothionein-2 expression (Burgos *et al.*, 2005). Both Cd and the polycyclic aromatic hydrocarbon fluoranthene have been found to have an impact on *L. rubellus* reproduction and gene expression (Svendsen et al., 2008;Long et al., 2009); meanwhile arsenic exposure has been found to both cause detrimental impacts on *L. rubellus* in a short-term exposure and an adaptation response after prolonged exposure at polluted sites (Langdon *et al.*, 1999). While it is known that *L. rubellus* phenotypes and some molecular biomarkers are impacted by toxic chemical exposures, less is known of the molecular epigenetic impacts of pollution on this species of earthworms. However, a limited number of studies have been carried out. For example, DNA methylation has been indicated as a possible biomarker when observing multiple metalloid impacts on earthworms in a study by Santoyo et al., 2011. In one study of the earthworm *L. rubellus*, collected from natural heavily polluted soils, it was found that DNA methylation levels were correlated with arsenic levels in soils in one cryptic lineage while this was not the case in another (Kille, Andre et al. 2013). In another study, this time using the

related earthworm species *Lumbricus terrestris*, long term exposure to low levels of Cd resulted in an increase in global DNA methylation, some remains of which were retained by the worms even when placed in clean soil (Šrut *et al.*, 2017).

In this study, the effects of long-term exposure on the *L. rubellus* DNA methylome are characterised to test the hypothesis that change in DNA methylation status forms a part of earthworm responses to pollutant exposure and that this is affected by the length of the exposure (a single lifespan versus many generations).

The global DNA methylation, as measured by msAFLP (methylation sensitive amplified length polymorphism), of earthworms *L. rubellus* was explored in organisms after a life-long laboratory exposure to three chemicals (the heavy metals arsenic, cadmium and the polycyclic aromatic hydrocarbon fluoranthene) in single chemical exposure studies in the laboratory.

In the second part of this experiment, the effects of prolonged exposure of pollution emitted by a cadmium/lead/zinc smelter located at Avonmouth in the South-West of the United Kingdom were studied by measuring epigenetic traits, specifically, DNA methylation. This was done using the methylation sensitive amplified fragment length polymorphism technique (msAFLP). The metal contamination arising from this smelter in the soils of the surrounding area has been present for many decades since the initiation of operations in the 1920s (Spurgeon and Hopkin, 1996). Therefore, this area has provided a setting for the long-term exposure of organisms to sustained high concentrations of heavy metals in soil.

4.2. Materials and Methods

4.2.1. Life-time exposures of *L. rubellus* to arsenic, cadmium and fluoranthene

The *L. rubellus* exposures to arsenic (As) in this experiment were carried out from a hatchling stage to adulthood over 280 days (the procedure and data bar DNA methylation results have been reported before by Anderson et al., 2013) **who carried out this part of the experiment**. The exposures to cadmium and fluoranthene were performed in the same way, except the cadmium exposure was over 20 weeks (140 days), while the fluoranthene exposure was over 44 weeks (308 days). The difference in exposure time was needed because at the highest concentrations of cadmium tested in the experiment some mortality of the earthworms was detected. Therefore, the exposure duration was shortened to ensure that there was a sufficient number of earthworms available in these higher concentrations for downstream analysis. Hence, it was not possible to allow

the exposure to continue for an extended length of time to allow full development of the earthworms. The same degree of mortality was not found for the fluoranthene treatments, therefore this exposure could be carried out to a time-scale to which worms in all treatments reached full adult weight.

The soil used for the exposure was a commercially available clay loam soil (Broughton Loams, Kettering, UK) amended with 3% dry weight of composted bark (LBS Horticultural, Colne, UK). The soil for the experiment was prepared the same way as described in Section 2.2.2.. The chemicals added were: arsenic in the form of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$, Sigma Aldrich, Bournemouth, UK), fluoranthene (99% purity) (Sigma Aldrich, Bournemouth, UK) and cadmium (applied as $CdCl_2$, Sigma Aldrich, Bournemouth, UK). Both arsenic and cadmium are water soluble. Hence, it was possible to add these two elements by dissolving the relevant salt into the water that was added to the prepared soil. Fluoranthene was added to the soil by preparing the compound into an acetone stock solution that was then added to the surface of the test soil. Top-up additional solvent was added to the control and lower concentration treatments at the time of spiking to ensure that all containers (including controls) received a similar amount of acetone. After allowing the residual acetone to evaporate (checked by assessment for residual wetting and smell), the spiked soils were then thoroughly mixed and water added to the test soil to bring the medium to 50% of water holding capacity. Unlike arsenic and cadmium, fluoranthene is subject to microbial degradation in soil with a measured half-life, therefore, at 16 weeks of treatment, the earthworms in this exposure were placed into newly prepared soils prepared the same way as the initial soils with the appropriate amounts of the chemical added.

The concentrations originally used were chosen to cover the full sublethal range of exposures, selected based on Svendsen et al., 2008 and Anderson et al., 2013. The following concentrations of chemicals (per dry weight soil) were successfully carried out throughout the experiment:

As: 3, 12 and 36 mg/kg

Cd: 13, 43.9 and 148 mg/kg

FA: 20.8, 31.2, 70.2 and 800 mg/kg

These concentrations were used for the methylation sensitive amplified length polymorphism analysis as a sufficient number of earthworms in these conditions survived the whole experiment for use in the molecular analysis. The arsenic exposure also involved a concentration of 125 mg/kg of

the metal, but survival in this treatment was too low to be used for further analysis (previously reported in Anderson et al., 2013).

To start the test, earthworms at a young hatchling stage were placed in individual 200 ml containers containing 250 g of control or spiked soil and 1 g (dry weight) horse manure (one worm per container, 25 replicates per each concentration) added to the surface of the test soil as a source of food. The manure used as food was spiked with the relevant concentration of test chemical by addition to the water used for wetting the manure to 80% water holding capacity for arsenic and cadmium or in acetone as described above for the soils for fluoranthene. The containers were then kept in 14^{o}C under constant light throughout the experiment. Every 28 days, earthworm survival, weight and developmental status were recorded. At these stages, an additional amount 1 g (dry weight) of appropriate horse manure was added, after removal of previous manure. This amount of manure was increased to 1.5 g once the earthworms had reached adulthood as indicated by the presence of a fully developed clitellum. At the end of the exposure, the earthworms were recovered from the test soil, their final weight recorded, and the earthworms then snap frozen in liquid nitrogen and then stored at -80^{o}C for later analysis. These samples were later ground to powder under liquid nitrogen and used for DNA extraction for the genotyping and methylation sensitive amplified fragment length polymorphism (msAFLP) analysis using the same procedure as described in Sections 1.2.8, 1.2.9, 1.2.10.

4.2.2. Earthworm and soil collection from a gradient of sites near the Avonmouth smelter

Adult *L. rubellus* with a fully developed clitellum and approximately 100 g soil samples were collected from six sites near a zinc, lead and cadmium smelter located in Avonmouth, UK and a control site located in an uncontaminated area 24 kilometres away from the smelter (See site map in Figure 4.1 and coordinates in Table 4.1, placed in the results section). The earthworms were identified according to phenotypic marks and this identification was later confirmed by genotyping using COI mitochondrial barcoding. The *L. rubellus* were collected onto a large volume own site soil and taken to the laboratory and placed in a 13°C controlled temperature room to be dissected and frozen the next day. Ten earthworms were collected from the most and least polluted sites while 7 -8 earthworms were taken from each location along the transect. The soil samples from each site were collected from the top layer of soil, (the description of the procedure has been published before by (Spurgeon and Hopkin 1995)). On return to the laboratory the site soil samples were dried prior to their use for analysis of

their physio-chemical properties including soil pH, loss on ignition and concentrations of trace metals measured by inductively coupled plasma mass spectrometry (ICP-MS).

4.2.3. Earthworm dissections and DNA extraction

It is established the methylation status of DNA can vary between different tissues within the same organisms (Christensen *et al.*, 2009). Hence collection of target tissues can result in more homogenous methylation profiles to allow the better detection of changes in the methylome in response to external stimuli. The target tissue that was selected for downstream analysis was the posterior alimentary canal. This tissue is responsible for digestion of food materials. However, in additional it is also surrounded by the chloragogen tissue of the earthworm. This area has a key metabolic function analogous to the liver of vertebrates and hind-gut gland of molluscs. The chloragogen tissue is known to be a target organ for chemical effects, including the sequestration of metals and the metabolism of organic chemicals (Morgan *et al.*, 2002). Therefore, a sample from the gut can be expected to show heightened responses to chemical exposures, such as gene expression change and associated changes in DNA methylation status. Further, in addition to this main tissue of interest, clitellum, head and tail samples were also collected and retained for use in additional analyses. The head samples were used for the isolation of DNA for earthworm genotyping and the tail samples were used for chemical analysis of pollutant content, meanwhile, the clitellum was not used for further analysis in the experiments described in this thesis.

Dissections were performed by cutting the skin of the anterior side of the earthworms, which was then pinned to the sides to allow access to the major internal organs including the posterior gut/chloragogen tissues. A centimetre of intestine below the clitellum was cut outplaced in a 1.5 ml Eppendorf tube containing approximately 10 times tissue volume of RNA*later*™-ICE (ThermoFisher Scientific, Gloucester, UK) to ensure long-term sample integrity and then immediately flash frozen in liquid nitrogen. The same procedure was then used to collected clitellum region, head (from prostomium to approximately segment 26) and the tail (all segments after the clitellum (approximately from segment 31 onwards)) samples. The samples were then kept at -80°C until required for the relevant downstream analyses. All equipment was cleaned in 70% ethanol between dissections to ensure that there was no cross contamination between individual specimens.

For the analysis of DNA methylation patterning using methylation sensitive amplified fragment length polymorphism (msAFLP), DNA was extracted from the posterior gut tissue sample. This tissue had been previously kept in RNA*later*™-ICE frozen tissue transition solution. In order to extract

DNA successfully, the tissue was first placed in PBS solution for 10 to 20 minutes in order allow some of the RNA*later*™-ICE solution to leach from the sample. The downstream DNA extraction steps were then performed the same way as for the ground up tissue in Section 2.2.8.

DNA barcoding and genotyping analysis using PCR and sequencing of the COI gene was performed the same way as described in Section 2.2.10.

4.2.4. Soil metal analysis

This analysis was performed on samples of the soils from a gradient of sites near the Avonmouth zinc and lead smelter in order to assess the heavy metal content in this complexly polluted soil.
Three samples of roughly 5 g of soil from each site were used for the analysis of metal content for this experiment. The method used is previously described by (Spurgeon et al. 2011), it is briefly described below.

The sample was homogenised per individual and 0.5 g measured using acid hydrolysis and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Perkin Elmer 4300DV, Cambridge, UK). The quality control was conducted using standard reference material ISE 192 (International Soil Exchange, The Netherlands). Following digestion, samples were analysed for V, Mn, Fe, Cu, Zn, P, S using ICP-OES and for Al, Ti, Ca, Cr, Co, Ni, As, Mo, Cd, Sb, Se, Ba, Hg, Pb, using ICP-MS. The values were expressed as mg/kg dry weight soil. Certified values for reference materials were well aligned with data in this analysis, they were within 10% of certified values for all analysed metals.

A sample of the same air-dried soil was used for determining the LOI (loss of ignition) and pH for soil organic content and acidity measurements. The LOI was measured by furnace combustion at 550°C (as described before by Allen, 1989). Soil pH was measured by an electrode from a 1:2.5 by weight soil/water mix using an appropriate calibration and reanalysis method to confirm measurement accuracy (Allen, 1989).

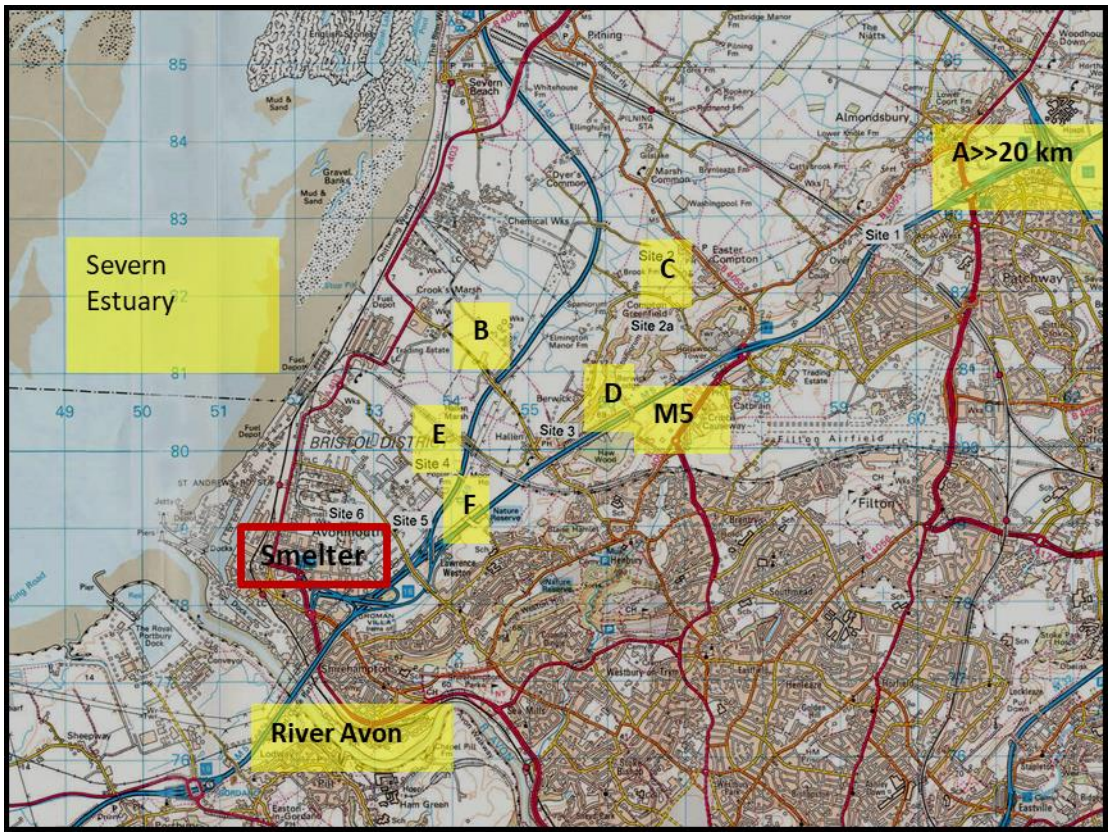


Figure 4.1. Map showing earthworm and soil sampling sites close to the Avonmouth zinc and lead smelter as well as significant landmarks nearby. The sites are named in alphabetical order from least to most polluted with zinc and other heavy metals.

4.2.5. Methylation sensitive amplified fragment length polymorphism (msAFLP) analysis

The protocol and downstream analyses used for the methylation sensitive amplified fragment length polymorphism analysis were adapted from Díaz-Freije et al., 2014 and Ardura et al, 2017. and Blouin et al., 2010).

The msAFLP experimental pipeline used in this experiment is as follows:

Following the extraction, the DNA samples were diluted to acquire similar concentrations of DNA in each sample and split into two aliquots. Each was then cleaved using two restriction enzymes, either EcoRI (Thermo Fisher Scientific, Birchwood, UK) and HpaII (Thermo Fisher Scientific, Birchwood, UK), or EcoRI and MspI (Thermo Fisher Scientific, Birchwood, UK). Both HpaII and MspI cut DNA at the same nucleotide sequence: 5'-CCGG-3', however, MspI is unable to cleave DNA at the recognition

site when the outer cytosine is either fully or hemi methylated while HpaII is blocked from cleaving the site if either cytosine is methylated at both strands. Neither restriction enzyme can cleave the site if both cytosines are methylated or if a polymorphism is present in the site (Ardura et al. 2017).

The mixtures including the DNA samples, appropriate restriction enzymes and CutSmart™ buffer (New England Biolabs, Hitchin, UK) were placed in 37^{o}C temperature for 3 hours to allow the digestion reaction to occur. A ligation reaction was then carried out in order to attach linkers to the cut DNA fragments which allow them to be amplified in further reactions. The linker sequences used were as follows:

EcoRI linker 1: CTCGTAGACTGCGTACC (Sigma Aldrich, Bournemouth, UK)
EcoRI linker 2: AATTGGTACGCAGTCTAC (Sigma Aldrich, Bournemouth, UK)

MspI/HpaII linker 1: GACGATGAGTCCTGAG (Sigma Aldrich, Bournemouth, UK)
MspI/HpaII linker 2: TACTCAGGACTCAT (Sigma Aldrich, Bournemouth, UK)

The ligation mix included: the cut DNA produced in the digestion reaction, linkers, T4 DNA Ligase (Thermo Fisher Scientific, Birchwood, UK), ATP (Thermo Fisher Scientific, Birchwood, UK), CutSmart™ buffer (New England Biolabs, Hitchin, UK) and deionised water. This mix was placed in 37°C temperature for three hours to allow the ligation reaction to occur. After this time, two amplification steps using the Polymerase Chain reaction (PCR) were performed. These were termed the pre-selective PCR and the selective PCR. The former enriches the amounts of target sequences needed for the detection of bands, the latter selects a smaller number of fragments (as the amount of AFLP fragments produced using genomic DNA can often be too great for effective analysis) and also attaches a dye in the form of 6-FAM reporter molecule 38 which is needed for the detection of bands by the DNA analyser.

The primers used in the pre-selective PCR were:
EcoRI-pre: GACTGCGTACCAATTC (Sigma Aldrich, Bournemouth, UK)
Msp-pre: GATGAGTCTAGAACGGA (Sigma Aldrich, Bournemouth, UK)

The primers used in the selective PCR step were:
EcoRI-AC: 6-FAM-GACTGCGTACCAATTCAC (Sigma Aldrich, Bournemouth, UK)
EcoRI-AG: 6FAM-GACTGCGTACCAATTCAC (Sigma Aldrich, Bournemouth, UK)

MspI-ATC: GATGAGTCTAGAACGGATC (Sigma Aldrich, Bournemouth, UK)
MspI-AG: GATGAGTCTAGAACGGAG (Sigma Aldrich, Bournemouth, UK)

Both PCR reactions included the following ingredients: ligated DNA, deionised water, deoxyribonucleotide mix (Applied Biosystems, Thermo Fisher Scientific, Birchwood, UK), Taq DNA polymerase including $MgCl_2$ (Sigma Aldrich, Bournemouth, UK), Taq PCR buffer (Sigma Aldrich, Bournemouth, UK) and the appropriate primers. The cycling conditions for both reactions were as follows:

Pre-selective PCR: 72°C for 2 min, 20 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 2 min, and a final step of 60°C for 30 min.

Selective PCR: 94°C for 2 min, 10 cycles of 94°C for 20 s, 66°C (decreasing by 1°C each cycle) for 30 s, and 72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, ending with 60°C for 30 min.

The resulting PCR products were then cleaned using ZR DNA Sequencing Clean-up Kit™ (Zymo Research, Cambridge Bioscience, Cambridge, UK), mixed with GeneScan™ 600 LIZ™ size standard v2.0 (Thermo Fisher Scientific, Birchwood, UK) and loaded into an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific, Birchwood, UK). Different profiles of cut fragments were observed by the presence of size specific bands in each sample that are detected within the capillary sequencer. The number and composition of bands varies depending on the locations of methylation marks on the DNA obtained from each individual earthworm. The DNA Analyser produced a report which was then used for carrying out msAFLP data analysis.

4.2.5.1. MsAFLP data analysis

The AFLP scoring and fragment analysis were performed using GeneMapper v.4.0 software (Applied Biosystems, Thermo Fisher Scientific, Birchwood, UK). The resulting scoring was analysed using the R package msap. A full explanation of this process can be found in Ardura et al, 2017. Briefly, using the data gained from the initial msAFLP analysis, the numbers of two types of the CCGG restriction sites can be accurately acquired – unmethylated ones, where both restriction enzymes cut the site, or partially methylated ones (either the internal or external C is methylated) where one of the restriction enzymes (HpaII or MspI) has cut the site and the other one has not. In theory, another

type of site, where hypermethylation has occurred and neither enzyme has been able to cut the restriction site, can be detected. However, this may also occur if a polymorphism is present in this site, therefore hypermethylation is possibly not the right answer a sufficient amount of times, especially as genetically diverse environmental samples were used in this experiment.

Therefore, four types of sites were identified from the data acquired from msap analysis, these were:

Type 1: both HpaII and MspI have cut (two fragments present);

Type 2: MspI has cut, HpaII has not;

Type 3: HpaII has cut, MspI has not;

Type 4: Neither enzyme has cut, either due to polymorphism or hypermethylation.

The global methylation level was calculated as the proportion of partially methylated loci over all scorable sites (partially and umethylated sites, excluding "hypermethylated" sites).
The analysed restriction loci were divided into Methylation-susceptible loci (MSL) or Non-methylated loci (NML), only those sites showing polymorphisms with at least two occurrences of each state were used. The MSL sites were used for assessing epigenetic polymorphisms and the NML sites for genetic ones. The msap package also carried out Principal coordinates analyses (PCoA) and analysis of molecular variance (AMOVA). Shapiro-Wilks test was performed to check for residual normality, Kruskal-Wallis test was performed to compare the groups. Spearman's rank test was performed to assess correlations. This analysis was done using Microsoft Office Excel and the R statistical software.

The msap package was also used to acquire the AFLP scores for genetic variability between the populations by employing the meth(false) option. This option scores all loci for genetic difference, not just NML, therefore providing a more statistically powerful measure of genetic diversity.

4.2.6. Other data analysis and data representation

Weight data for the As, Cd and fluoranthene exposed earthworms was tested for normality using Shapiro Wilks test and then the testing for statistically significant differences between the different treatment groups was carried out with ANOVA at the final growth stage of each treatment. The correlations of soil metal content with distance from the Avonmouth smelter were carried out using

the Spearman's rank correlation test. This test was used as it does not require a linear association of data. These analyses were carried out using the R statistical software.

4.3. Results

4.3.1. Life-time exposures of *L. rubellus* to arsenic, cadmium and fluoranthene

4.3.1.1. Arsenic

The phenotypic traits of the Arsenic (As) exposed *L. rubellus* used for the analysis have been previously reported by Anderson et al., 2013**.** Mortality was not affected in earthworms exposed to any of the As concentrations tested. Significant effects of As exposure on earthworm growth was, however, found. The final weights of arsenic exposed earthworms was significantly affected at 280 days for the 36 mg/kg and the 125 mg/kg exposure (see Figure and Anderson et al., 2013).

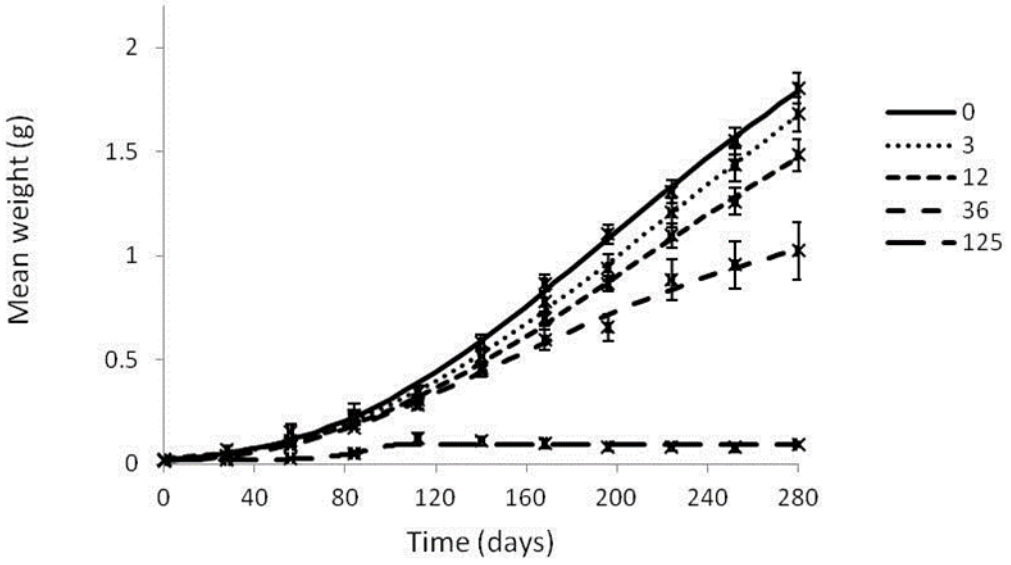


Figure 4.2. Mean weight (+/- SE) for *L. rubellus* exposed to a range of arsenic concentrations in soil, over 280 days at each 28-day time interval, with points appearing proximal to relative growth curves. Fitted curve correspond to best fit Gompertz growth curves estimated based on mean time interval weights. Figure previously reported by Anderson et al. 2013. 25 replicates of a single hatchling for each treatment were established at the beginning of the experiment, the mortality experienced by these is reported by Anderson et al. 2013.

4.3.1.1.1. Effects of arsenic on global DNA methylation as determined by msAFLP

Of the total of 77 loci found using msAFLP analysis, 55 (71%) were methylation susceptible loci and 22 (29%) were no methylation susceptible. The respective proportions of the four types of msAFLP sites found can be seen in Figure. ϕST analysis showed no significant differences between the

exposed groups using comparisons between either AFLP or MSL loci (ϕST = 0.0426; *p* = 0.104 and ϕST = -0.0216; *p* = 0.7038, respectively). The results can be seen in Figures 4.3; 4.4; 4.5) .

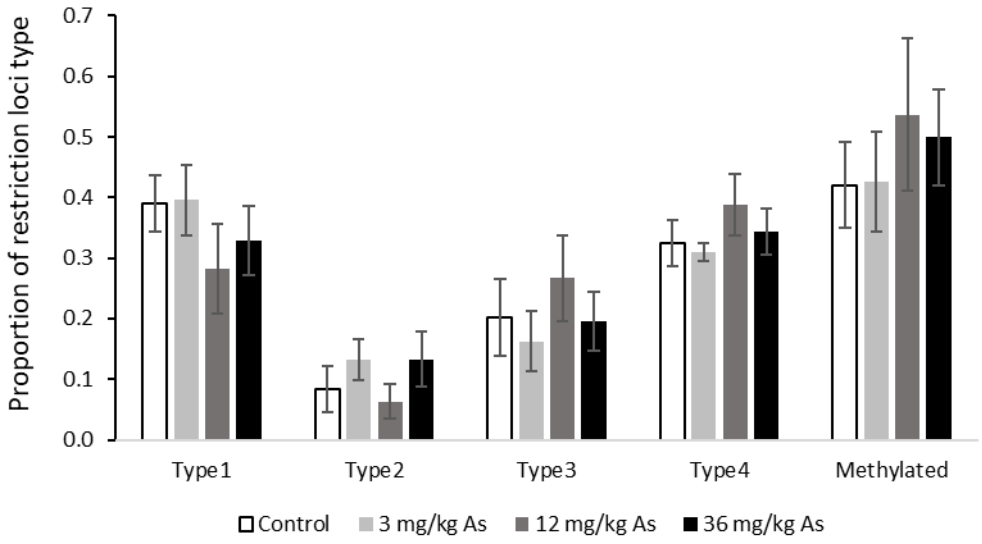


Figure 4.3. DNA methylation in methylation-sensitive loci detected from the analysed specimens of *L. rubellus* exposed for 280 days throughout their full development to a range of concentrations of arsenic (As). Type 1 to 3 are, respectively: no methylated, methylation of internal C, methylation of external C or hemimethylation, and hypermethylation or mutation in restriction site (of the 5'-CCGG-3' sequence). Methylated: Global methylation level estimated following Nicotra *et al*, as proportion of (Type 2 + Type 3 loci / Type 2+Type 3 + Type 1(scorable loci)).

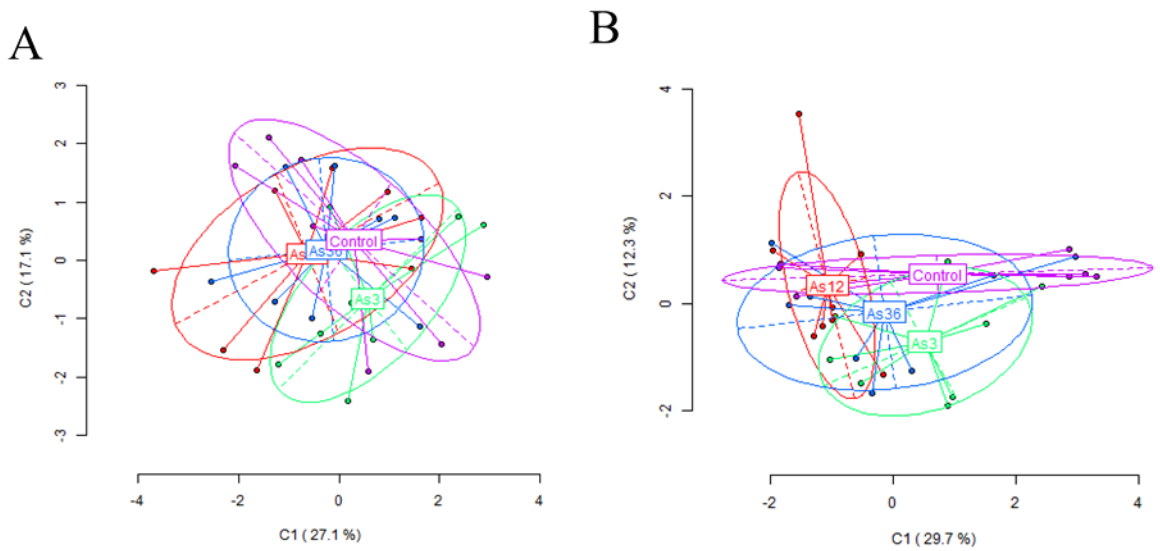


Figure 4.4. Principal Coordinate Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3' for *L. rubellus* in this experiment were exposed to 3, 12 and 36 mg/kg per dry weight soil arsenic (As) throughout their development for 280 days starting from an early juveniles stage. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP).

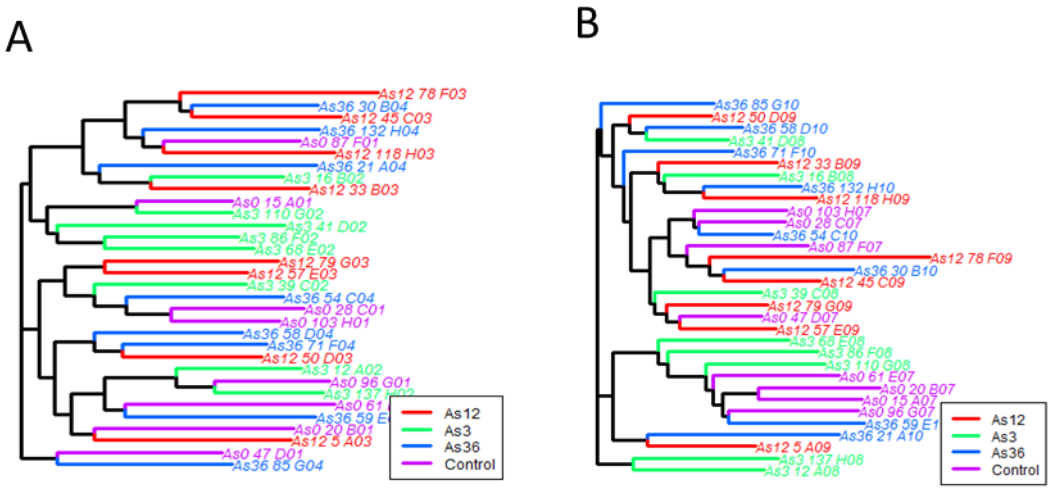


Figure 4.5. Neighbour Joining trees of the detected methylation and genetic polymorphism site distances in *L. rubellus* at the sequence 5'-CCGG-3' for *L. rubellus* in this experiment were exposed to 3, 12 and 36 mg/kg per dry weight soil arsenic (As) throughout their development for 280 days starting from an early juveniles stage. The Panel A shows the epigenetic variation (methylation-sensitive loci) and the Panel B shows the genetic variation (AFLP).

4.3.1.2. Cadmium

L. rubellus growth was reduced by exposure to cadmium at all concentrations tested. Divergent growth rates were evident for 148 mg/kg after 4 weeks exposure and for 13 and 43.9 mg/kg from 8 weeks onwards (See Figure 4.6). The growth weight of earthworms was significantly affected at all treatment levels after140 days of exposure (ANOVA: 13 mg/kg: $f = 9.59$, $p < 0.05$; 43.9 mg/kg: $f = 11.9$, $p < 0.01$; 148 mg/kg: $f = 18.9$, $p < 0.001$). At the 139 day stage of exposure higher mortality was visible in the 148 mg/kg treatment, therefore the whole exposure was cut short at this stage.

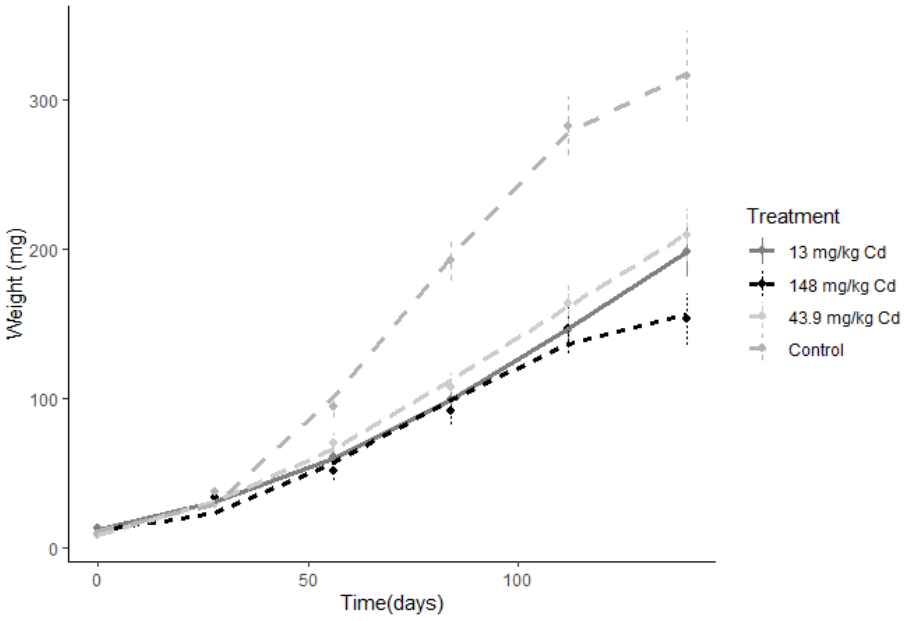


Figure 4.6. Mean ±SD weights (mg) of *L. rubellus* exposed to different concentrations of cadmium (Cd) from a young juvenile stage throughout development for 20 weeks (140 days). Fitted curves correspond to best fit linear models with polynomial function curves estimated based on mean time interval weights. The following sample sizes were used in the experiment: Control $n = 52$; 13 mg/kg $n = 40$; 43.9 mg/kg $n = 40$; 148 mg/kg $n = 40$..

4.3.1.2.1. Effects of cadmium on global DNA methylation as determined by msAFLP

192 AFLP loci were detected for the *L. rubellus* used in the exposure to Cd. Of these loci, 127 (66%) were methylation susceptible and 65 (34%) were not methylation susceptible. Neither the AFLP nor MSL site comparisons indicated a significant difference between the exposed groups ($\phi ST = 0.03751$; $p = 0.0847$ and $\phi ST = 0.07266$; $p = 0.1094$, respectively). However, a significant difference ($\chi 2 = 9.2448$, $p < 0.01$) in the proportions of type 4 sites (where no bands were detected, either due to full methylation or a polymorphism) between the control and the 13 mg/kg exposure was found (See

Figures 4.7; 4.8; 4.9).

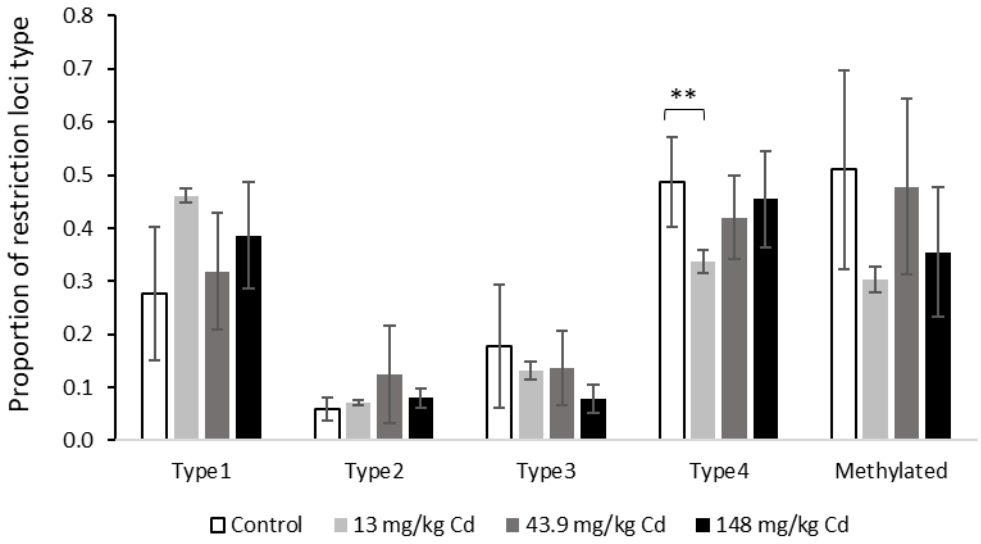


Figure 4.7. DNA methylation in methylation-sensitive loci detected from the analysed specimens of *L. rubellus* exposed for 140 days throughout their full development to a range of concentrations of cadmium (Cd). Type 1 to 3 are respectively: no methylated, methylation of internal C, methylation of external C or hemimethylation, and hypermethylation or mutation in restriction site (of the 5'-CCGG-3' sequence). Methylated: Global methylation level estimated following Nicotra *et al*, as proportion of (Type 2 + Type 3 loci / Type 2+Type 3 + Type 1(scorable loci)).

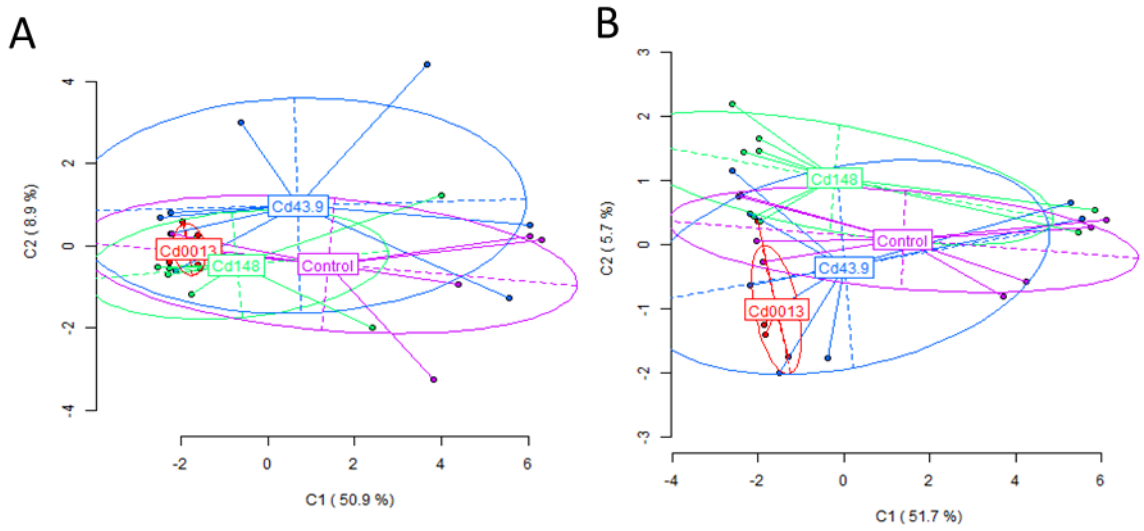


Figure 4.8. Principal Coordinate Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3' exposed for 140 days to 13,43.9 and 148 mg/kg per dry weight soil cadmium (Cd) throughout development. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP). The *L.rubellus* in this experiment were exposed starting from an early juvenile stage.

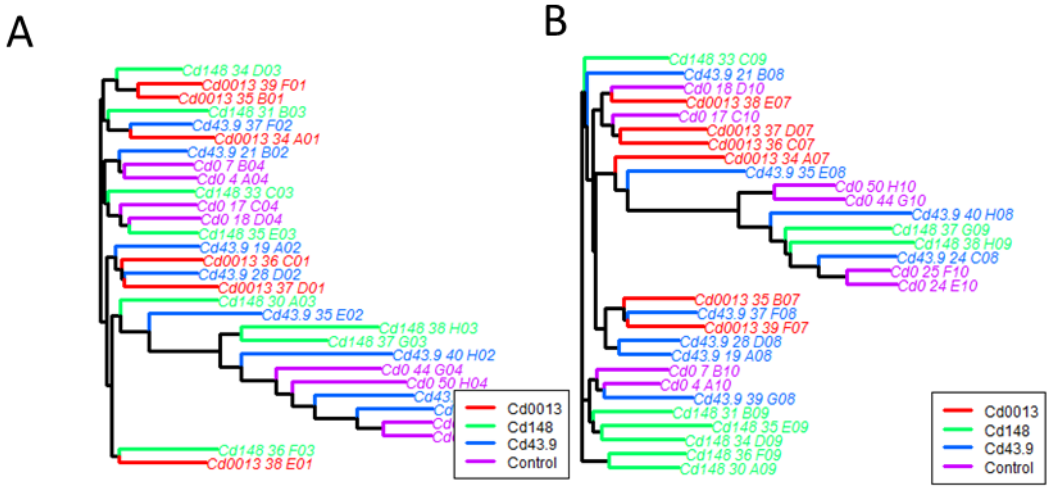


Figure 4.9. Neighbour Joining trees of the detected methylation and genetic polymorphism site distances in *L. rubellus* at the sequence 5'-CCGG-3' exposed for 140 days to 13,43.9 and 148 mg/kg per dry weight soil cadmium (Cd) throughout development. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP) on the right. The *L.rubellus* in this experiment were exposed from an early juvenile stage.

4.3.1.3. Fluoranthene

There were no effects of fluoranthene exposure on survival at any of the concentrations analysed. Further, until 112 days of exposure, significant differences in average weights were not observed. Thereafter from 140 days, the highest concentration (800 mg/kg) resulted in a significant negative effect on earthworm weights compared to the controls (ANOVA *f*-ratio = 23.7, $p < 0.001$). The negative effect at the highest exposure concentration was related to the change to freshly spiked soil after 112 days. Interestingly, the older juveniles showed a more pronounced response than the freshly hatched juveniles to the highest fluoranthene concentration. After this initial effect, the 800 mg/kg group slowly recovered, such that by 240 days individuals at this treatment reached similar average weights to controls. The 20.8 mg/kg exposure showed similar growth weights to the control group up until the 212 days and thereafter remained lower than in the other treatments, although this difference was not significant (ANOVA *f*-ratio = 4.06 , $p >0.05$). See Figure 4.10.

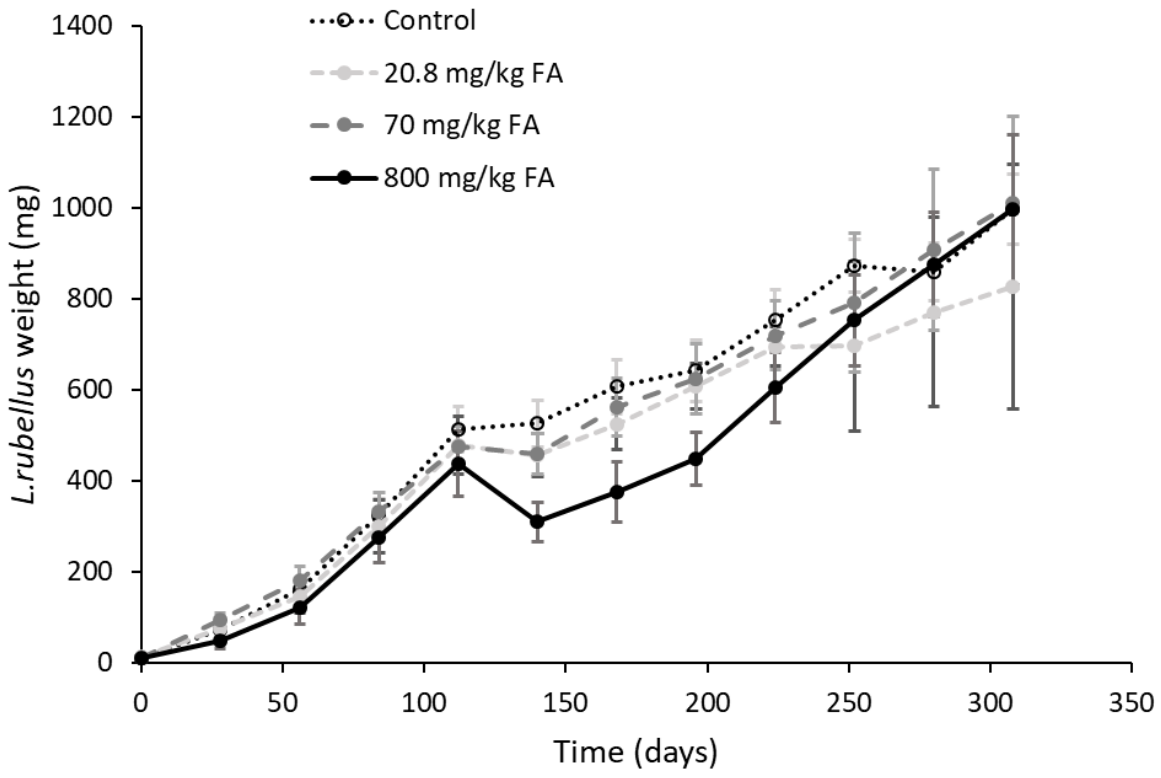


Figure 4.10. Mean±SD weights (mg) of *L. rubellus* exposed to different concentrations of fluoranthene (FA) from a young juvenile stage throughout development for 44 weeks (308 days). Age 0 is the time at which hatched juveniles were first exposed to the different soils. A growth

model was not applied to this graph in order to better portray the dip in average weights at the 140 day stage. The following sample sizes were used in the experiment: Control *n* =22; 20.8 mg/kg *n* = 16; 70 mg/kg *n* = 11; 800 mg/kg *n* = 7 (excluding samples with mortality and/or inconsistent records).

4.3.1.3.1. Effects of fluoranthene on global DNA methylation as determined by msAFLP

A total of 150 AFLP sites were found in earthworms exposed to fluoranthene. Of these sites 114 (76%) were methylation sensitive and 36 (24%) were non- methylation sensitive loci. AFLP profiles showed no significant differences between treatments (ϕST =0.0982; *p* <0.01). However, a significant effects of fluoranthene on msAFLP exposure was found ((ϕST = 0.0351; *p* <0.05). When comparing the proportions of the different types of AFLP sites, multiple significant differences were observed (See Figure 4.11). Most notably, fluoranthene appeared to have an impact on the amount of Type 2, 3 and 4 sites. Effects on Type 2 and Type 3 sites showed contrasting trends with Type 2 loci decreasing in frequency with concentration while Type 3 loci frequency increased with increasing fluoranthene concentration. (Figures 4.11;4.12;4.13)

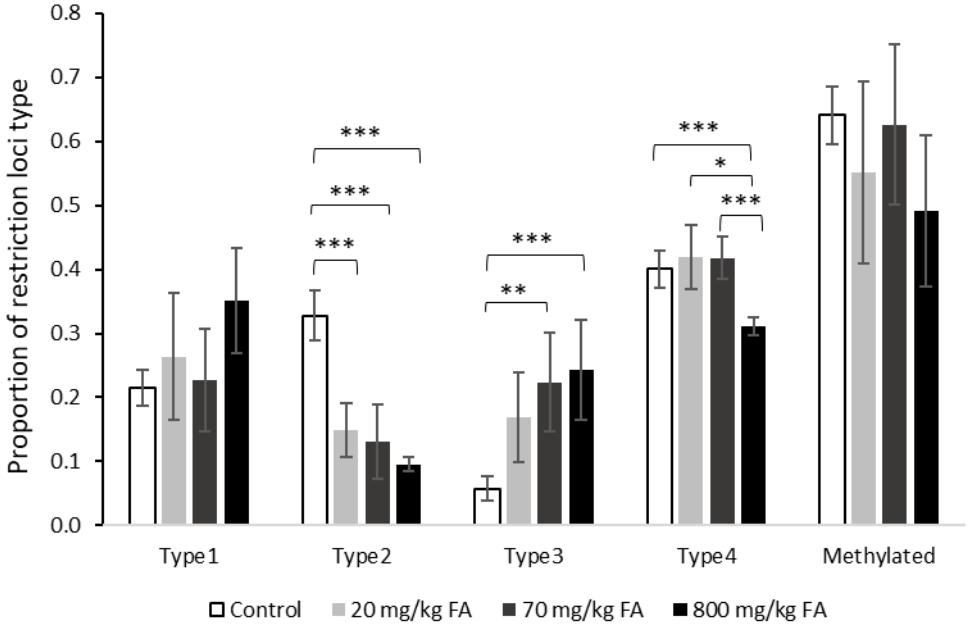


Figure 4.11. DNA methylation in methylation-sensitive loci detected from the analysed specimens of *L. rubellus* exposed for 308 days throughout their full development to a range of concentrations of fluoranthene (FA). Type 1 to 3 are respectively: no methylated, methylation of internal C, methylation of external C or hemimethylation, and hypermethylation or mutation in restriction site (of the 5'-CCGG-3' sequence). Methylated: Global methylation level estimated following Nicotra *et al*, as proportion of (Type 2 + Type 3 loci / Type 2+Type 3 + Type 1(scorable loci)).

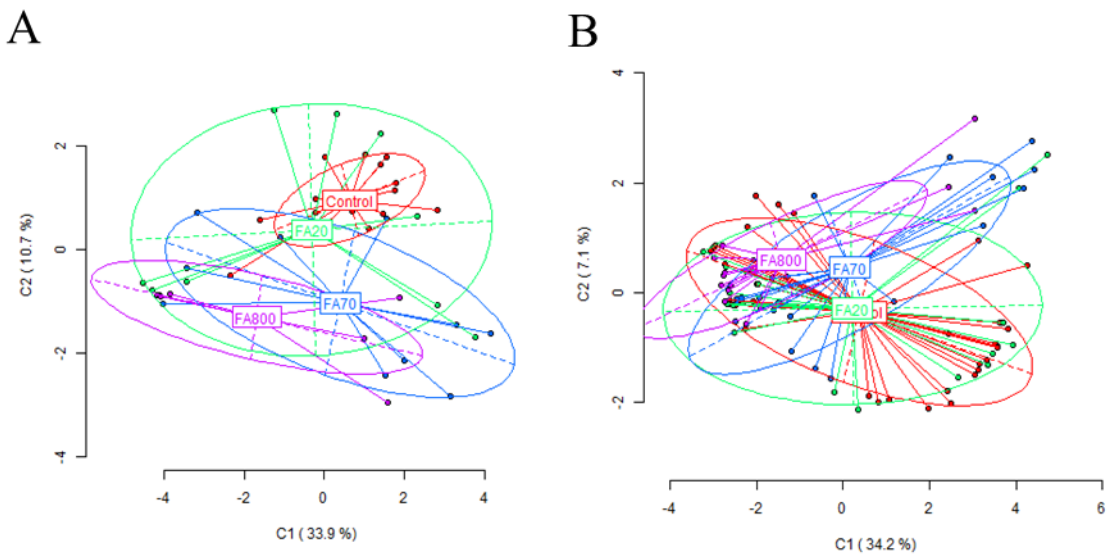


Figure 4.12. Two-dimensional visualization of the Principal Component Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3'. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP) on the right. The *L.rubellus* in this experiment were exposed to 20, 70 and 800 mg/kg per dry weight soil fluoranthene (FA) for 308 days throughout their development starting from an early juvenile stage.

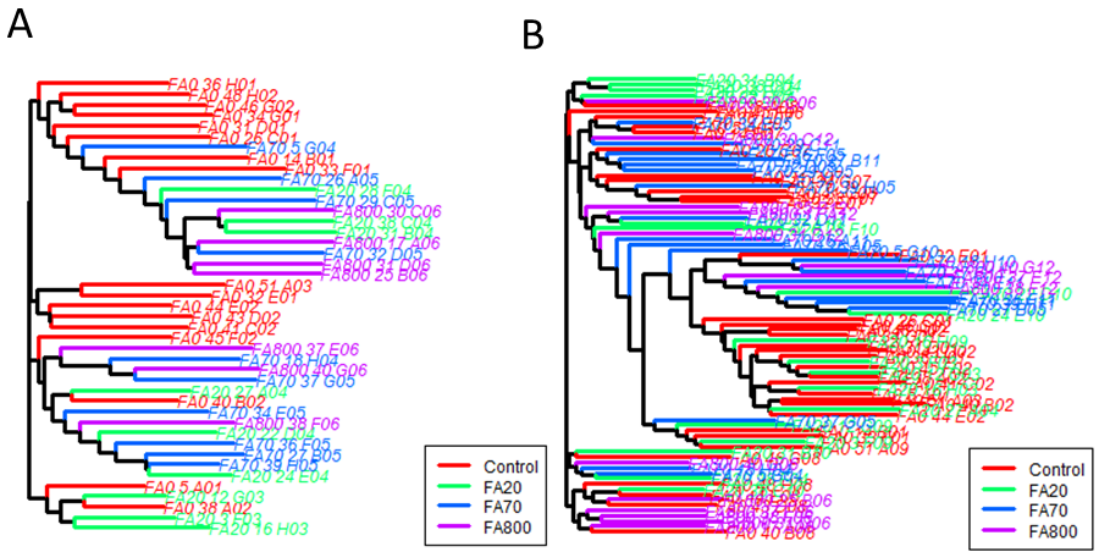


Figure 4.13. Neighbour Joining trees of the detected methylation and genetic polymorphism site distances in *L. rubellus* at the sequence 5'-CCGG-3'. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP) on the right. The *L.rubellus* in this experiment were exposed to 20, 70 and 800 mg/kg per dry weight soil fluoranthene (FA) for 308 days throughout their development starting from an early juvenile stage.

4.3.2. Characterisation of earthworms and soils collected from a gradient of sites near the Avonmouth zinc and lead smelter

4.3.2.1. Soil and earthworm physiochemistry

The code for gradient site names was chosen to be in alphabetical order from the least polluted control site (A) to the most polluted site (F) based on the sum of concentrations for the metals lead, zinc, mercury, antimony and cadmium. Site names also contain the pH value and average concentration of zinc (Zn) for each site. Site $C_{Zn315}^{pH6.43}$ contained more of the toxic metals than $B_{Zn166}^{pH6.57}$, despite being further away from the smelter. This difference is likely due to factors such as wind patterns and external pollution sources which influence local soil metal concentrations (Colgan *et al.*, 2003).

Locations and soil pH and organic content (as detected by loss of ignition) for each of the sites are given in Table 4.1. Sites A, B, C and F showed similar pH levels of between 6.34 and 6.68 while the soil from site D was somewhat more alkaline at 7.07 and site E was more acidic at 5.42. Overall there was a difference in soil pH of two units across sites. However, this difference was not directly correlated to the distance from the smelter. Thus pH, which is known to have a potential effect on earthworm physiological responses, did not covary in a consistent manner with distance from the smelter and, hence, metal pollution status. Soil organic matter content was relatively high in all soils (>10% in all cases). The three sites closest to the smelter had the highest loss on ignition. Hence there is some degree of covariance of organic matter levels with distance and, therefore, potential metal pollution status, although this is characterised by an effectively binary response (e.g. distant sites relatively low, proximal sites relatively high). The concentrations of all of the analysed metals found in the soils can be seen in Table 4.2.

Table 4.1. Characteristics of the soils collected from a gradient of sites near the Avonmouth zinc and lead smelter.

Site	OSGR	Distance to smelter (km)	pH (median)	% Loss of ignition ± SE
$A_{Zn145}^{pH6.34}$	Control	24.5	6.34	11.1 ± 1.83
$B_{Zn166}^{pH6.57}$	ST 541 816	3.2	6.57	13.7± 1.3
$C_{Zn315}^{pH6.43}$	ST 569 821	5.4	6.43	19.4± 0.3
$D_{Zn879}^{pH7.07}$	ST 560 807	2.8	7.07	22.7± 4.6
$E_{Zn1740}^{pH5.42}$	ST 539 802	1.9	5.42	23.8± 2.5
$F_{Zn3533}^{pH6.68}$	ST 540793	1.4	6.68	23.4± 1.9

Ordinance Survey Grid Reference (OSGR), distance to smelter, soil acidity (pH) and organic content (as detected by loss of ignition, mean ±SE) are shown. The site names also show the concentrations of zinc (Zn) found in each of the sites.

Zinc (Zn), cadmium (Cd), mercury (Hg) and lead (Pb) all showed a significant pattern of increase in concentrations from site $A_{Zn145}^{pH6.34}$ to $F_{Zn3533}^{pH6.68}$ (see Figure 4.14). This pattern was as expected as all these metals were expelled by the Avonmouth smelter (Spurgeon and Hopkin, 1996).

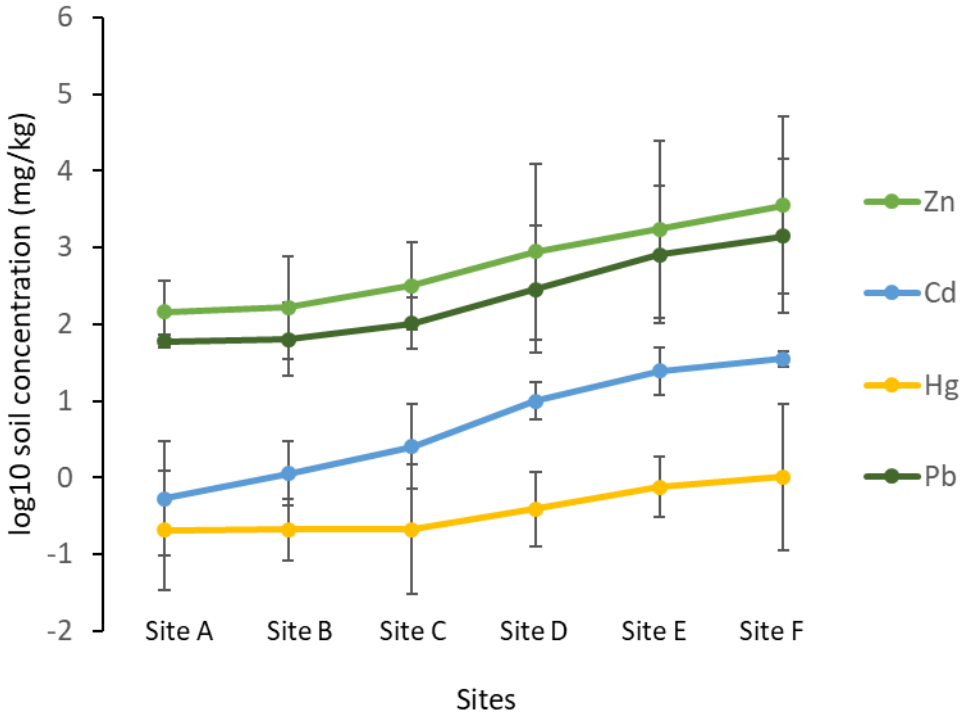


Figure 4.14. Log_{10} concentrations (mg/kg) (mean ± SD) of key metals in soils from a gradient of sites near the Avonmouth zinc and lead smelter. These metals show a statistically significant negative correlation with the distance from the smelter (R = -0.943, p = 0.0167). The sites are named in alphabetical order from the least to most polluted. The sample sizes from each site used in this analysis were the following: A n = 12; B n = 7; C n = 7; D n = 8; E n = 8; F n = 12.

Table 4.2: Amount of metals found in soil samples from a gradient of sites around the Avonmouth zinc and lead smelter (mg/kg dry weight, mean± SD).

	Aluminium	Phosphorus	Sulphur	Calcium	Titanium	Vanadium	Manganese	Iron	Cobalt	Nickel	Copper	Zinc	Selenium	Molybdenum	Cadmium	Antimony	Barium	Mercury	Lead	Chromium	Arsenic
Site	mgAl/kg	mgP/kg	mgS/kg	mgCa/kg	mgTi/kg	mgV/kg	mgMn/kg	mgFe/kg	mgCo/kg	mgNi/kg	mgCu/kg	mgZn/kg	mgSe/kg	mgMo/kg	mgCd/kg	mgSb/kg	mgBa/kg	mgHg/kg	mgPb/kg	mgCr/kg	mgAs/kg
$A_{Zn145}^{pH6.34}$	16600± 1769	1100± 30	679± 36	4370± 91	217± 56	33.9± 2.89	110o± 100	32907± 1290	19.4± 1.2	45.9± 3.4	37.5± 1.9	145± 7	0.882± 0.011	1.58± 0.0681	0.529± 0.0327	0.893± 0.08	114± 5	0.206± 0.027	59.1± 1.5	30.4± 2.1	18± 0.5
$B_{Zn166}^{pH6.57}$	10800± 600	590± 23	1080± 20	47100± 3100	28.3± 1.5	27.6± 1.86	568± 91	27800± 1500	9.79± 0.61	23.3± 1.2	6.49± 1.99	166± 22	0.495± 0.049	0.787± 0.0330	1.12± 0.146	0.740± 0.0748	53± 12	0.209± 0.155	63.6± 9.1	24.7± 0.6	10.7± 0.1
$C_{Zn315}^{pH6.43}$	16700± 2000	1210± 70	746± 19.	4800± 950	109± 10	29.1± 1.30	956± 79.3	23200± 2100	12.8± 1.1	26.5± 3.1	20.0± 2.8	315± 14	0.526± 0.032	0.825± 0.0297	2.54± 0.0781	0.958± 0.0450	80± 5.95	0.212± 0.02	102± 5	29.8± 3	7.19± 0.31
$D_{Zn879}^{pH7.07}$	16007± 1000	3010± 260	1570± 236	27200± 4200	74.1± 3.4	24.7± 0.929	955± 90.8	26300± 1200	12.9± 0.9	27.8± 1.7	47.3± 2.82	879± 201	1.06± 0.13	1.15± 0.118	9.97± 2.97	2.35± 0.131	168± 12	0.387± 0.105	285± 46	24.3± 1.9	9.1± 0.94
$E_{Zn1740}^{pH5.42}$	13003± 870	1590± 10	974± 204	5550± 1520	36.6± 5.5	30.4± 0.513	490± 72.6	29200± 200	11.5± 0.7	26.3± 2.	56.6± 7.9	1740± 200	2.02± 0.28	1.22± 0.0985	24.5± 4.21	5.33± 0.485	59.7± 3.4	0.751± 0.167	802± 61	28.5± 1.6	17.3± 2.4
$F_{Zn3533}^{pH6.68}$	20233± 4844	1507± 40.4	4463± 116	36700± 7217.3	122± 60.5	45.5± 6.52	838± 83	34033± 651	17.1± 1.12	39.9± 3.86	146.3± 4.93	3533± 206	4.17± 0.198	3.05± 0.0681	35.0± 1.65	9.45± 0.180	352.3± 87.8	1.01± 0.0121	1413± 103	39.2± 4.75	29.8± 0.361

The average metal concentrations found in earthworm tissues can be seen in Table 4.3. No statistically significant correlations could be established for the concentrations of metals with distance from the smelter. For example, when testing using the Spearman's rank correlation test, the *R* value for zinc was -0.428 ($p > 0.05$) and for lead it was -0.7 ($p > 0.05$). However, the earthworms from the most polluted sites contained significantly higher levels of copper, zinc, selenium, cadmium and arsenic (see Table 4.3) as detected by one way ANOVA.

Site / Metal	Copper	Zinc	Selenium	Molybdenum	Cadmium	Antimony	Lead	Chromium	Arsenic
	mgCu/kg	mgZn/kg	mgSe/kg	mgMo/kg	mgCd/kg	mgSb/kg	mgPb/kg	mgCr/kg	mgAs/kg
$A_{Zn145}^{pH6.34}$	1.65± 0.399***F	89.9± 21.3***F	0.223± 0.0616 ***F	0.169± 0.0649	0.369± 0.133***F	0.0460± 0.0560	1.018± 0.648***F	0.284± 0.201	0.238± 0.179**F
$B_{Zn166}^{pH6.57}$	1.78± 0.375**F	99.4± 67.1***F	0.194± 0.102 **F	0.0400± 0.0222	0.832± 0.839***F	0.0400± 0.0695	-	-	1.33± 1.85
$C_{Zn315}^{pH6.43}$	1.49± 0.302***F	78.2± 23.9***F	0.182± 0.064 ***F	0.156± 0.0339	1.65± 0.729 ***F, ***A	0.0482± 0.0455	0.189± 0.587	0.188± 0.155	0.0982± 0.104**F
$D_{Zn879}^{pH7.07}$	2.17± 1.39**F	161± 109	0.506± 0.469 *F	0.119± 0.0483	3.28± 3.41 ***F, *A	0.0031± 0.0814	1.10± 2.48	0.0901± 0.107	0.198± 0.120**F
$E_{Zn1740}^{pH5.42}$	1.59± 0.734***F	178± 89.1	0.488± 0.187 **F,***A	0.0802± 0.0341	15.9± 8.96 ***A	-	13.5± 10.2***A, *F	-	0.438± 0.479**F
$F_{Zn3533}^{pH6.68}$	3.23± 3.42	211± 69.3	1.23± 0.672	0.148± 0.0825	15.0± 5.42	-	17.7± 27.5	0.441± 0.646	4.53± 3.03

Table 4.3. Mean ± SD concentration (mg/kg dry weight tissue) of metals found in earthworms collected from five sites near the Avonmouth zinc and lead smelter and a control site. The sites are named in alphabetical order from least to most polluted (from A – control site to F – most polluted site). The significance values are * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$ followed by the site the group is significantly different to (A for the control site $A_{Zn145}^{pH6.34}$, F for the most polluted site, $F_{Zn3533}^{pH6.68}$)

4.3.2.2. Genotyping results

Comparison and alignment against reference sequences from the BLAST NCBI database indicated that two individuals aligned to the reference sequences for *Lumbricus terrestris*, two to *Lumbricus festivus* and the species identity of another specimen could not be clearly established, as it showed a 91% sequence similarity to both *Allolobophora hrabei* and *Eisenia oreophila*. These individuals were removed from further analysis. The remaining individuals all aligned with reference sequences for *L. rubellus* confirming the phenotypic identification. The majority (26 individuals) of the *L. rubellus* collected belonged to *L. rubellus* haplotype subgroup A3, 8 belonged to haplotype subgroup B and another 2 to haplotype subgroup A1 (See Figure 4.15). The phylogenetic distances between the two A lineages, A1 and A3 were 3.7% while the distances from A1 and A3 to B were 14.9% and 15.6%, respectively, indicating that the two A subgroups are inter lineage haplotypes, while the A and B sub-groups represent different lineages. The two A lineages separated from the reference sequence of *E. fetida* by 32.8% (A1) and 33.2% (A3), while the B lineage was separated form *E. fetida* by 32.1%. This was calculated using the Kimura 2 parameter method.

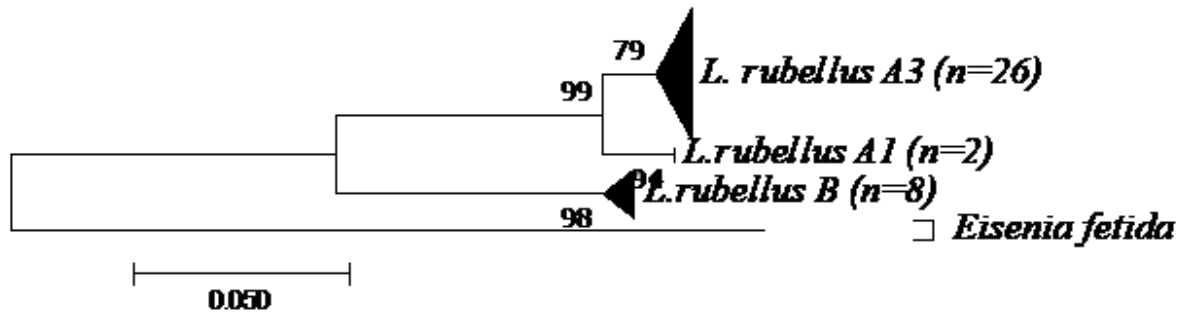


Figure 4.15. Evolutionary relationships between the *L. rubellus* collected from a gradient of sites near the Avonmouth zinc and lead smelter. Evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.479 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method in MEGA7 and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 216 positions in the final dataset.

The *L. rubellus* lineages were distributed unevenly between the different sites. Seven of the nine control individuals used for the molecular analysis belonged to lineage B, the only other B individual came from site $D_{Zn879}^{pH7.07}$.

To assess the relationships between lineages in order to understand the extent to which earthworms

from different haplotypes need to be treated separately within the downstream analysis, msAFLP analysis was performed, using all of the successfully genotyped individuals for whom msAFLP data was available to establish if the lineage background may have an impact on DNA methylation. This was found not to be the case. No clear, significant differences could be found between the distributions of methylation sites between the lineages of *L. rubellus* ($\phi ST = 0.05083$; $p = 0.177$. When comparing the lineages for AFLP markers, similarly, no clear differences could be observed ($\phi ST = 0.0219$; $p = 0.245$) (Figure 16).

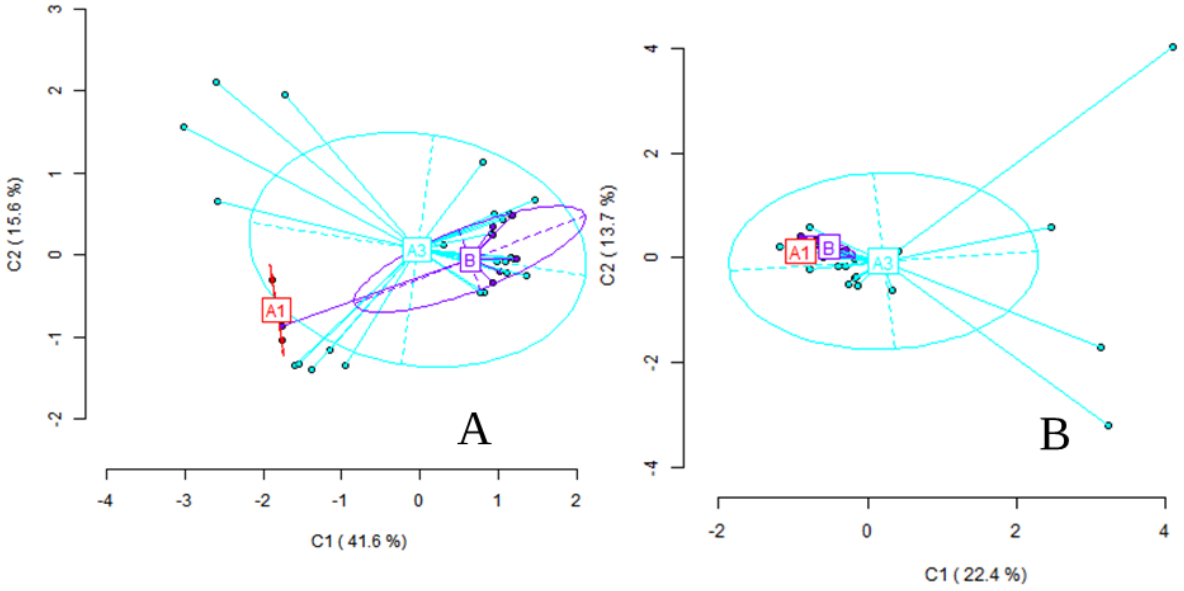


Figure 4.16. Principal Coordinate Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3' for *L. rubellus* were collected from polluted sites along a transect near the Avonmouth zinc and lead smelter (UK). Panel A shows the epigenetic variation (methylation-sensitive loci) and Panel B shows the genetic variation (AFLP). The earthworms are separated into lineages according to COI barcode sequences.

These findings are in contrast to those of Kille et al (2013) who showed a clear separation between A and B lineages for AFLP and msAFLP lineage earthworms as detected by both COII mitochondrial barcoding and AFLP (Kille *et al.*, 2012). This work, however, also reported the existence of hybrid earthworms whose mitochondrial markers indicated earthworms belonging to one lineage, but for which molecular AFLP markers showed a pattern intermediate between those for the A and B lineages. Andre et. al (2010) also found individuals clearly belonging to either lineage A or B, while some showed intermediate genotypes (Andre *et al.*, 2010). The contradictory results between the current study and the two previous studies may be due to the relatively small number of individuals in each genotyped group used in the experiment reported in this chapter, only seven B lineage

individuals were successfully genotyped in comparison to the 24 A3 lineage individuals. The result reported in this chapter may also be in part due to the broader geographical distribution of the sampling sites. It is possible that individuals collected for the current study belonged to the hybrid populations reported earlier and that is the reason why clear differences between the lineages could not be established using the AFLP markers. There is clearly some complexity in the relationship between the mitochondrial and nuclear genotypes among *L. rubellus haplotypes*, that similar to that for *E. fetida*, warrants further investigation (Plytycz *et al.*, 2018).

4.3.2.3. msAFLP comparisons between different sites

A total of 140 loci was found during the AFLP analysis. Of these, 57 (41%) were identified as methylation sensitive loci (MSL) and 83 (59%) were identified as non-methylation sensitive loci (NML). The Pairwise ϕST results showed no statistically significant differences between the sampling sites for either the MSL or NML loci (ϕST = 0.0763; p = 0.052 and ϕST = 0.01479; p = 0.1513, respectively), but a significant difference was present between the sites when comparing the genetic differences by exploring all loci within the AFLP analysis (ϕST = 0.0701; p = 0.0054) (See Figures 4.17;4.18;4.19). The significance found for all AFLP loci, but not the NML is likely to be related to the greater number of sites available for the former analysis.

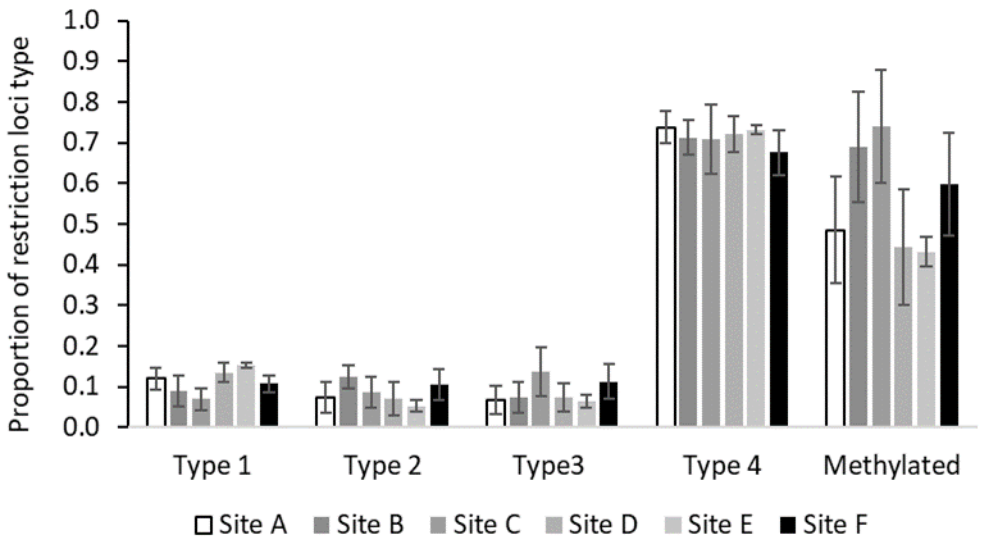


Figure 4.17. DNA methylation in methylation-sensitive loci detected from the analysed specimens of *L. rubellus* collected from five sites around the Avonmouth zinc and lead smelter and a control site (The naming was done in alphabetical order from the least to most polluted for zinc, with A being the control site and F the most heavily polluted). Type 1 to 4 are respectively: no methylated, methylation of internal C, methylation of external C or hemimethylation, and hypermethylation or mutation in restriction site (of the 5'-CCGG-3' sequence). Methylated: Global methylation level estimated following Nicotra *et al*, as proportion of (Type 2 + Type 3 loci / Type 2+Type 3 + Type 1(scorable loci)).

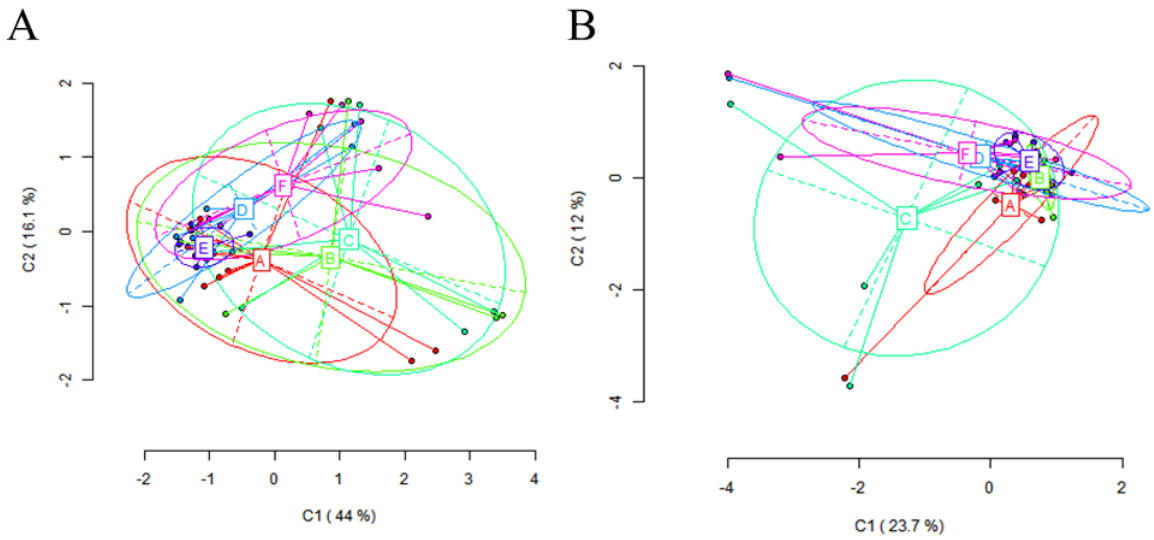


Figure 4.18. Principal Coordinate Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3'. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP) of *L.rubellus* collected from polluted sites from a transect near the Avonmouth zinc and lead smelter (UK).

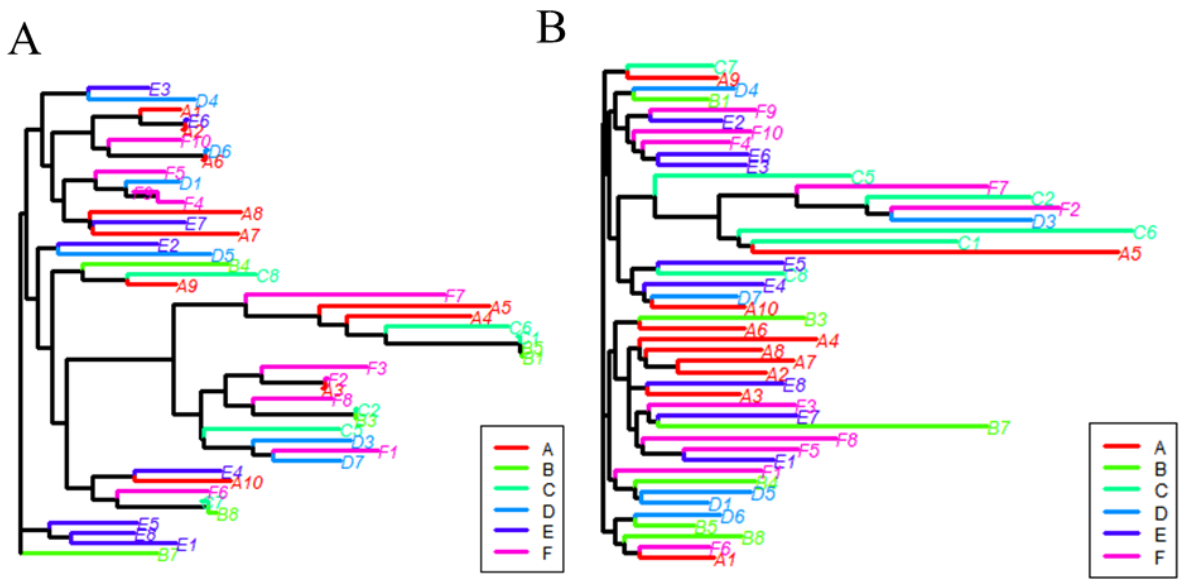


Figure 4.19. Neighbour Joining trees of the detected methylation and genetic polymorphism site distances in *L. rubellus* at the sequence 5'-CCGG-3'. The figure A shows the epigenetic variation (methylation-sensitive loci) and the figure B shows the genetic variation (AFLP of *L.rubellus* collected from polluted sites along a transect near the Avonmouth zinc and lead smelter (UK).

4.4. Discussion

The findings in this chapter show an interesting difference in molecular responses of earthworms when exposed to different classes of toxic chemicals and also the complexities of exploring wild populations of genetically diverse organisms.

Earthworms phenotypically identified as *L. rubellus* have been previously shown to represent two different lineages, A and B, which have been observed to clearly separate when comparing both mitochondrial DNA sequences and AFLP polymorphisms (Kille *et al.*, 2012). In the current field study, the earthworms collected from the smelter gradient separated into A and B lineages using the mitochondrial COI marker, but not by the AFLP loci or DNA methylation polymorphisms. Between the collection sites, no significant differences were found for earthworms from either lineage based on the DNA methylation, but there were significant genetic differences between sites as detected by AFLP. The absence of separation of AFLP data between the two lineages may have been due to the presence of hybrid individuals or more inbreeding in populations used in previous experiment (Anderson *et al.*, 2013). Individuals from site $C_{Zn315}^{pH6.43}$ were genetically distinct from individuals at all other sites. This site was also notable because, even though it was further from the smelter than site $B_{Zn166}^{pH6.57}$ (by 2.2 km), it was more contaminated with key heavy metals (zinc, lead, cadmium, copper) indicating an alternative route and/or source of pollution which might have acted as a driver of genetic separation at this site.

It has been reported before that immigrating organisms contain more DNA methylation variation than established populations and that DNA methylation variability positively correlates with inbreeding in a songbird species (Liebl *et al.*, 2013). In another study DNA methylation has been found to increase with inbreeding, potentially contributing to inbreeding depression (Vergeer *et al.*, 2012). The lack of clear separation into the AFLP profiles for lineages A and B observed in the current study may be because of the population histories of the *L. rubellus* at the sites studied. For example, if changes in DNA methylation are a marker of recent invasive events, it is possible that the populations studied currently are simply more established and more genetically diverse than those reported by Kille et al., 2013b and Andre et al., 2010. These studies were also carried out in mine sites where it is likely recolonization has occurred, which has likely been accompanied by botttleneck events. Kille et al (2013b) also reported a methylation difference only between the individuals of the lineage B, but in the current study majority of lineage B *L. rubellus* were collected in the control site and only one individual was collected from the more polluted sites, therefore not enough

earthworms belonging to this lineage were present in this study to establish a difference. In the laboratory study described in Chapter 4, after a 30 day long transplant using earthworms and soils from the $A_{Zn145}^{pH6.34}$ and $F_{Zn3533}^{pH6.68}$ sites there was an observable difference in DNA methylation patterns as detected by msAFLP. However, this experiment was run using only lineage A individuals, therefore it is likely that DNA methylation pattern changes are affected by the soils polluted by the Avonmouth smelter but are only visible in comparisons involving more genetically similar earthworms.

Earthworm growth was affected at the highest arsenic concentration analysed (36 mg/kg per dry weight soil). However, arsenic had no impact on DNA methylation as detected by msAFLP and no AFLP genetic marker differences could be found between the treatment groups. In a study reported by Kille *et al* (2013b) DNA methylation was affected by arsenic concentrations in soils in tolerant populations of *L. rubellus* collected from sites in an arsenic polluted mine. The concentrations found in the mine site soils were much higher than those used in the current experiment. For example, one of the control sites contained 310 mg/kg arsenic and the highest polluted soil 19200 mg/kg arsenic (Kille *et al.*, 2012), as compared to the highest concentration explored for DNA methylation effects in this chapter, 36 mg/kg. Hence, the long-term exposure at the mine site was to different arsenic concentrations than used in this study, although further work would be needed to understand the differences in bioavailability between the mine site and spiked soil. In the Kille *et al* (2013b) study the methylation differences were only observed when comparing lineage B individuals. In the current laboratory arsenic exposure earthworm, only 15 of the 63 successfully genotyped individuals were lineage B with the others belonging to lineage A (as reported in Anderson et al., 2013). The lineage B individuals were spread across the treatments. Therefore, even if DNA methylation was altered in these individuals after a life time exposure, the number of lineage B representatives in each treatment was too small to establish this pattern of response within the study.

The cadmium exposure affected earthworm growth at all of the exposure levels studied. Despite this effect, this metal had no impact on msAFLP profiles. This exposure was cut short due to mortality, therefore different results may be reported if the exposure had been carried out for its full intended length of 280 days. In another study which used coelomocyte samples of the earthworm *Lumbricus terrestris*, an adult exposure to 10 mg/kg Cd resulted in significantly altered DNA methylation profiles in individuals exposed for 4 weeks, but this difference was less apparent after 12 weeks in this study (as well as a replicate experiment reported in the same publication) (Šrut *et al.*, 2017). A 14 day long 50 mg/kg Cd exposure on *L. rubellus* and *L. terrestris* has been observed to induce the expression of metallothionein genes, which are responsible for regulating DNA methylation

(Drechsel *et al.*, 2017). Therefore it is possible DNA methylation in *L. rubellus* is indeed affected by cadmium, but that this is a short-term response and perhaps is induced in adults rather than if the exposure is carried out in juveniles from a freshly hatched stage as done here.

Fluoranthene had no effect on the growth of newly hatched juveniles, but the highest concentration of 800 mg/kg resulted in significantly diminished growth in earthworms at the age of 140 days, 28 days weeks after the worms had been placed in freshly spiked soil to account for fluoranthene degradation. When exploring the results of msAFLP, the treatment groups separated at a significant degree in terms of both differences in methylation and AFLP polymorphisms. However, the methylation differences were more significant, therefore it is most likely the DNA methylation differences are present due to fluoranthene exposure and not or only partially due to inherited differences. Furthermore, a concentration dependent effect on DNA methylation by fluoranthene was detected. Thus, the highest concentration treatment, 800 mg/kg, separated most from controls, followed by 70 mg/kg. The 20 mg/kg exposure largely overlapped with the control group. An effect of fluoranthene, on global DNA methylation status in a living organism has not been reported in any other study to date. However, fluoranthene has been found to impact gene expression in adult *L. rubellus* exposed for 28 days (Svendsen *et al.*, 2008). Exposure to fluoranthene has also been associated with higher expression of a cytochrome P4501A gene expression in chick embryos and associated promoter DNA methylation (Brandenburg and Head, 2018). As DNA methylation has an impact on gene expression (Jung and Pfeifer, 2015), it is possible it affects both molecular markers, although it is to be elucidated if fluoranthene has an effect on gene expression which results in DNA methylation or alters DNA methylation which results in an alteration of gene expression or if it is in fact a mixture of both effects. There is also evidence of fluoranthene causing the formation of DNA adducts (Babson *et al.*, 1986), which may alter chromatin structure which may in turn affect DNA methylation.Previous research on other polycyclic hydrocarbons (PAHs) (namely, benzo[*a*]pyrene) has found that they form DNA adducts, preferentially in DNA regions with higher levels of cytosine methylation and that prenatal exposure may have an impact on global DNA methylation (Herbstman *et al.*, 2012). Therefore it is possible fluoranthene elicits a similar effect in earthworms and possibly other organisms, this would make it a fruitful area of research.

4.5. Conclusion

In the study reported here, exploration of DNA methylation after toxic exposures for whole developmental periods and for many generations in real life exposures has revealed some interesting observations. Fluoranthene, a polycyclic hydrocarbon, was shown for the first time to have an effect on organisms' DNA methylation levels. Meanwhile, arsenic and cadmium did not show any effect on DNA methylation despite having been observed to illicit one in previous studies. Meanwhile, no differences in methylation levels were observed in individuals collected from a gradient of variously heavy metal polluted sites.

Chapter 5. Molecular mechanisms of adaptation to a highly polluted environment by the earthworm *L. rubellus* as detected by RNAseq.

5.1. Introduction

The Avonmouth zinc smelter near Bristol, UK, ceased its operations in 2003 after seven decades of work which caused the release of large amounts of atmospheric pollution of zinc, lead, arsenic and other metals to the surrounding area (Sims *et al.*, 2009). Ten years later soil in the area around the smelter was still heavily polluted (unpublished results), therefore it is fertile ground for exploration of the effects of long-term metal pollution on terrestrial species.

The earthworm *L. rubellus* is a sentinel species in ecotoxicological studies of metal pollution (Spurgeon *et al.*, 2004). An extremophile population of these earthworms has adapted to living in the highly contaminated soil near Avonmouth zinc and lead smelter and is one of only three species of earthworms able to survive in the more metal polluted soils, alongside *Lumbricus castaneus* and *Lumbricus terrestris* (Spurgeon and Hopkin, 1996). The molecular adaptations behind this success are so far unknown. Exploration of another population of *L. rubellus* adapted to heavy metal pollution near a mine has revealed results which imply that both genetic mutations and DNA methylation may play a role in their adaptation. A genetically diverse lineage of the earthworm appeared to have alternate patterns of DNA polymorphisms as a result of long term exposure to high levels of pollution, while a more inbred lineage contained alternate patterns of DNA methylation (Kille et al., 2012). These were detected using the AFLP (amplified fragment length polymorphism) and msAFLP (methylation sensitive amplified fragment length polymorphism) techniques, which can indicate the adaptive processes at a genome wide scale for both genetic and DNA methylation polymorphisms. *L. rubellus* has a (unpublished) sequenced genome which provides the opportunity to explore genome wide molecular mechanisms of adaptation to highly polluted environments by employing next generation sequencing assays. In this experiment, RNAseq of alternatively expressed genes will be used in order to understand which cellular processes are involved in the adaptation.

There is a growing amount of research on the genome wide molecular effects of metal pollution on a variety of species. For example, lead exposure to radishes (*Raphanus raphanistrum subsp. sativus)* resulted in changes in the expression of a large number of transcripts (detected by RNAseq), with upregulation in genes involved in cell wall protection response, glutathione pathway and downregulation in genes involved in carbohydrate metabolism (Wang *et al.*, 2013). In an experiment using the springtails *Orchesella cincta* adapted to mine site pollution, genes involved in structural integrity of the cuticle, anti-microbial defence, calcium channel-blocking, sulphur assimilation and chromatin remodelling were upregulated due to Cd exposure and adaptation to metal contamination and carbohydrate metabolic processes, Ca^{2+}-dependent stress signalling, redox state, proteolysis and digestion were downregulated after exposure in control animals (Roelofs et al. 2009). More than 500 alternatively expressed genes were found in dark septate endophytes exposed to cadmium (Zhao et al., 2015) while in a study which looked at the transcriptome of the microalga *Chlamydomonas acidophila* treated with copper it was found that less than fifty genes had altered expression despite the LD50 concentration of copper used (Olsson *et al.*, 2015). Hence, there is convincing species wide evidence of significant transcriptional remodelling in response to metal exposure.

To date, the studies of gene expression change in response to metal mostly do not expressly look at the role of epigenetic alterations in changes in gene expression. There are, however, some indications that these mechanisms may be important. In the microalga *C. acidophila* a DNA methyl transferase and two histone deacetylases were downregulated in response to copper. This implies a change in chromatin and DNA methylation after the exposure (Olsson *et al.*, 2015). In another study of Cd exposed maize plants, genes related to chromatin assembly were also significantly affected (Peng et al., 2015). In the above cited study on springtails chromatin alteration related genes were found to be upregulated as a result of heavy metal exposure, both in the short and the long-term (Roelofs et al. 2009).

Some metal ions can also have a direct impact on the enzymes related to chromatin regulation. For example, histone deacetylases require zinc ions in order to catalyse deacetylation while excess zinc has been found to inhibit this reaction (Polsinelli and Yu, 2018). Iron has been found to affect lysine demethylases activity (Ganesan, 2018). Therefore, metal pollution may have a biochemical impact on the functions of proteins affecting epigenetic marks. In order to explore the genome wide gene expression changes and underlying epigenetic alterations in the extremophile *L. rubellus* population, a common garden reciprocal transplant experiment was carried out using individuals from the heavily polluted environment and a control site, exposing earthworm from each population to both their own

and the alternative site soil. Epigenetic analysis using msAFLP and gene expression analysis using RNAseq techniques were used on the samples to explore the global DNA methylation levels and to compare gene expression of adapted and control *L. rubellus*.

5.2. Materials and Methods

The experiment was carried out with *L. rubellus* and soil collected from a site close to the Avonmouth zinc smelter and a clean site located in the region, but outside the polluted area. The two groups of earthworms and soil will be referred to as polluted and control throughout the text. A map with the locations of both sites can be seen in Figure 4.1, where they are referred to as sites A (control) and F (Polluted). The *L. rubellus* and soils were collected on a minimum of 10 kg of own site soil in sandbags and were taken to the laboratory and placed in a 13^{o}C controlled temperature room until used for the experiment.

5.2.1. Soil preparation

The soils used for the earthworm exposures (minimum 50 kg) were collected from the top 5 cm of soil from both sites and returned to the laboratory. The experimental design was based on a reciprocal transplantation experimental design in which earthworms from each site were both maintained on their own soil, as well as being transferred to soil from the alternate site. Based on the use of a single control and polluted site, the design generated four different treatment combinations for assessment (Control earthworms: Control soil (CC); Control earthworms: Polluted soil (CP); Polluted earthworms : Control soil (PC); Polluted earthworms : Polluted soil (PP)). To prepare the soils for use in the study, both the polluted and control soils were air dried and then frozen at -20^{o}C to remove the indigenous earthworm fauna. Soils were sieved through a 2 mm mesh to remove larger stones, plant debris and the majority of any earthworm cocoon present. Samples of the original soil were oven dried to establish the original moisture content, which was 33% for both soils. After drying and screening, 500g of each soil was placed in each 500 ml container and distilled water added to raise soil moisture content to the value determined for the field soil.

5.2.2. Reciprocal transplant experiment

For the transplant exposure, a total of 144 individuals, 72 from polluted, 72 from control soil were used for the experiment. Within the design, 36 individuals were exposed to each soil, with 18 pairs exposed to each of the four population-soil treatment combinations. To investigate the time course of the effect invoked on transplantation, 6 replicate pairs of earthworms (total 12 individuals) were

sampled from each population and soil combination after 3, 30 and 90 days of exposure. Therefore, there were 12 treatment groups sampled representing exposure and time each with 6 replicate containers containing up to 12 individuals for each treatment combination and time point (see Table 5.1).

Table 5.1. *L. rubellus* transplant experimental groups and sampling times used for the experimental study		
3 days	30 days	90 days
Control worms, Control soil (3dCC) (n = 12)	Control worms, Control soil (30dCC) (n = 12)	Control worms, Control soil (90dCC) (n = 12)
Control worms, Polluted soil (3dCP) (n = 12)	Control worms, Polluted soil (30dCP) (n = 12)	Control worms, Polluted soil (90dCP) (n = 12)
Polluted worms, Control soil (3dPC) (n = 12)	Polluted worms, Control soil (30dPC) (n = 12)	Polluted worms, Control soil (90dPC) (n = 12)
Polluted worms, Polluted soil (3dPP) (n = 12)	Polluted worms, Polluted soil (30dPP) (n = 12)	Polluted worms, Polluted soil (90dPP) (n = 12)

For the exposure, the *L. rubellus* pairs were kept at 13^{o}C in a constant temperature facility under a 12 h: light, 12 h: dark regime. The earthworms were fed once a month at day 0, 30 and 60 with 1.5 g (dry weight) of horse manure per individual (horse manure prepared the same way as described in Section 2.2.3.2. At each sampling point, the surviving earthworms were retrieved from the soil, washed in deionised water and weighed prior to dissection. The dissections were carried out the same way as described in Section 4.2.3 and the obtained fragments stored in 1.5 ml Eppendorf tubes, snap frozen in liquid nitrogen and stored in – 80^{o}C to obtain samples for the analysis of both gene expression using RNA sequencing and DNA methylation status, as well as tissue metal concentrations. To provide an assessment of the effects of exposure on reproduction, the soils from each container were wet sieved to allow the number of laid cocoons to be counted. The number of cocoons was comparted to the number of surviving earthworms to allow the cocoon production rate (cocoon/worm/week) to be calculated.

The transplant groups are thus referred to in the rest of the chapter: CC – Control earthworms : Control soil; CP - Control earthworms : Polluted soil; PC – Polluted earthworms : Control soil; PP - Polluted

earthworms : Polluted soil. Where necessary, the number days the individuals were exposed for during the experiment was added before the treatment combination name to identify the sample time and type (e.g. 30dCC).

5.2.3. DNA extraction and genotyping

The frozen samples collected from the anterior end of the earthworms were ground up and their DNA isolated and the mitochondrial COI gene sequenced the same way as described in Sections 2.2.8, 2.2.9 and 2.2.10. This was done using samples from all of the transplant groups.

5.2.4. Methylation sensitive amplified length polymorphism (msAFLP) analysis

The msAFLP analysis was done using DNA extracted from the intestinal tissues (the same way as described in Section 4.2.3) of earthworms from all twelve transplant groups using all of the individuals in each group. The msAFLP reactions and analysis were carried out as described in Section 4.2.5.

5.2.5. RNA extraction and sequencing

The dissected intestinal samples were placed in RNA*later*™-ICE frozen tissue transition solution (ThermoFisher Scientific, Gloucester, UK) for storage, cut up and cleaned to get rid of soil and other contaminants, placed in TRI reagent™ (Applied Biosystems, Thermo Fisher Scientific, Birchwood, UK) and homogenized using a pestle. The RNA was then extracted using the Direct-zol™ RNA MiniPrep (Zymo Research, Cambridge Bioscience, Cambridge, UK) extraction kit according to the producer's instructions, it was then cleaned using RNA clean & concentrator™ (Zymo Research, Cambridge Bioscience, Cambridge, UK). RNA quality was checked using Qubit (Thermo Fisher Scientific, Hemel Hemsptead, UK) and it was kept on dry or regular ice between analyses. The isolated RNA was stored at -80°C before sequencing with an Illumina HiSeq4000 machine at the Wellcome Trust Cancer Research Centre in Oxford. Raw reads were trimmed with Trimmomatic v0.36 (Bolger et al., 2014) in paired end mode, read quality was subsequently assessed with Fastqc v0.11.7. 24 intestine samples from the 30 day exposure group were used for RNA seq analysis, six individuals were used from each exposure group.

5.2.6. Data analysis of survival reproduction and weight data.

The survival and reproduction data obtained was tested for normality using the Shapiro-Wilks test and a 2-way ANOVA with two levels was used to assess statistically significant differences between the groups. This analysis was run using the R statistical software.

5.2.7. RNASeq data analysis

This part of the analysis was carried out using the process is described in detail below. The overall scheme can be seen in Figure 5.1.

5.2.7.1. Phylogenetic analysis using full mitochondrial sequences

Reads belonging to mitochondrial DNA were extracted using BBTools' v37.10 mapPacBio (Khan et al. 2012) utility to map the trimmed reads to a reference mitochondrial genome with low stringency. Different mitochondrial genomes were used as a reference for this process corresponding to the lineage (A and B) corresponding to that derived using the initial COI genotyping. Consensus sequences for aligning the samples to reference were created using SAMtools v1.5 and BCFtools v1.7 (Li *et al.*, 2009) to produce a full mitochondrial genome. The mitochondrial genomes were then annotated using MITOS and full COI sequences were extracted for posterior phylogenetic analysis using MEGA7 software (Kumar *et al.*, 2016).

5.2.7.2. Phylogenetic analysis using nuclear gene sequences

The sequences used for nuclear phylogenetics were derived from the output of evaluation of individual transcriptome assemblies produced using BUSCO v3.0.2 (Waterhouse et al., 2017). Contigs identified as containing complete, single copy orthologs in all the samples were used. The longest reading frame was identified and translated using TransDecoder's v5.5.0 in order to produce nucleotide and corresponding amino acid sequences. These sequences were then used to construct phylogenetic trees using a concatenated alignment of the genes using MEGA7 (Kumar et al., 2018).

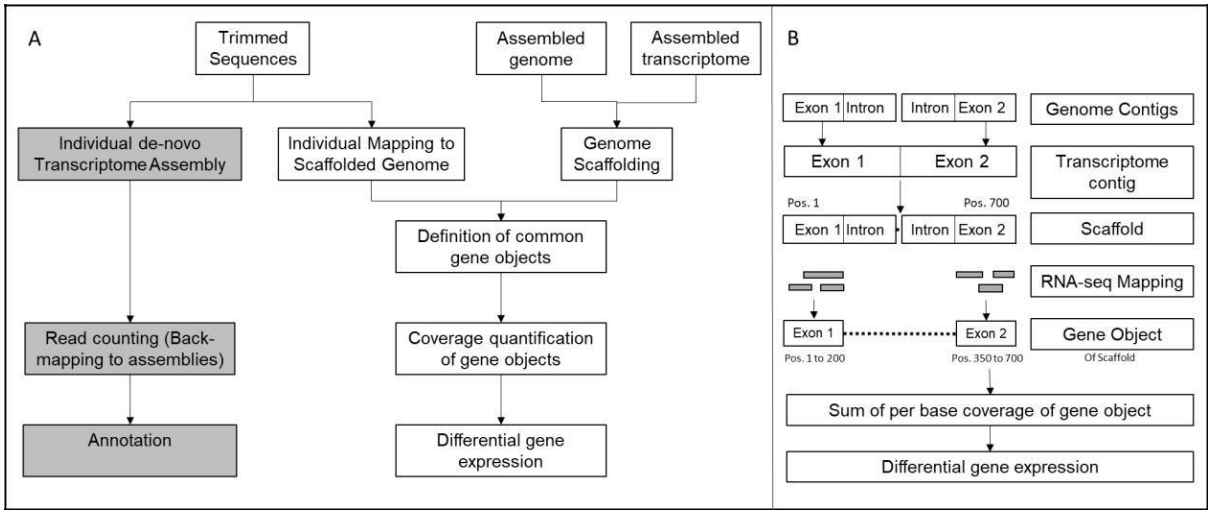


Figure 5.1 . Explanatory diagrams of the approaches followed for gene object creation. (A) Conceptual scheme of the approach of gene object creation. Main approach that produced the presented results in white, secondary approach in grey. (B) Simplified explanatory diagram of the main approach at the sequence level.

5.2.7.3. Gene object creation

Due to the complexity of the data which contained very genetically diverse earthworm RNA sequences, it was necessary to use different pipelines to create common gene objects to allow the quantification of the expressed genes. In the current study, a "gene object" is defined as a sequence or a set of coordinates that map onto gene features in a common reference sequence. A summary of the informatic workflow used into order to obtain gene objects for comparable differential expression across the *L. rubellus* samples is provided in Figure 5.1.

In order to join fragments of genes that may be located on several different contigs, a pre-existing *L. rubellus* lineage B genome assembly was scaffolded to a lineage B transcriptome assembly composed of several tissues of 5 individuals using L_RNA_Scaffolder and PEP_scaffolder (Xue *et al.*, 2013). The necessary alignments were produced using Blat v. 36x2 (Kent, 2002) and the resultant assemblies were evaluated using BUSCO. BlobTools (Laetsch et al., 2017) was used for the evaluation of coverage, GC content, and potential matches for annotation.

Trimmed sequences from each sample were mapped individually, using BBMapper v37.10 (Bushnell, 2014), onto the scaffolded genome at low stringency conditions, with maximum allowed intron length of 100,000 bp, all ambiguously mapped reads were retained. The potential transcripts were assembled and conglomerated into a list of commonly found non-redundant transcripts using Stringtie v1.3.5 (Pertea et al., 2016). After this, several possible transcripts of varying lengths and numbers of exons per "gene" were attained, the longest version of the assembled transcripts were kept as gene objects. Lastly, the SAMtools' v1.5 (Li *et al.*, 2009) bedcov function was used to quantify abundance, extracting the sum of per base coverage of the regions described by the gene objects from the sample-scaffolded transcriptome alignments.

5.2.7.4. Differential Expression analysis

The R package DESeq2 (Love *et al.*, 2014) was used to carry out pairwise comparison tests of differential expression between the different exposure groups. This was done using the SARTools DEseq2 workflow. (Varet *et al.*, 2016). The Benjamini-Hochberg method was applied to produce *p*-

values to establish significant differences between the sample groups. These values were adjusted for a 0.05 false discovery rate (Varet *et al.*, 2016).

5.2.7.5. Gene object annotation

TransDecoder v5.5.0. (Haas *et al.*, 2013) was used to extract the sequences defined by the common gene objects from the scaffolded genome and to translate and predict the longest most likely reading frame. The resulting peptide sequences were queried against the SwissProt database (Downloaded Feb 2019) using blast v2.4.0 (McGinnis and Madden, 2004) with an e-value cut-off of <1xE-10. The hits with the lowest e-values were used to annotate the gene objects.

5.2.7.6. Functional analysis

Functional analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 webtool (Huang *et al.*, 2009) was carried out with differentially expressed transcripts. The relevant gene ontology terms were visualized with ReViGO (Supek *et al.*, 2011). Search and reporting for epigenetically relevant genes was carried out using Microsoft Office Excel. Venny (Oliveros,2007-2015) was used for comparing gene expression differences.

5.3. Results

5.3.1. Soil physiochemistry results

The soil metal content, pH and organic content of both the polluted and control sites can be seen in Tables 4.1 and 4.2, where these are referred to as Site A (Control) and Site F (Polluted). The soil pH content for the control soil was 6.34 and for the polluted soil it was 6.68, therefore this is unlikely to have a great effect on the resident earthworms. The metal content showed huge differences between the two soils, with, for instance, the amount of zinc in control soil being 145±7 mg/kg (per dry weight soil) while in the polluted soil it was 3533±206 mg/kg, therefore the two soils provide sufficiently contrasting environments for experimentation.

5.3.2. Survival

There was a 100% survival rate within all groups after 3 and 30 days and after 90 days for the CC earthworm. The CP and PC earth worms had an 83.3% survival rate while PP earthworms had a survival rate of 91.7% at the 90 day exposure. No significant differences in survival between the groups were observed (ANOVA, $p>0.05$) at any of the three time points. As there were only twelve individuals in each group, the absence of a significant effect between treatments may not exclude the possibility of

a weak effect on survival in a similar experiment conducted at larger scale. However, that an effect was not observed in this case does suggest that 1) the experimental conditions used were suitable for earthworm survival; 2) that the polluted soil did not contain metal levels at concentrations sufficient to cause substantial mortality in the control *L.rubellus*, at least not within 90 days; and 3) that the adaptations accumulated by earthworms from the polluted site did not greatly reduce their ability to survive in a less polluted environment.

5.3.3. Cocoon production

The average cocoon number per *L. rubellus* pair was lower among the pollution adapted individuals after 90 days of exposure in comparison to control *L. rubellus* (See Table 5.2). However, as there were only 12 earthworms per group, the sample size was not sufficient to establish a trend. Consequently, no significant differences in cocoon production between the groups were observed (ANOVA, $p>0.05$), indicating that neither incubation of control individuals in the polluted soil nor of the adapted individuals in clean soil had a clear effect on reproduction. It is unknown if any other effects on other reproductive traits might have been caused in the experiment as testing of cocoon viability was not carried out.

5.3.4. Weight differences

There was no significant difference in weight change among the groups sampled after 3 and 30 days exposure (2- way ANOVA). At these stages, all groups gained a small amount of weight (see Table 5.2). After 90 days, only the control *L. rubellus* placed in polluted soil had not on average lost weight. This unexpected finding might be an indication of an adaptation mechanism in which the earthworms increase their metabolism and, thus, consume more food in order to cope with the increased metal exposure. It is also feasible that this is due to avoidance behaviour, that the earthworms might have consumed rather more of the organic content rich manure than the polluted soil.

Table 5.2. Average weight change per individual compared to initial weight and cocoon production of *L. rubellus* collected from a polluted and control site either maintained on own site soil or transplanted onto either control or polluted site soil.					
Treatment	Weight difference (g) after 3 days	Weight difference (g) after 30 days	Cocoon count after 30 days (cocoon/worm/ 30 days)	Weight difference (g) after 90 days	Cocoon count after 90 days (cocoon/worm/ 30days)
CC	+0.0687±0.0372	+0.0053±0.0799	4.42±1.74	-0.205±0.289	4.73±2.19
CP	+0.0361±0.0419	+0.0067±0.0467	4.17±1.57	+0.012±0.151	4.38±3.14
PC	+0.0589±0.0194	+0.0615±0.0403	3.92±2.27	-0.17±0.1755	2.72±1.65
PP	+0.0885±0.0302	+0.0703±0.0455	3.42±1.66	-0.193±0.132	2.47±1.39
Each value (mean ±SD) calculated on the basis of six replicates with two *L. rubellus* per replicate, the weight differences were calculated by the equation: weights after – weight before = difference. Statistical significance calculated comparing control group (CC) with each treatment. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$.					

5.3.5. Earthworm metal accumulation

Mean tissue concentrations for a range of metals in the tails of *L. rubellus* from the experimental treatment groups with associated significant differences between treatments, are presented in Table 5.3. Higher tissue concentrations of zinc, lead, copper, cadmium and arsenic were present in the earthworms exposed and/or sampled from the polluted soil. After only 3 days of exposure to polluted soil the CP earthworms had levels of zinc which were almost four times higher, levels of cadmium which were ten times higher and levels of lead which were seventeen times higher than in the CC individuals. The PC *L. rubellus* retained higher concentrations of cadmium, zinc, lead and arsenic in tissues than the CC earthworms even after being placed in control soil for 30 days. Interestingly, these individuals achieved similar levels of copper to CC worms within three days, but still had higher levels of arsenic than both CC and CP individuals even after 30 days, suggesting different mechanisms for the handling of these element in this species. The PP earthworms had higher amounts of arsenic, zinc and cadmium than the CP *L. rubellus* even after 30 day exposure indicating slow toxicokinetics. These findings imply that the adaptation strategy of the earthworm population living near Avonmouth does not include a mechanism for faster elimination of trace metals found in the soil. However, there is the potential that elevated sequestration, potentially in a toxicologically unavailable fraction, may play a role in adaptation to increased metal exposure.

Table 5.3. Mean ± SD concentration (mg/kg dry weight tissue) of metals found in *L. rubellus* earthworm tissues after a transplant experiment from the CC, CP, PC, PP treatment for 3 and 30 days.

Site / Metal	Control worms, Control soil (CC)		Control worms, Polluted soil (CP)		Polluted worms, Control soil (PC)		Polluted worms, Polluted soil (PP)	
	3 days	30 days	3 days	30 days	3 days	30 days	3 days	30 days
Copper mg Cu/kg	4.96 ± 0.97	3.89 ± 1.25	10.1 ± 3.94 ***CC	11.3 ± 5 ***CC	4.83 ± 0.74	3.08 ± 1.16	13.7 ± 3.18 ***CC	9.13 ± 4.36 ***CC
Zinc mg Zn/kg	111 ± 37.5	147 ± 113	388 ± 121 ***CC	340 ± 150 ***CC	182 ± 84.7 *CC	234 ± 98.7	486 ± 141 ***CC	471 ± 250 ***CC
Selenium mg Se/kg	0.32 ± 0.1	0.47 ± 0.27	1.16 ± 0.24	0.6 ± 0.17	1.37 ± 0.46	1.52 ± 0.56	1.97 ± 0.61	3.02 ± 2.01
Molybdenum mg Mo/kg	0.27 ± 0.07	0.31 ± 0.16	0.43 ± 0.11	0.34 ± 0.08	0.21 ± 0.03	0.21 ± 0.03	0.32 ± 0.07	0.319 ± 0.1
Cadmium mg Cd/kg	0.44 ± 0.19	0.61 ± 0.44	4.9 ± 1.28 ***CC	3.42 ± 1.46 ***CC	9.38 ± 4.13 ***CC	13.07 ± 6.84 ***CC	15.89 ± 8.25 ***CC	18.1 ± 14.3 ***CC
Lead mg Pb/kg	5.16 ± 1.33	3.39 ± 1.56	89.1 ± 46.3 ***CC,*PP	106 ± 59.23 ***CC	9.68 ± 2.12 ***CC	9.62 ± 4.51 ***CC	134 ± 53.2 ***CC	89.1 ± 57.6 ***CC
Chromium mg Cr/kg	2.73 ± 0.73	1.66 ± 0.9	1.8 ± 0.67	2.11 ± 0.79	2.7 ± 0.59	1.27 ± 1.07	2.18 ± 0.6	1.56 ± 0.65
Arsenic mg As/kg	1.73 ± 0.57	1.31 ± 0.55	2.4 ± 0.68 *CC	2.29 ± 0.73 **CC	7.3 ± 2.6 ***CC	5.02 ± 2.2 ***CC	7.1 ± 2.62 ***CC	9.64 ± 7.19 ***CC

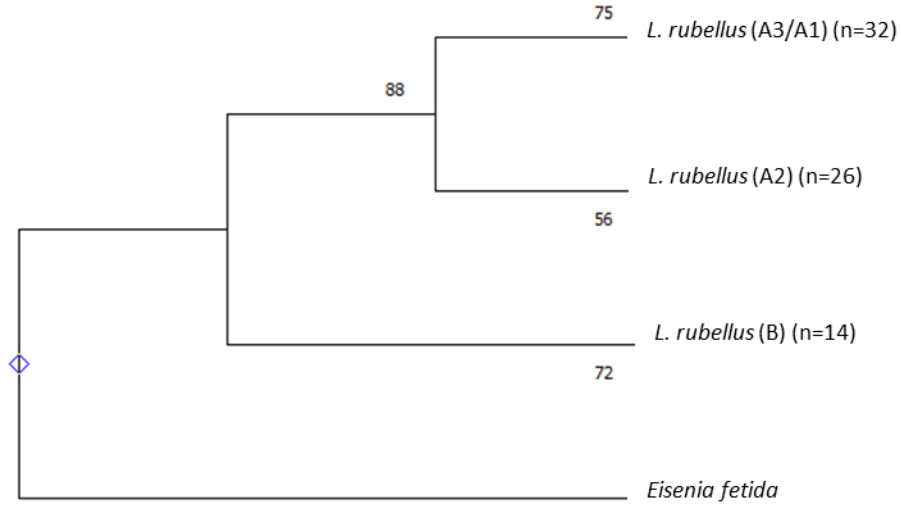
The significance values are * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$ followed by the treatment the group is significantly different to.

5.3.6. COI phylogenetic analysis

Phylogenetic analysis of the successfully genotyped individuals indicated that four earthworms were not *L. rubellus* and instead belonged to the species *Aporrectodea calignosa*, *Lumbricus festivus*, *Lumbricus terrestris* and a miscellaneous *Lumbricidae sp*. These were removed from further analysis. Of the transplant *L. rubellus* individuals, 14 were found to belong to the *L. rubellus* lineage B and 78 to the lineage A. These two *L. rubellus* lineages have been previously described to have very different AFLP profiles and to respond to pollution stress differently in terms of global DNA methylation changes as detected by msAFLP (Kille *et al.*, 2012). Given the high numbers for Lineage A, it was decided to perform the msAFLP analysis only on individuals from this lineage and to exclude lineage B due to the low sample size. In the experiment reported in Chapter 4, no msAFLP effects due to lineage background were observed, however, as the number of individuals used in the current study allowed the exclusion of the B individuals, it was decided to do so to avoid potential lineage issues with the interpretation of the results.

The phylogenetic relationships between the lineages found in this experiment can be seen in Figure 5.2. The COI analyses indicated that the Lineage B *L. rubellus* were a more homogenous clade for this marker than lineage A. The greater similarity of COI sequence among lineage B, compared to lineage A *L. rubellus* is consistent with previous findings (Andre *et al.*, 2010). There was an average 10.6% difference in COI sequences between lineage A and B individuals consistent with their previous identification as separate cryptic linages. There was a maximum 4.6% difference between the A sublineages, commensurate with previous finding of this group as a complex single clade (Andre *et al.*, 2010).

All lineage B individuals were collected at the control site, while the lineage A *L. rubellus* contained individuals from both the control and polluted sites. This possibly implies the reduction in genetic diversity in the polluted site due to previous bottleneck events either due to the presence of the heavy metal pollutants or potential other causes. In a previous study, (Spurgeon et al. 2016) it was shown that the lineage composition of *L. rubellus* populations was significantly related to site soil properties, notably pH and organic matter composition. It is possible that *L. rubellus* lineage A is more successful in colonising sites due to the higher diversity within this lineage in this area and are therefore more likely to exhibit the phenotypic plasticity needed to be present under the environmental condition prevailing at the sites. The greater genetic diversity found in this lineage may be an explanation for this success.



	L.rubellus A2	*L. rubellus* A3/A1	*L. rubellus* B	*L. rubellus* A
L. rubellus A2				
L. rubellus A3	0.046			
L. rubellus B	0.096	0.101		0.106
E. fetida	0.208	0.267	0.185	0.252

Figure 5.2. Phylogenetic relationships between *L. rubellus* clades based on COI sequencing
The phylogeny was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.32937259 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 95 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 55 positions in the final dataset. The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Analyses were conducted using the Jukes-Cantor model. The analysis involved 95 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 55 positions in the final dataset. The sequences used for this analysis were of the mitochondrial COI gene. The individuals analysed came from a transplant experiment where *L. rubellus* had been collected from a heavily polluted site near the Avonmouth zinc smelter and a control site and transplanted between soils from sites for 3,30 and 90 days.

5.3.7. Global DNA methylation and DNA polymorphism differences between the treatment groups

In the earthworms transplanted for 3 days, a total of 242 restriction loci was found using msAFLP analysis, 63 (26%) of these were methylation susceptible loci (MSL) and 179 (74%) were no methylation susceptible loci (NML). The R package msap used for running the msAFLP data produced results of Phi coefficient statistical analysis (ϕST) (Horst, 1954;Šrut et al., 2017). ϕST analysis showed a significant difference between the exposed groups using comparisons between AFLP, but not MSL loci (ϕST = 0.05965; p <0.0001 and ϕST = -0.009017; p = 0.6427, respectively). Therefore, significant differences between the genetic backgrounds of the 3 day transplantation groups (CC, CP, PC and PP) could be detected, but no significant differences were observed between the DNA methylation profiles of these groups.

For the *L. rubellus* transplanted for 30 days, the analysis of methylated and non-methylated loci indicated significant differences in both DNA methylation and the DNA polymorphism profiles different between the treatment groups. The significance values for the AFLP profile differences were ϕST = 0.02 and p = 0.02, while these values for the comparisons between the DNA methylation profiles were ϕST = 0.051 and p = 0.004. These were calculated using a total of 557 AFLP loci, 139 (25%) of these methylation sensitive and 418 (75%) NML. As visible in Figure 5.3, the CC group can be clearly distinguished from the other three groups in terms of their DNA methylation profile, with the PP and PC groups spate and overlapping and the CP group placed mid-way between the PP and PC and the CC groups. A similar pattern cannot be seen based on AFLP profiles. This implies that the 30 day transplant groups show clearly distinguishable DNA methylation patterns between the CC and PP groups and that the CP group has likely retained some patterns characteristic to its Control background but has also moved towards a more similar pattern to the PP earthworms. As reported in the physiochemistry results, the PC group had retained a significant fraction of the metal present in tissues. This suggests that the similarity of the DNA methylation profile of the PC group to the PP earthworms may reflect the genetic background, exposure history and metal retention of the transplant PC individuals.

A total of 604 AFLP loci, 168 (28%) of these MSL and 436 (72%) NML were found in the 90 day samples. The visual representations of the relationships between the genetic difference and the DNA methylation profiles can be seen in Figures 5.3;5.4. In the 90 day transplant groups, a clear picture of DNA methylation patterning is not seen between the treatment groups. Thus, although a somewhat

similar pattern where the CC group was different from all of the other groups, whilst the PP,PC and CP groups clustered together was observed, the differences in DNA methylation profiles were not statistically different between groups (ϕST = 0.03866 ; *p*= 0.057), The *p* value was though close to the significance threshold. The genetic AFLP differences present between the transplant groups were statistically significant to a greater degree than for the 30 day sampled earthworms ϕST = 0.02275 and *p*= 0.0043, it is possible that this genetic differentiation interacted with the MSL profiles and in a more genetically homogenous populations a significant difference between the DNA methylation profiles could be found for the 90 day exposure group.

When comparing the proportions of the amounts of different sites exhibiting hemimethylation, no statistically significant differences could be observed (See Figure 5.5).

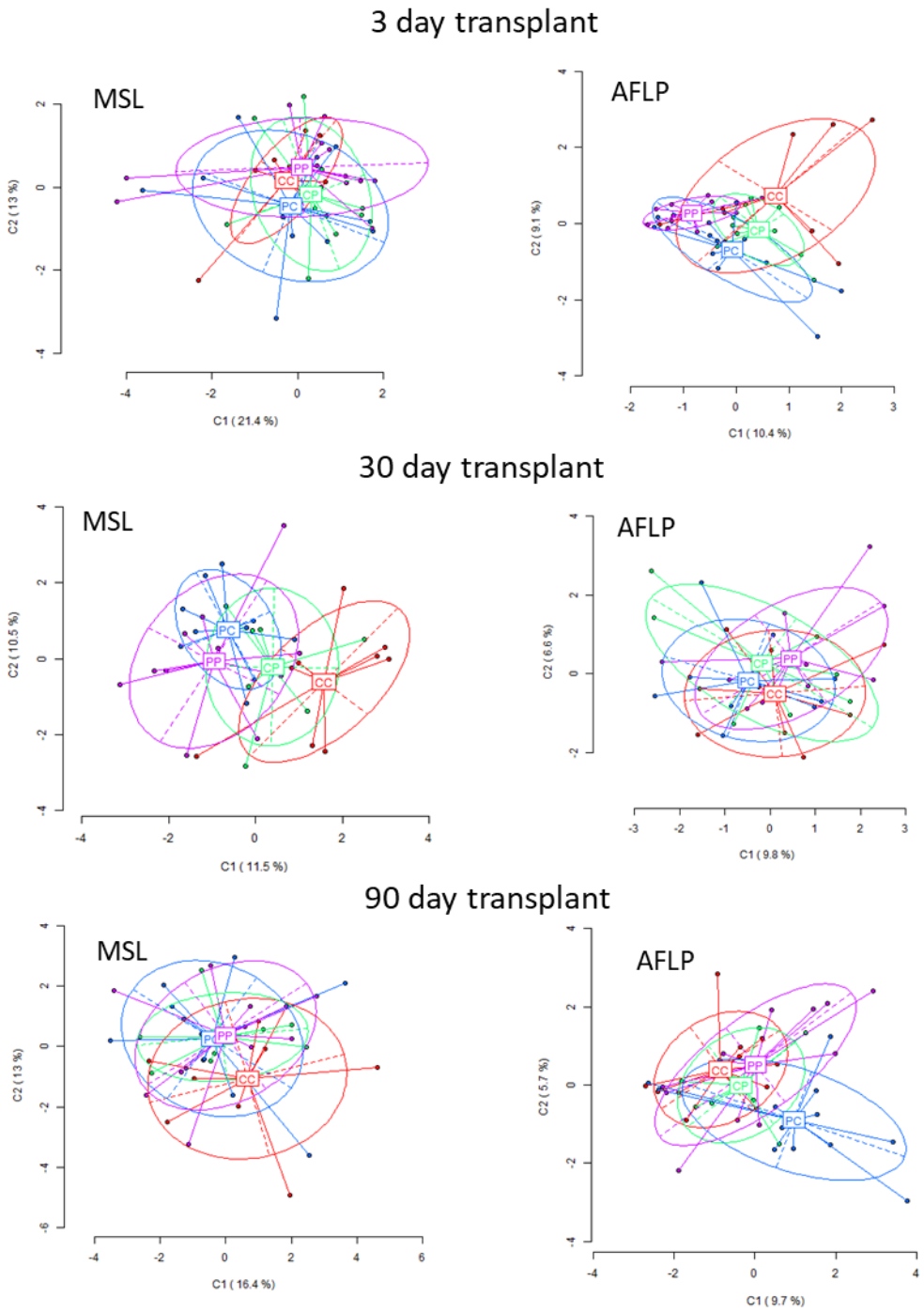


Figure 5.3. Principal Coordinate Analysis (PCoA) of the detected methylation and genetic polymorphism site patterns in *L. rubellus* at the sequence 5'-CCGG-3 collected from and transplanted between heavily polluted (P) soil collected near the Avonmouth zinc and lead smelter and control (C) soil for 3, 30 and 90 days in treatment groups CC, CP, PC, PP. The figures show the epigenetic variation (MSL, methylation-sensitive loci) and the genetic variation (AFLP).

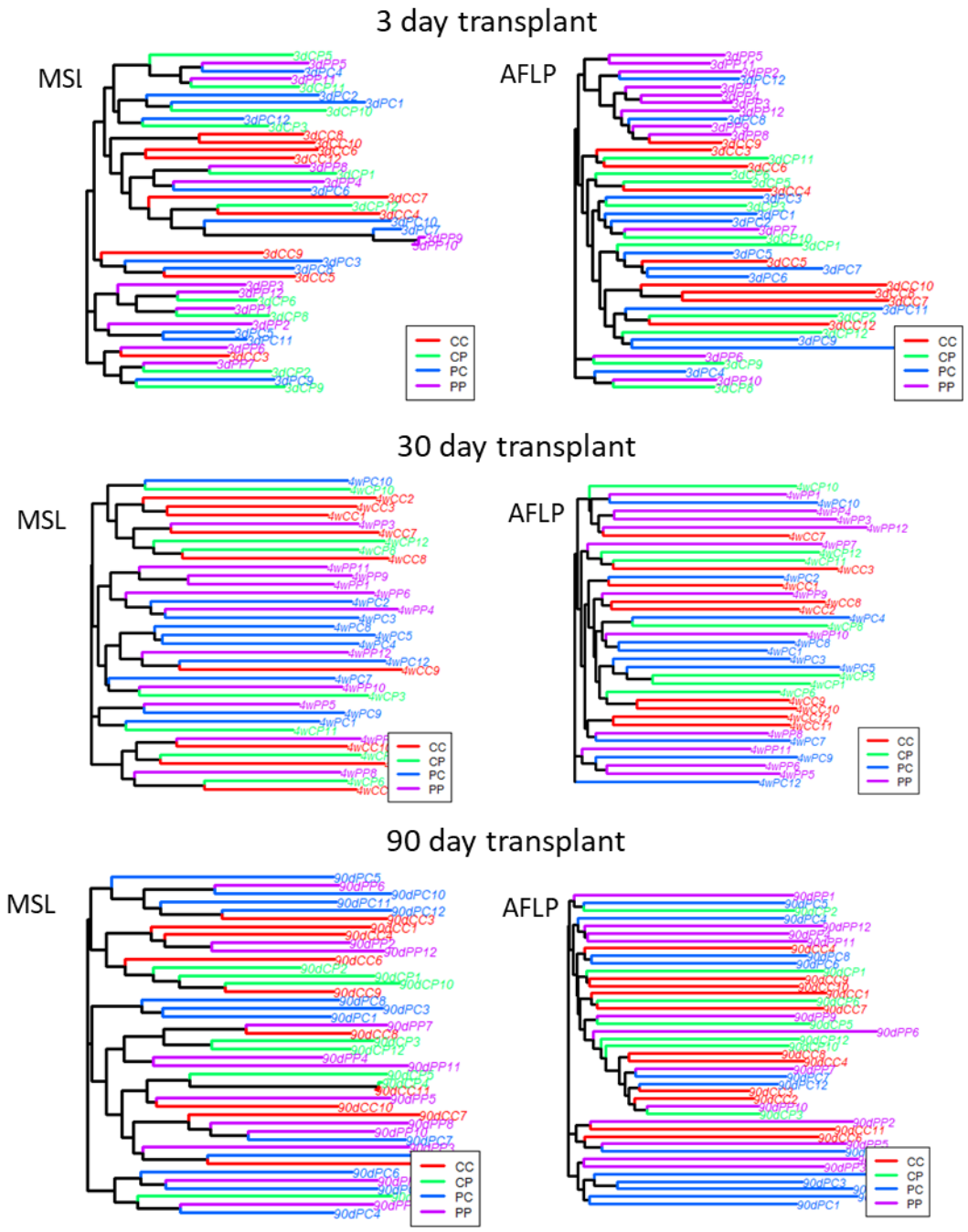


Figure 5.4. Neighbour Joining trees of the detected methylation and genetic polymorphism site distances in *L. rubellus* at the sequence 5'-CCGG-3'. In this experiment the earthworms were collected from and transplanted between heavily polluted (P) soil collected near the Avonmouth zinc and lead smelter and control (C) soil for 3, 30 and 90 days in treatment groups CC, CP, PC, PP. The figures show the epigenetic variation (MSL, methylation-sensitive loci) and the genetic variation (AFLP).

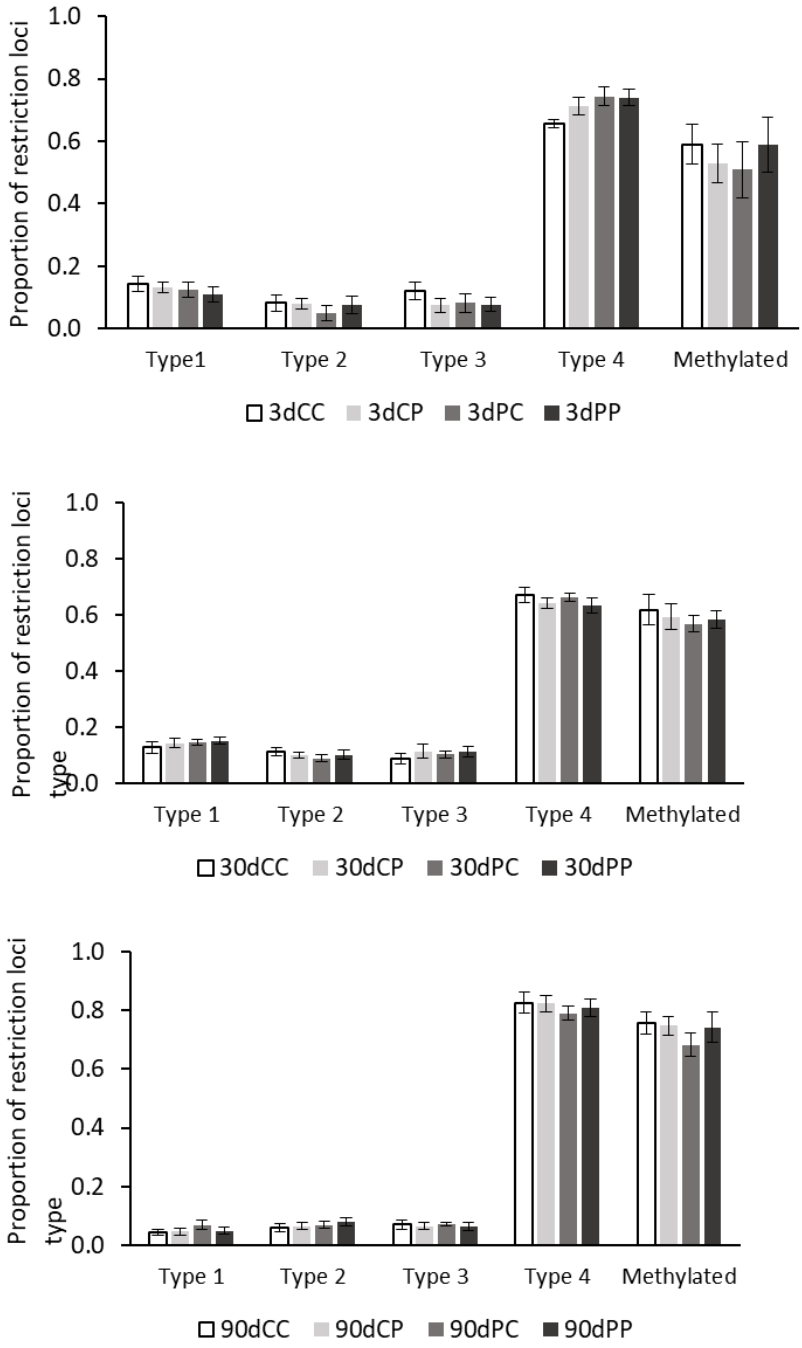


Figure 5.5. DNA methylation in methylation-sensitive loci detected from the analysed specimens of *L. rubellus* collected from a heavily polluted (P) site near the Avonmouth zinc smelter and a control (C) site and transplanted to soils from the sites in a laboratory exposure for 3, 30 and 90 days. The transplant codes are: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil. Type 1 to 3 loci are respectively: no methylated, methylation of internal C, methylation of external C or hemimethylation, and hypermethylation or mutation in restriction site (of the 5'-CCGG-3' sequence). Methylated: Global methylation level estimated following Nicotra *et al*, as proportion of (Type 2 + Type 3 loci / Type 2+Type 3 + Type 1(scorable loci)).

5.3.8. RNASeq analysis of the 30 day transplant groups

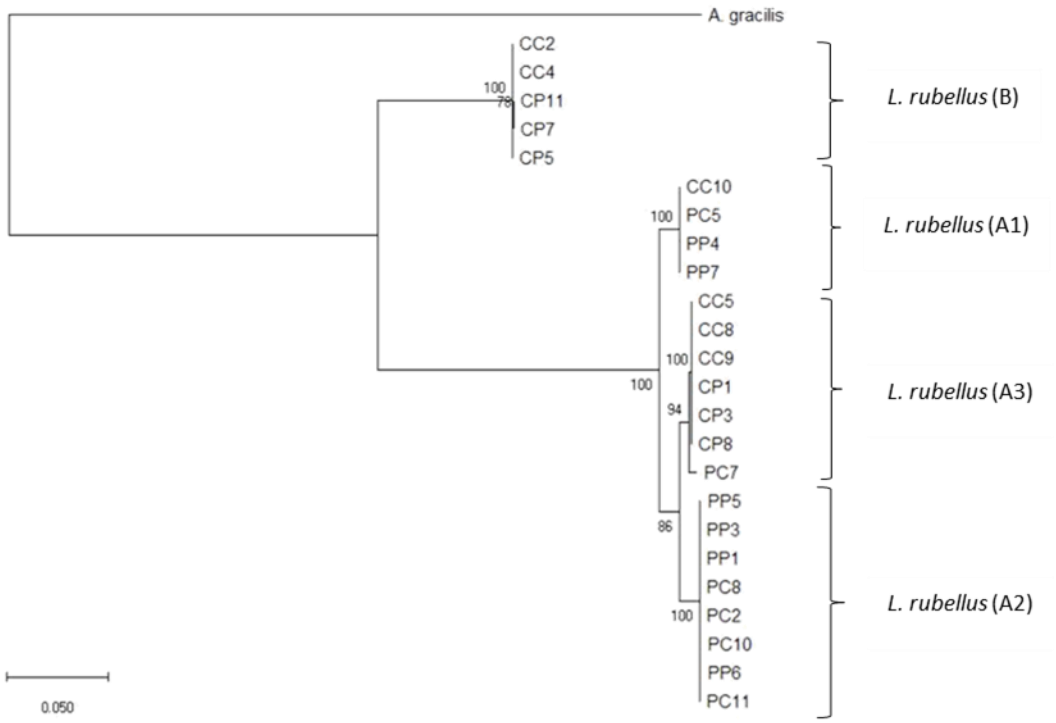
The 30 day transplant group was used for the RNASeq analysis to explore gene expression in order to observe the pollution adaptation mechanisms potentially utilised by *L. rubellus* in response to elevated trace metal exposure. This time point was selected due to the global DNA methylation differences reported above as the aim was to further explore the role played by of epigenetic mechanisms and transcriptional responses in pollution adaptation.

5.3.8.1. Raw data processing and quality assessment

An average number of 26 Million raw reads of paired end data was produced for each sample, 95% of this remained paired after trimming. *De-novo* transcriptome assemblies produced 110,732 transcripts and 70,505 genes (transcript encoding longest isoform ORF) on average with a N50 of 1552 and 1142 bp respectively. When mapped back to their individual assemblies, 93.9% was the lowest overall alignment rate with an average 26% and 60% of paired reads aligning once and more than once respectively.

5.3.8.2. Mitochondrial phylogeny

The phylogenetic analysis of COI sequences extracted from mitochondrial assemblies showed that the two *L. rubellus* lineages, A and B, separated clearly, with A containing multiple sublineages and B being more genetically homogenous in agreement with the provisional COI amplicon genotyping discussed above. The distances between A sub lineages and B ranged between 14% and 15% and distances between A sub-lineages ranged between 1.7%-3% consistent with the COI results (See Figure 5.6). The individuals from the two sites showed some separation, with all of clade A2 individuals coming from the polluted site.



	L.rubellus A_Y	*L.rubellus A_X*	*L.rubellus A_Z*	*L.rubellus B*
L.rubellus A_Y	-			
L.rubellus A_X	0.026	-		
L.rubellus A_Z	0.03	0.017	-	
L.rubellus B	0.146	0.149	0.152	-

Figure 5.6. Phylogenetic analysis by Maximum Likelihood using Hasegawa-Kishino-Yano model and a discrete Gamma distribution (5 categories). Highest log likelihood (-3932.40) tree with the percentage of trees (out of 50) in which the samples clustered together is shown. Branch lengths measure substitutions per site. Lineages and possible sub lineages are labelled and a mean between group distance matrix (Tamura 3 parameter model +G (5)) of these groups is shown including standard error estimates above the diagonal. Gaps and missing data were eliminated.

5.3.8.3. Nuclear phylogeny

A phylogenetic tree was derived from four sequences of conserved nuclear genes. These were identified in a BUSCO evaluation of Trinity transcriptome assemblies and listed in OrthoDB as peptidase (mitochondrial processing) alpha, tetratricopeptide repeat, ribosome production factor 1 homolog and protein phosphatase 2A activator, regulatory subunit 4. The individual peptide and nucleotide sequences were too conserved to yield an informative tree, therefore a concatenation of the four genes was carried out in order to produce a nuclear provisional phylogeny (Figure 5.7). The *L. rubellus* A lineage samples varied and showed low support values, but lineage B formed a

highly supported cluster consistent with the one seen in mitochondrial phylogenetic analysis. In terms of population history, some separation could be seen between the polluted and the control individuals, but this was less prominent than when looking at the COI genotyping results. This is likely due to the effect of recombination on the diversity of the nuclear genomes as opposed to the non-recombinant mitochondrial DNA.

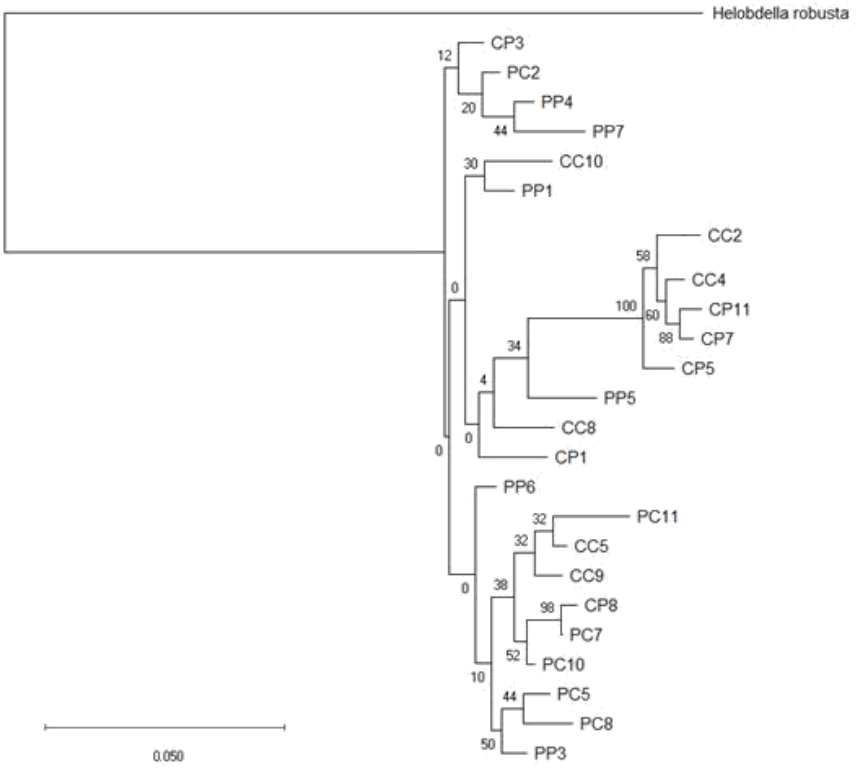


Figure 5.7. Phylogenetic analysis by Maximum Likelihood using Tamura-Nei model. Highest log likelihood (-22599.28) tree with the percentage of trees (out of 50) in which the samples clustered together is shown. Branch lengths measure substitutions per site. A total of 6112 positions were included in the analysis

5.3.8.4. Gene object construction

Evaluation of assembly completeness (BUSCO) was performed on the *L. rubellus* genome with no scaffolding or when scaffolded using either the *de novo* transcriptome or predicted peptides (representing longest open read frames (ORFs)). Peptide scaffolding showed limited enhancement (Table 5.4) whilst an additional twenty-three complete BUSCO genes were found when RNA scaffolded genome was performed using the transcriptome, most of these were new single copy genes that were previously fragmented. The graph presented in Figure 5.8 shows the GC content and coverage of the RNA scaffolded contigs together with the phylum of the contigs' best blast hit. The most common animal phylum to which the reads were assigned to was Annelida, but a large

number of reads was assigned to different groups and many, generally short contigs did not show significant homology when using blast (E exceeding $1x10^{-10}$).

Table 5.4.. Results of BUSCO evaluation of genome and scaffolded assemblies using 978 genes. Numbers of genes belonging to the assigned groups are shown.				
	Single copy	Duplicated	Fragmented	Missing
Genome	650	235	41	52
PEP	650	234	42	52
L_RNA	670	238	25	45

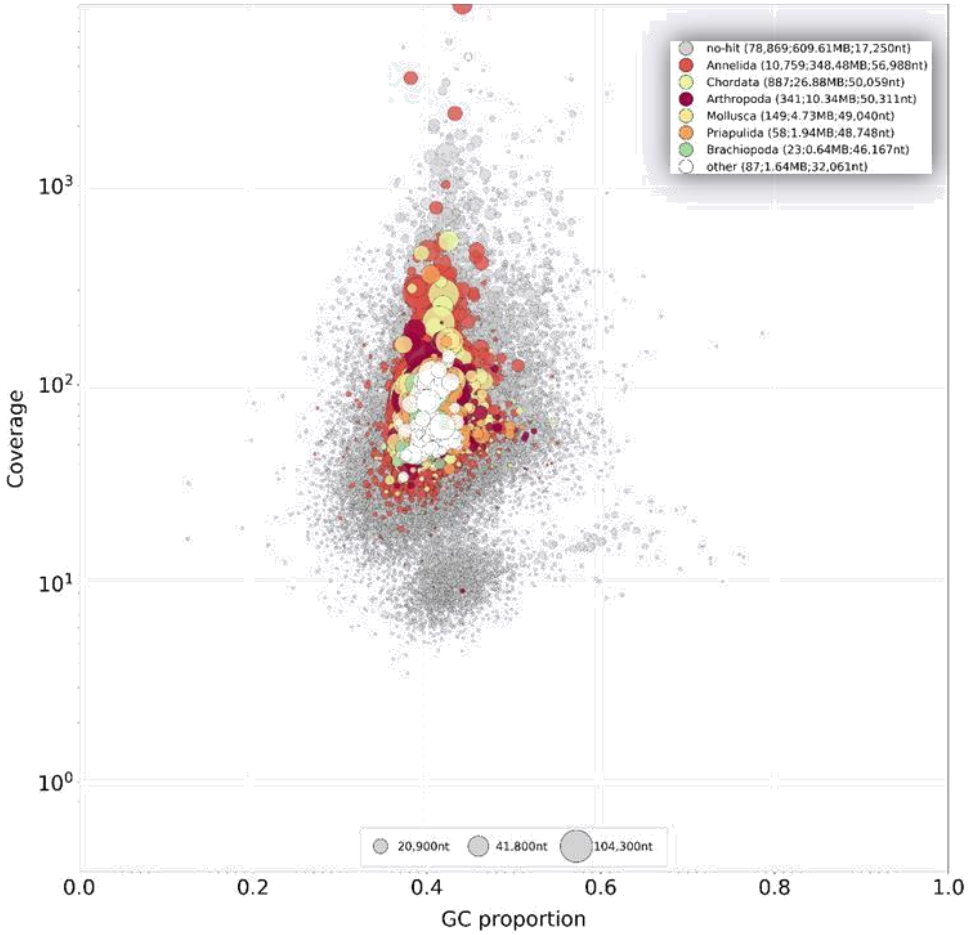


Figure 5.8. Plot of GC-content (%) vs coverage of the contigs in the RNA scaffolded *L. rubellus* genome. Diameter of the dots represents contig size, colour represents the phylum of the hits matched through blast as shown in the legend.

5.3.8.5. Mapping

The trimmed reads were mapped onto the RNA scaffolded genome. The average overall mapping rate was 90%, however, the mapping rate for the sample PP4 was only 61%. For this sample, both paired and unpaired reads had similar mapping reductions compared to the others (61% and 58% respectively). Individuals belonging to lineage B mapped slightly better than lineage A, ranging from 91% to 97% and 87% to 91%, respectively.

5.3.8.6. Gene object generation

The Individual assembled transcript numbers were on average 73,453 and varied between 53,802 and 97,633 across each assembled individual *L. rubellus* transcriptome. Initially, after merging 301,557 transcripts were found, this number was then reduced to 107,231 by filtering for the longest transcripts. These were then available for use as gene objects for downstream differential expression analysis.

5.3.8.7. Differential gene expression analysis

The per base gene object coverage counts were used to test differential expression between the different transplant groups. DESeq2 analysis was carried out twice to assess variability within the data, one with both the lineage A and lineage B individuals, the other with only worms belonging to the A lineage (figure 5.9). The cluster dendrograms and principal component analysis (PCA) show different clustering patterns between these two analyses. In the cluster dendrogram which included data from lineage B individuals (figure 5.9 A), the B individuals cluster in a separate branch from the rest of the group and look very different to the others. Similarly, principal component (PC) 2 analysis, which explained 17% of the variability in the PCA (Figure 5.9 B), also appeared to separate the samples by lineage rather than condition. It is PC1 and PC3 analysis of the samples excluding data from lineage B individuals (figure 5.9 F) that showcased the clearest clustering by condition, with CC, transplanted and PP individuals at the top, middle and bottom respectively. As a result of these observations, in further analysis only results from differential analysis of the lineage A dominant lineage A clade, excluding B lineage individuals, is presented.

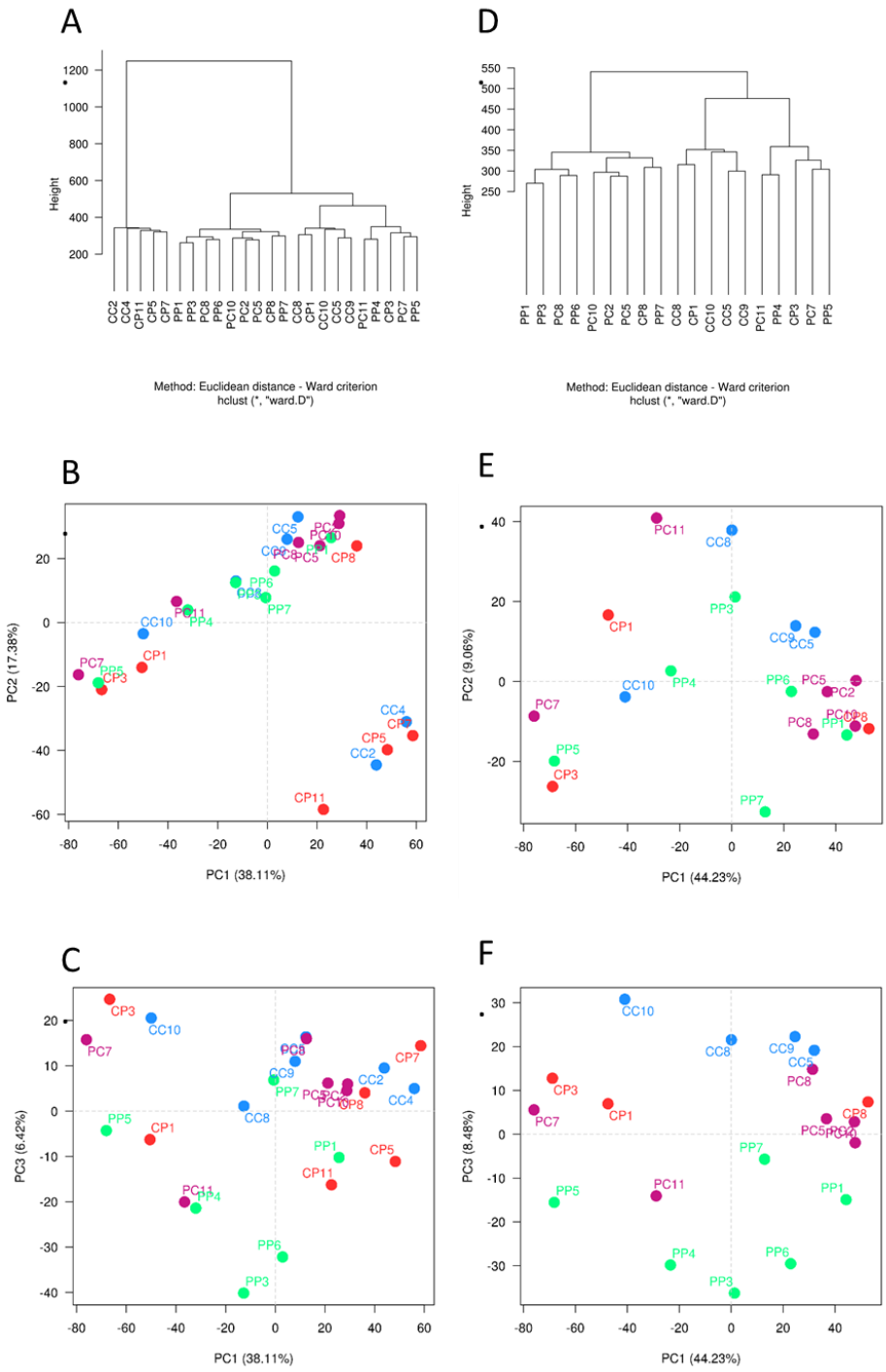


Figure 5.9. Cluster dendrograms and principal component analysis showing variability within the expression data produced by DESeq2 during analysis including lineage B individuals (A, B, C) and after excluding these (D, E, F). PCA plots in panels B and E include principal components (PC) 1 and 2. Panels C and F include principal components 1 and 3. Variance explained by the principal components is shown as percentage.

5.3.8.8. Differentially expressed gene counts between comparisons

The comparisons were carried out with the control earthworms/soils as reference, so, when the CC group was present in the comparison, it was always used as the reference, when the PP group was compared to CP and PC groups, the latter two treatment groups were used as the references. In the CP versus PC comparison, CP was used as the reference, so upregulated genes in this comparison were upregulated in the PC group compared to CP. The numbers of differentially expressed genes for all tests as outputted by DESeq2 can be seen in Table 5.5. MA and volcano plots in Figures 5.10 and 5.11 show differences in expression between the tested conditions for comparisons that showed the greatest differences between groups. The full annotated gene lists can be seen in Appendix 1a.

The comparison between the CC and PP groups produced the greatest number of significantly differentially expressed genes at 5590 (p<0.05), with 2143 genes remaining after elimination of false positives by applying a Benjamini-Hochberg adjusted p<0.05 (BH $p$adj)). The significant transcriptomic remodelling observed in this comparison is unsurprising as these individuals have different exposure histories both in the short and the long term and are somewhat separated in terms of genetic background (as reported above).

The CC vs PC comparison also identified a large number of differentially expressed genes between the treatment groups, despite the fact that the earthworms were kept in unpolluted soil prior to sampling. These two treatments, however, involve individuals drawn from populations with somewhat different genetic backgrounds. Additionally, the PC individuals retained a large amount of heavy metal pollutants in their tissues which may have continued to have an impact on these earthworms even after 30 days in control soil. This may possibly explain the large amount of gene expression variation between the PC and CC worms. At the same time, the comparison between CC and PC, while producing a very large number of differentially expressed genes 3354 [364] (p<0.05 [BH adj$p$<0.05]), produced considerably fewer differentially expressed genes than the CC versus PP comparison, therefore the common recent soil environment of the PC and CC groups also played a large role.

This role was underlined by the large number of differentially expressed genes in the comparison of the CC and CP earthworms (3694 [392] (p<0.05 [BH adj$p$<0.05])) and the relatively large number present in the PC and PP comparison (2312 [65] (p<0.05 [BH adj$p$<0.05])). Furthermore, the lowest number of differentially expressed genes was found when comparing the CP and PP individuals

(1571 [70] (*p*<0.05 [BH adj*p*<0.05])), therefore the recent environment, particularly when containing heavy pollution, resulted in homogenising the gene expression profiles of the two populations.

The recent exposure to pollution appeared to be slightly more resource costly for the polluted earthworms as in the PC versus PP comparison considerably more (1490 [46] (*p*<0.05 [BH adj*p*<0.05])) genes were upregulated than downregulated (822 [19] (*p*<0.05 [BH adj*p*<0.05])).

Table 5.5. Count table of differentially expressed genes. Test and reference groups used to test for differential expression shown as (test vs reference) and the resulting up, down and total differentially expressed gene counts with respect to the reference condition. The reference group in the comparisons is always the one coming first in the alphabet (e.g. CP vs PC, CP is the reference)

Test	Up	Down	Total
CC vs PP	2851 [1009]	2739 [1134]	5590 [2143]
PC vs PP	1490 [46]	822 [19]	2312 [65]
CC vs CP	1857 [156]	1837 [236]	3694 [392]
CC vs PC	1621 [197]	1733 [167]	3354 [364]
CP vs PC	954 [54]	1169[71]	2123 [125]
CP vs PP	873 [40]	698 [30]	1571 [70]

Number of differential genes *p*<0.05 [BH *p*adj<0.05]

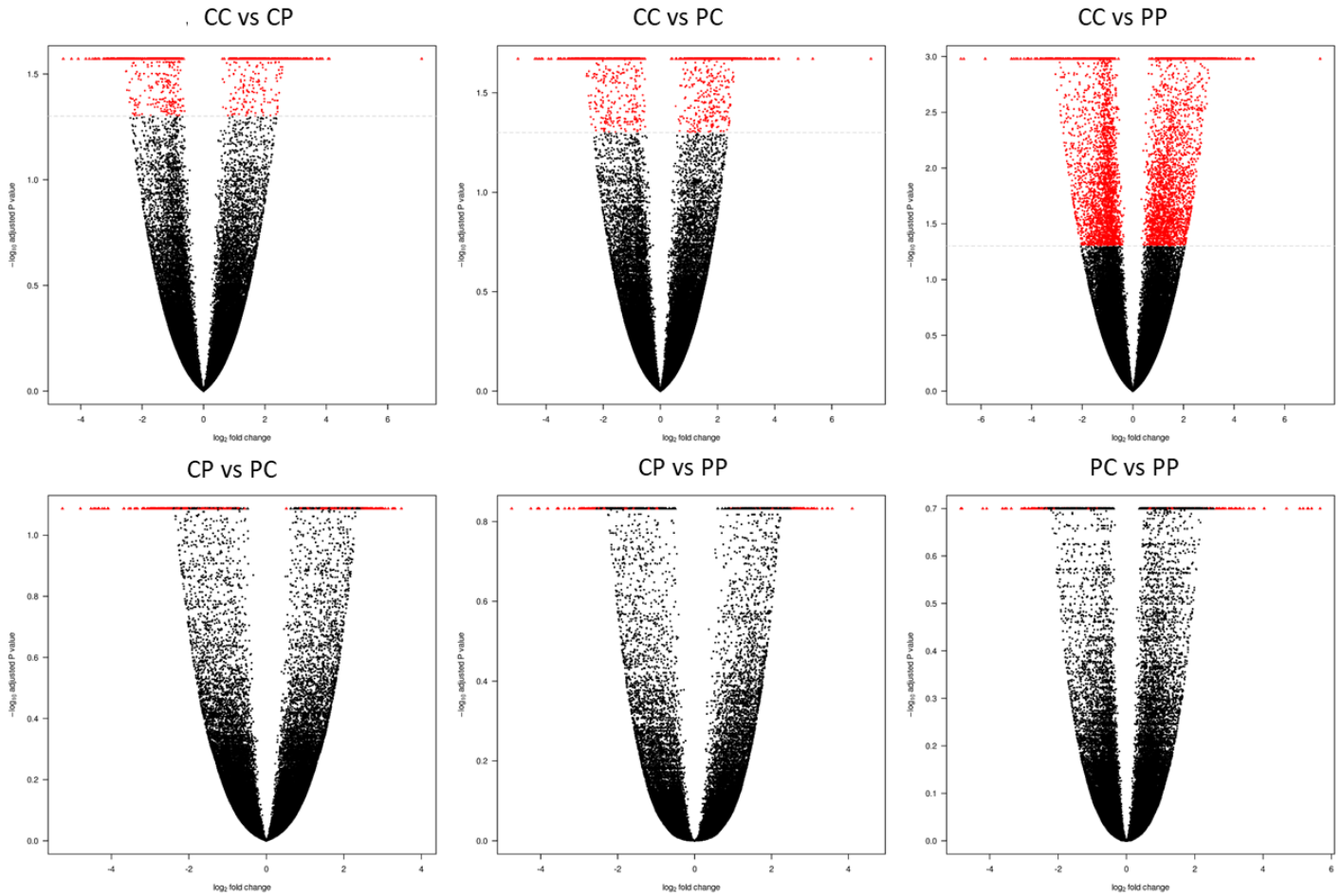


Figure 5.10. Volcano plots showing expression differences (as detected by RNASeq) between reciprocal transplantation groups of *L. rubellus*. Groups were collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby. The site names refer to: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil.Genes significantly (BH adjusted p-value <0.05) down or upregulated shown in red.

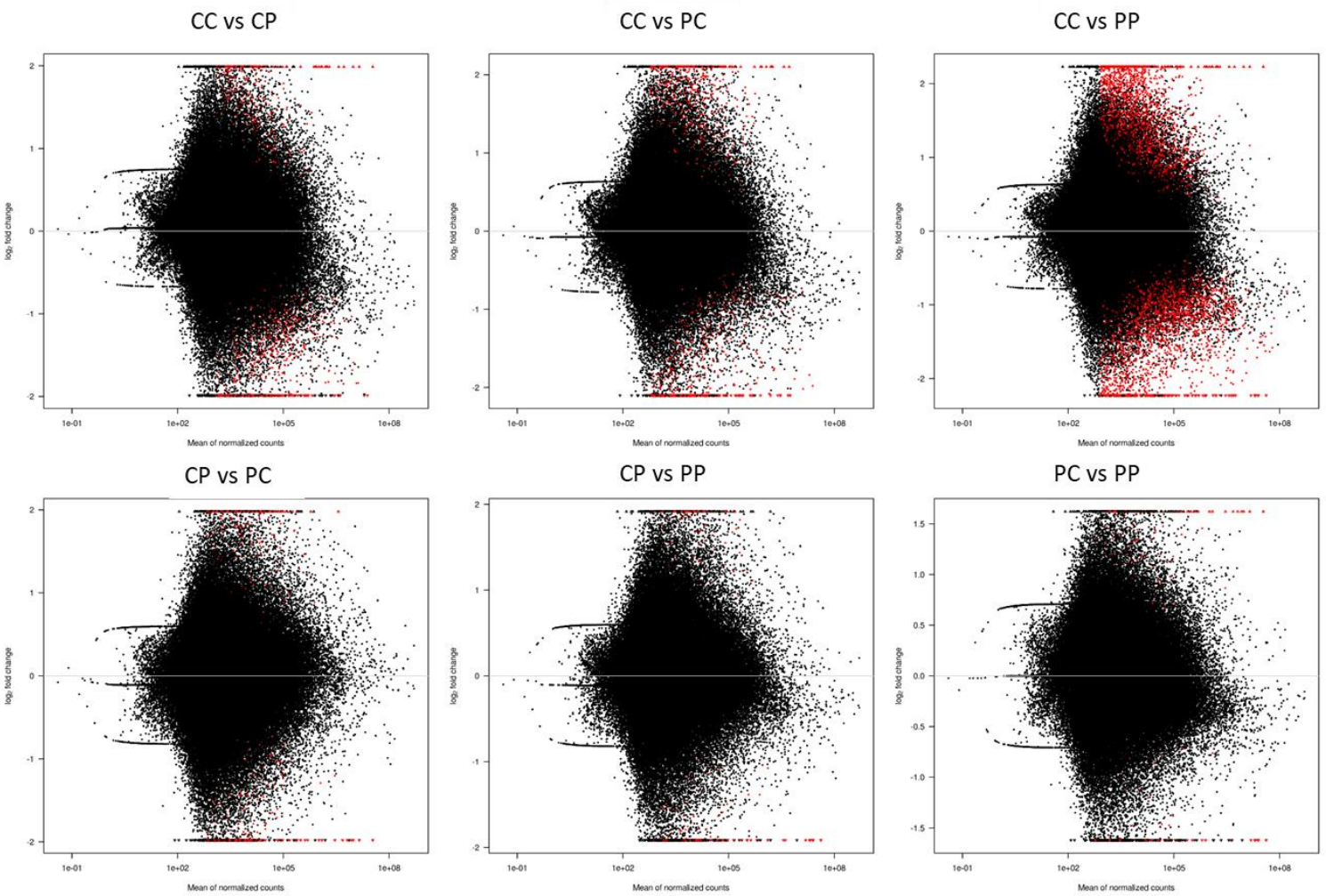


Figure 5.11. MA plots showing expression differences (as detected by RNASeq) between reciprocal transplantation groups of *L. rubellus*. Groups were collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby. The site names refer to: CC-Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil.Genes significantly (BH adjusted p-value <0.05) down or upregulated shown in red.

5.3.9. Comparisons in gene expression differences between the treatments

Gene expression overlap between the treatment comparisons using successfully annotated transcripts indicated that only 12.7% of the genes differentially expressed (using BH adj*p* values) between the CC and PP groups were also differentially expressed between CC and CP, while 69% of the genes differentially expressed between CC and CP were also differentially expressed between CC and PP groups (See Figure 5.12). This implies that, while substantial similarities between the mechanisms utilised for handling heavy metal pollution are similar for both long- and short-term exposed *L. rubellus*, differences in genetic background and history of exposure likely result in different mechanisms being used for handling pollution stress. However, the genetic background likely accounted for only a relatively small fraction of the observed difference in response. When looking for overlap between differentially expressed genes between CC and PC, CC and PP, CP and PP and CP and PC groups, only 9 (2.5%, 0.4%, 12.9% and 7.2% of total differentially expressed genes in each comparison, respectively) were found to overlap (See figure 5.12). This indicates that the recent soil environment had a huge impact on gene expression profiles and the PC group also employed some specific mechanisms to adapt to living in the control soil.

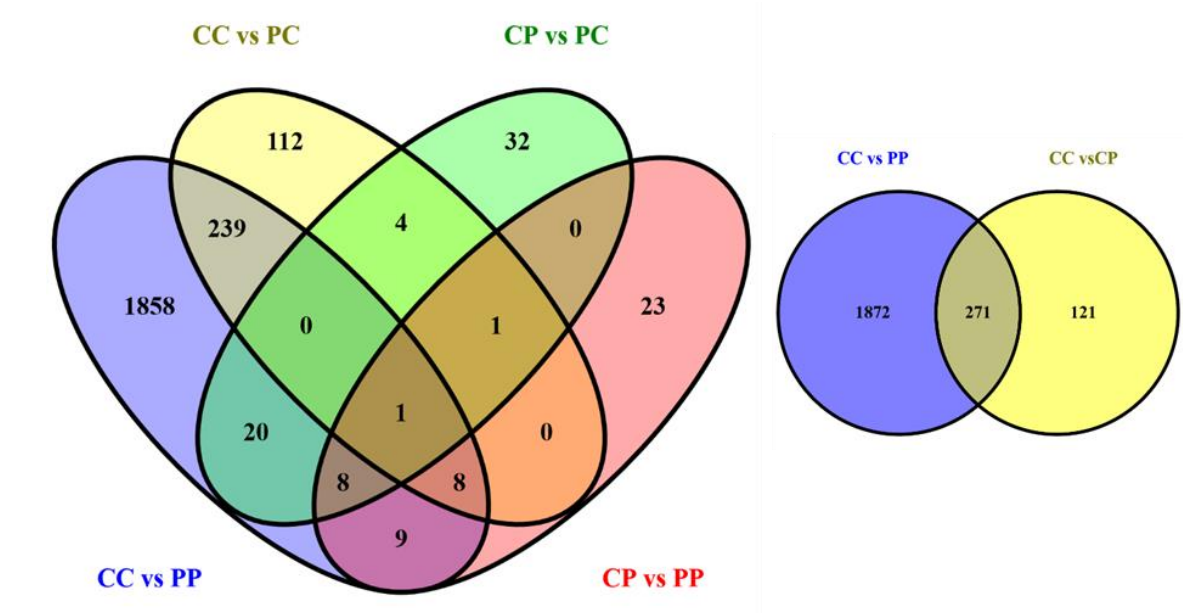


Figure 5.12. Overlaps between significantly differentially expressed genes (as detected by RNASeq) between reciprocal transplantation groups of *L. rubellus.* Groups were collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby. The site names refer to: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil.

5.3.10. Functional analysis

The functional enrichment analysis by DAVID produced significant raw *p* values for multiple Gene Ontology (GO) terms for all of the comparisons between treatment groups. The up- and downregulated genes were analysed separately as not much overlap was found between these groups. Filtering for differentially expressed genes to remove false positives by employing the BH adj*p*<0.05 significantly reduced the number of genes available for enrichment analysis by DAVID and therefore yielded a minimal number of enriched GO terms/pathways. Since enrichment analysis does not rely on individual genes but exploits the appearance of multiple genes associated with a common process or pathway it has been shown to be appropriate to include genes lists where false discovery correction has not been applied (García-Campos *et al.*, 2015). Therefore, differential gene list displaying *p*<0.05 were used for enrichment analysis of GO terms using DAVID. Related GO terms were identified and grouped using semantic similarity using REVIGO, and analysed under the headings of Biological process, Cellular component and Molecular function (See Appendix 1b).

The results of KEGG pathway analysis carried out using DAVID produced many significantly altered pathways when exploring the raw *p* values, but only a few terms that remained significant after correction using the Benjamini equation (See Appendix 1c). The gene ontology enrichment analysis confirmed that, at least in terms of individual genes and enriched GO terms, the different transplant groups used different mechanisms in order to cope with heavy metal pollution. However, more fundamental cellular processes were found to be commonly alternatively expressed between the groups when exploring molecular function terms.

5.3.10.1. Biological processes

As can be seen in Figures 5.13, 5.14, 5.15, 5.16, little overlap in the produced groupings could be found when comparing the biological process enrichment in the CC vs PP and the CC vs CP comparisons. Upregulation of protein phosphorylation was the only significantly affected biological process that was found to be common between both comparisons

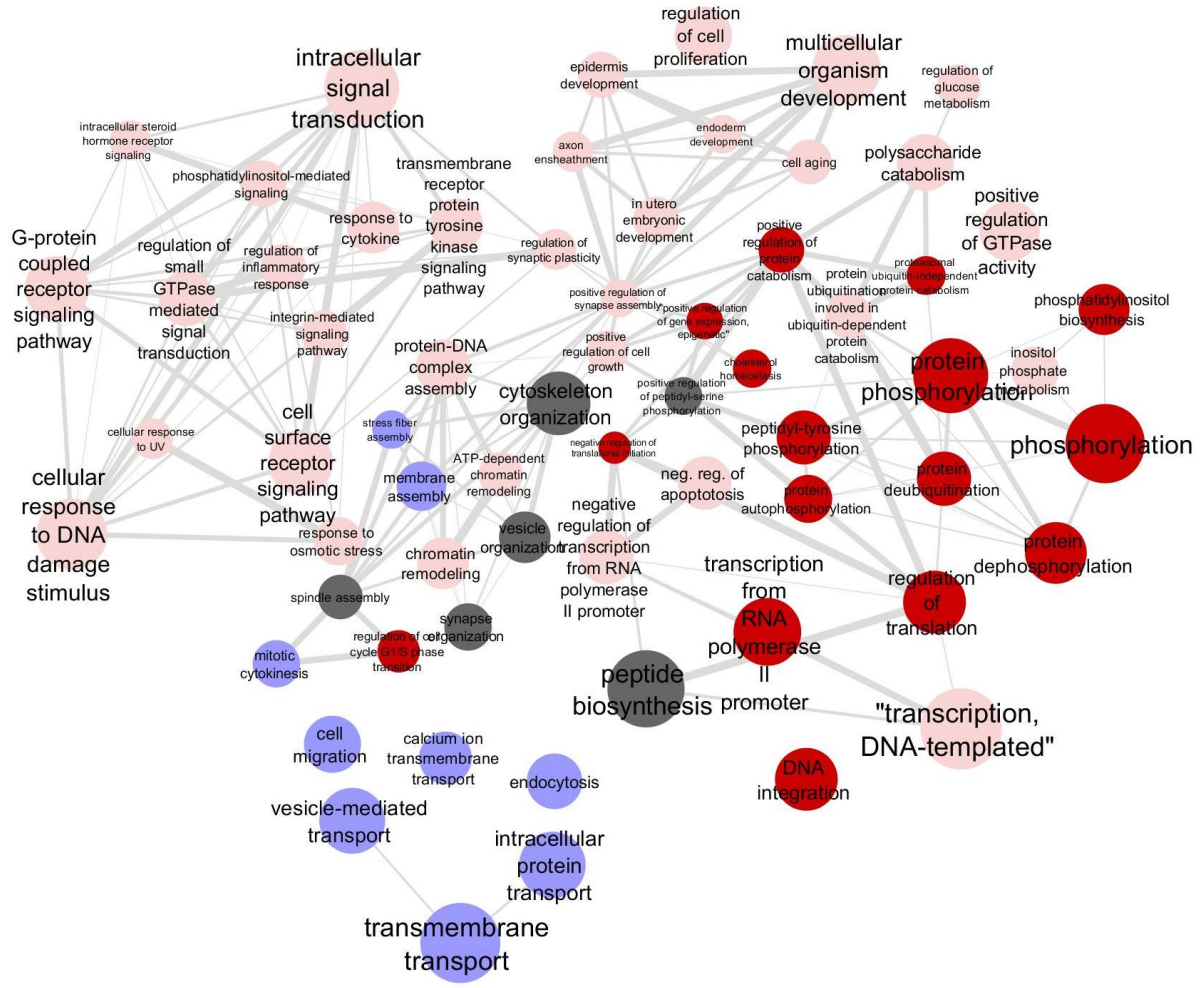


Figure 5.13. Biological processes upregulated in *L. rubellus* earthworms collected from a heavily polluted soil near the Avonmouth zinc smelter (Bristol, UK) and kept in soil from this area in a laboratory setting for 30 days (PP group) compared to *L.rubellus* collected from a control site and kept in control soil for 30 days (CC group). The nodes are coloured according to their groupings (as output by REVIGO). The colours refer to: Pink – intracellular signal transduction; Purple – cell migration; grey – cytoskeleton organization; red – protein phosphorylation. The edge thickness indicates the weight of the interactions between the GO terms.

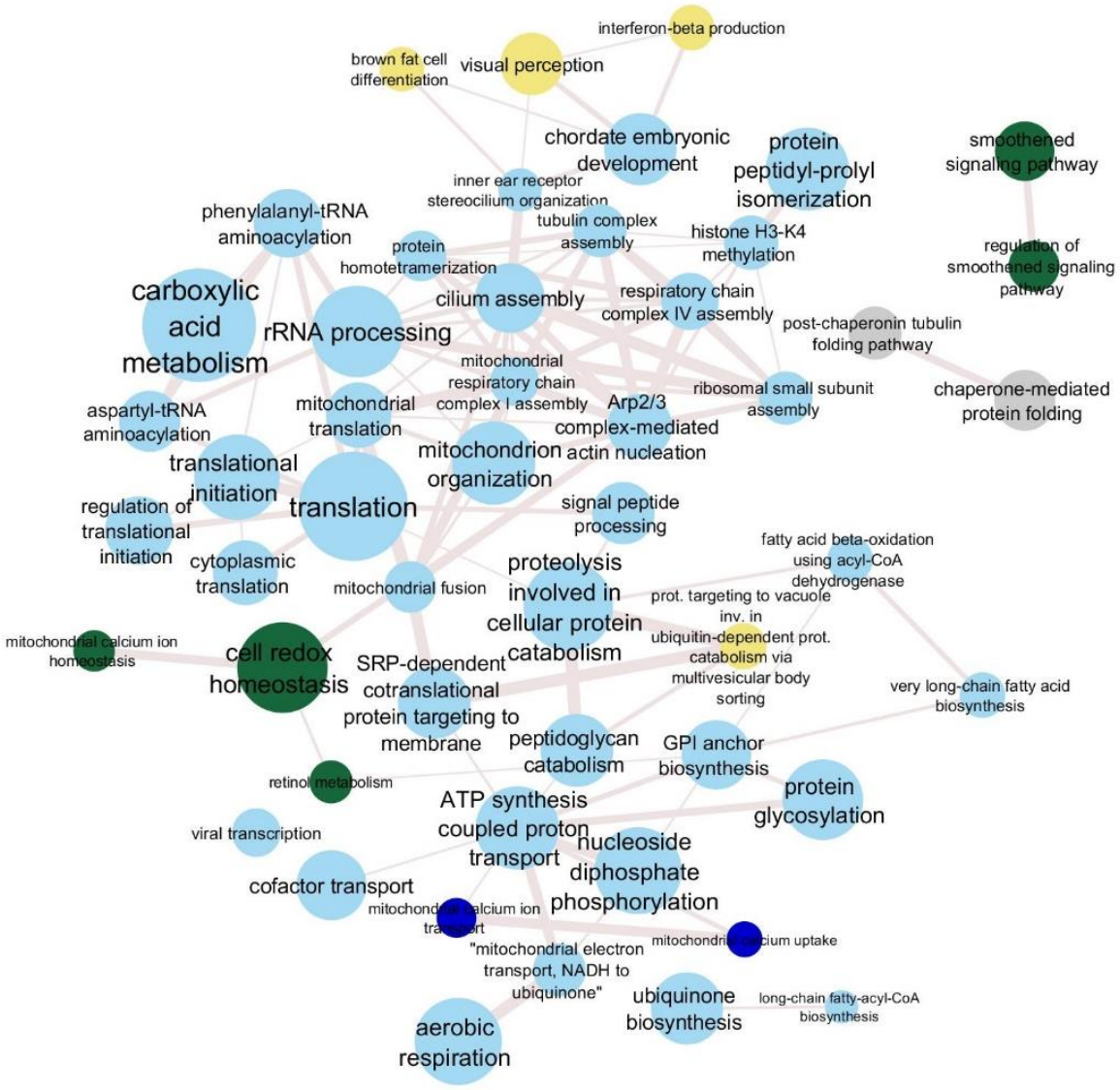


Figure 5.14. Biological processes downregulated in *L. rubellus* earthworms collected from a heavily polluted soil near the Avonmouth zinc smelter (Bristol, UK) and kept in soil from this area in a laboratory setting for 30 days (PP group) compared to *L.rubellus* collected from a control site and kept in control soil for 30 days (CC group). The nodes are coloured according to their groupings (as output by REVIGO). The colours refer to: light blue – mitochondrial translational initiation; green – cell redox homeostasis; dark blue – mitochondrial calcium uptake; dirty yellow – chordate embryonic development; grey – chaperone-mediated protein folding. The edge thickness indicates the weight of the interactions between the GO terms.

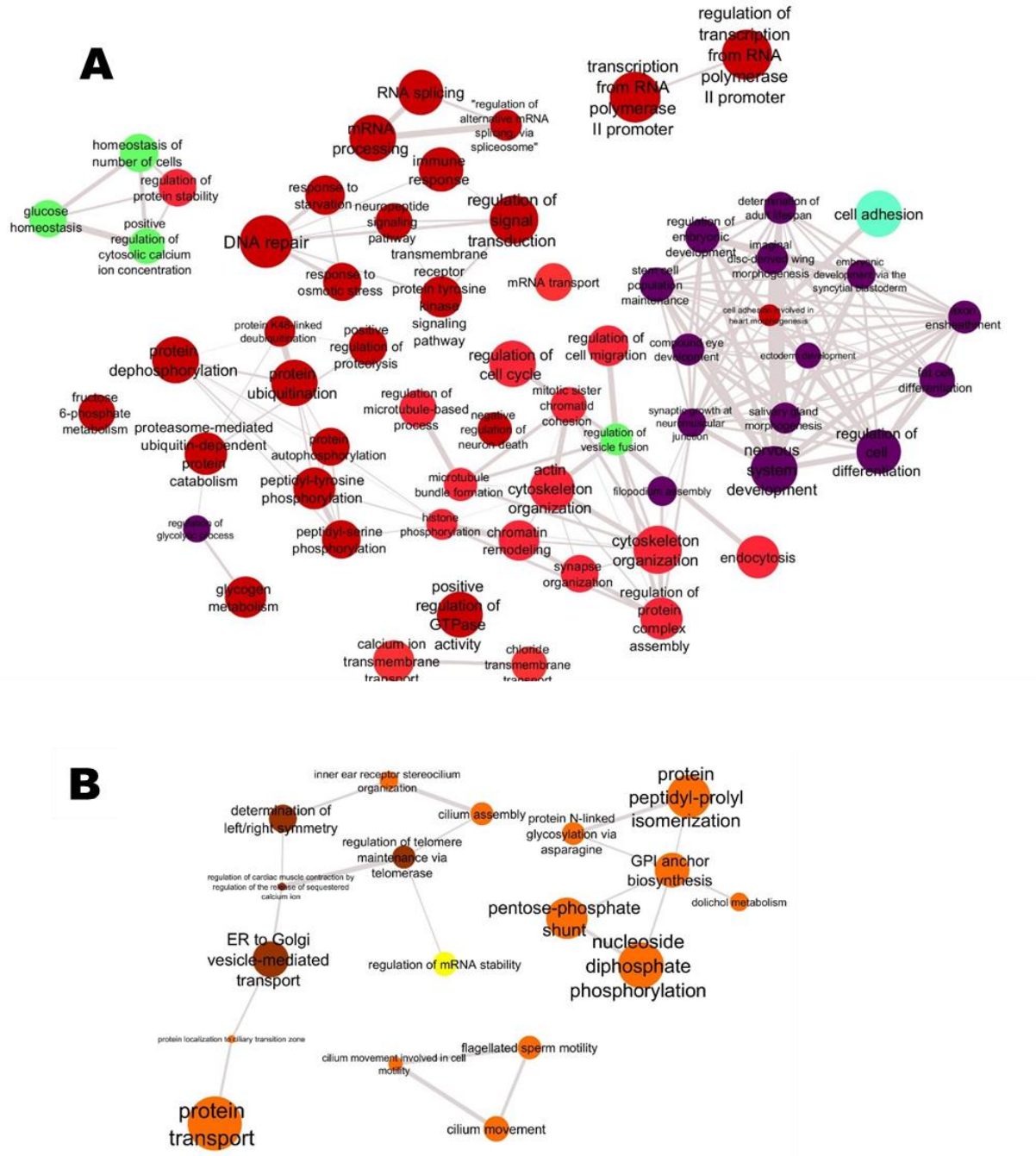


Figure 5.15. Biological processes up- (A) and down- (B) regulated in *L. rubellus* earthworms collected from a control site and kept in polluted soil collected near the Avonmouth zinc smelter (Bristol, UK) in a laboratory setting for 30 days (CP group) compared to *L.rubellus* collected from the control site and kept in control soil for 30 days (CC group). The nodes are coloured according to their groupings (as output by REVIGO). The colours refer to: red – protein phosphorylation; dark purple – open tracheal system development; dark pink – mitotic sister chromatid cohesion; light green – triglyceride homeostasis; turquoise – cell adhesion; orange – cilium assembly; brown – determination of left/right symmetry; bright yellow – smoothened signalling pathway. The edge thickness indicates the weight of the interactions between the GO terms.

5.3.10.2. Molecular functions and cellular components

The semantic groupings of molecular function GO terms differentially expressed between the comparisons of experimental conditions can be seen in Figure 5.16. These showed a rather complex picture, with few terms overlapping between comparisons. After conglomerating the molecular function term semantic groups further, some patterns could be elucidated in the nature of the transcriptional response to transplantation of the two populations on own and alternate site soils (see Table 5.6 and Appendix 1e).

Table 5.6. Summation of differently expressed GO term molecular function semantic groups divided into broader cellular processes.

Cellular process	CC vs CP		CC vs PC		CC vs PP		CP vs PC		CP vs PP		PC vs PP	
Transmembrane proteins, detoxification and metal ion binding proteins	-2	13	-1	31	-5	10	-11	13	-3	7	-1	10
Chromatin change and gene expression	-4	22	-4	4	-8	12	-8	21	-6	5		15
Translation and protein degradation				7	-13				-3		-1	1
Energy metabolism	-1						-6		-5			8
Cellular structure		22	-1			31	-23	22		13		27

The numbers denote the number of up (red) and down (blue) regulated semantic groups of differentially regulated GO terms in a data set comparing the gene expression as detected by RNASeq between groups *L. rubellus* collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby. The site names refer to: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil. The reference group in the comparisons is always the one coming first in the alphabet (e.g. for CP vs PC, CP is the reference)

5.3.10.2.1. Transmembrane transport related genes were differentially expressed due to metal pollution

The REVIGO output of molecular function-related GO term divisions into semantic groups revealed that ATPase activity, coupled to transmembrane movement of substances, was upregulated in the CC vs CP comparison and both up-and down-regulated in the CP versus PC comparison. In the CP vs PC comparison, Wnt-activated receptor activity was downregulated. Sodium-dependent L-ascorbate transmembrane transporter activity was upregulated in the CC vs PC comparison, as were cell adhesion molecule binding and G-protein coupled receptor activity. Signal transducer activity was

both up- and downregulated in the CC vs PP comparison and voltage-gated anion channel activity and voltage-gated chloride channel activity were down and upregulated, respectively, in this comparison. Cation channel activity and receptor activity were upregulated in the CP vs PP comparison, and organic anion transmembrane transporter activity was downregulated in this same comparison. Results from cellular component enrichment terms also showed that GO terms linked to membrane, extrinsic component of membrane, endosome, endomembrane system, integral component of plasma membrane and other related locations were differentially expressed as a result of heavy metal exposure in the same set of comparisons (See Figure 5.17, Appendix 1d). This is consistent with expectations, as changing signal transduction and binding to the toxic pollutants are ways in which it would be expected for organisms to cope with such a stress.

5.3.10.2.2. Metal ion binding and detoxification-related genes were differentially expressed between the transplant groups

A single GO term falling into the semantic group of "alcohol binding" was downregulated in the CC vs CP and the CC vs PC comparisons. In the CC vs CP comparison, toxin transporter activity was also downregulated. In both the CC vs PC and CP vs PP comparisons 2 GO terms falling under the semantic group of calcium ion binding were upregulated. Selenium binding and 2 iron, 2 sulphur cluster binding GO terms were downregulated in the CC vs PP comparison and manganese ion binding was downregulated in the PC vs PP comparison. GO terms related to zinc ion binding were upregulated in the CC vs CP, CC vs PP and PC vs PP comparisons, while in the CP vs PC comparison, 6 zinc ion binding-related GO terms were downregulated and four were upregulated.

5.3.10.2.3. Chromatin and gene expression related genes were differentially expressed in the transplant groups

Genes involved in chromatin change and gene expression were generally upregulated due to pollution, especially in the more recently exposed individuals (e.g. the CC vs CP comparison).Multiple GO terms related to chromatin binding were upregulated in the CC vs CP, CC vs PP, CP vs PC and PC vs PP comparisons while one was downregulated in the CP vs PC comparison. Histone acetyltransferase activity was upregulated in the CC vs CP and CP vs PC comparisons. Core promoter binding and core promoter sequence-specific DNA binding related GO terms were upregulated in the CC vs CP, CC vs PC, CP vs PC and CP vs PP comparisons. Transcription cofactor activity and transcription factor activity, sequence-specific DNA binding were upregulated in the CC vs CP, CC vs PC, CC vs PP, CP vs PC and PC vs PP comparisons. Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding were downregulated in the CP vs PC and CP vs PP comparisons while

nucleoside diphosphate kinase activity was downregulated in the CC vs CP, CC vs PC and CC vs PP comparisons.

5.3.10.2.4. Translation, protein modification and degradation related genes were differentially expressed between groups

In the CC vs PP downregulated gene list, three KEGG pathways related to the ribosome were significantly altered. Multiple ribosome and rRNA binding related groups were downregulated in the CC vs PP, CP vs PP and PC vs PP group comparisons.

Cysteine-type endopeptidase activity and endopeptidase inhibitor activity were upregulated in the CC vs PC comparison while unfolded protein binding was downregulated in the CC vs PP group.

Meanwhile, translation and GO terms linked to the ribosome were downregulated in the PP group and protein degradation upregulated in the PC individuals (See Figure 5.16), which may possibly mitigate the effects of changes in gene expression in earthworms that had been exposed to pollution for long periods of time and perhaps helps energy preservation. It may also point to some other mechanism helping the long-term exposed earthworms to cope with heavy metals.

5.3.10.2.5. Energy metabolism related genes were differentially expressed between the groups

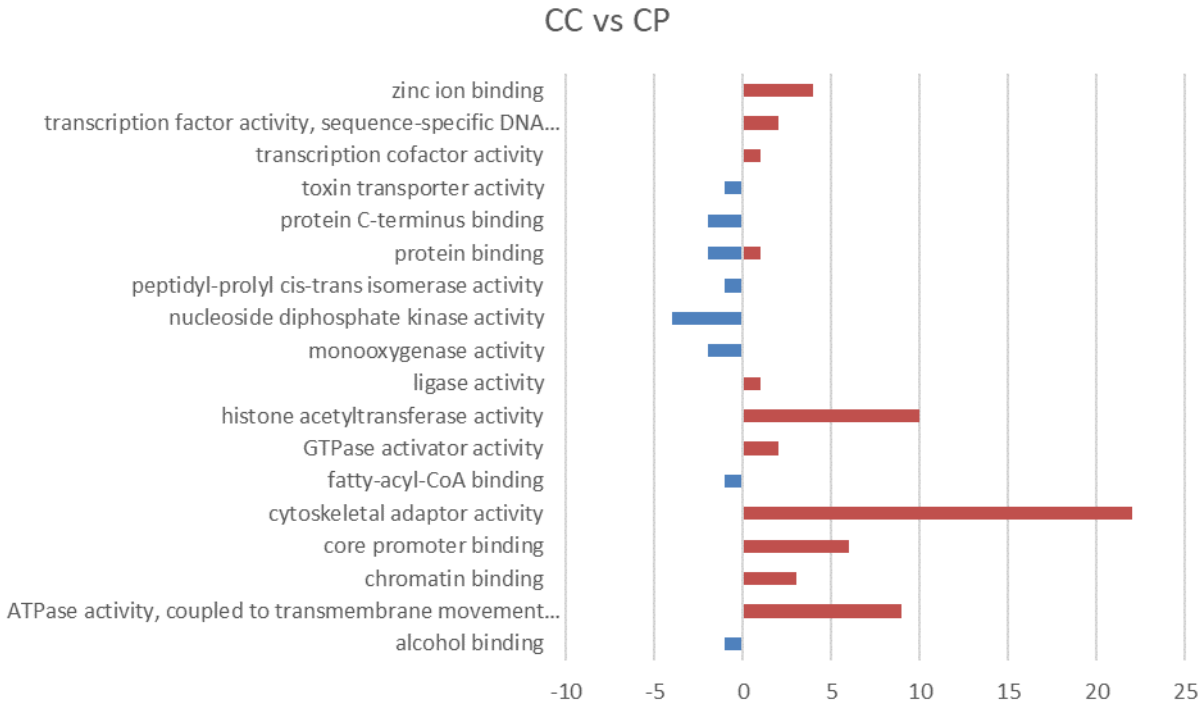
In the CC vs CP comparison, a KEGG Metabolic pathway was significantly downregulated. fatty-acyl-CoA binding was downregulated in the CC vs CP group, acetyl-CoA carboxylase activity was upregulated in the PC vs PP group, glycogen phosphorylase activity was downregulated I the CP vs PC group, acyl-CoA hydrolase activity was downregulated between the CP and PP groups.

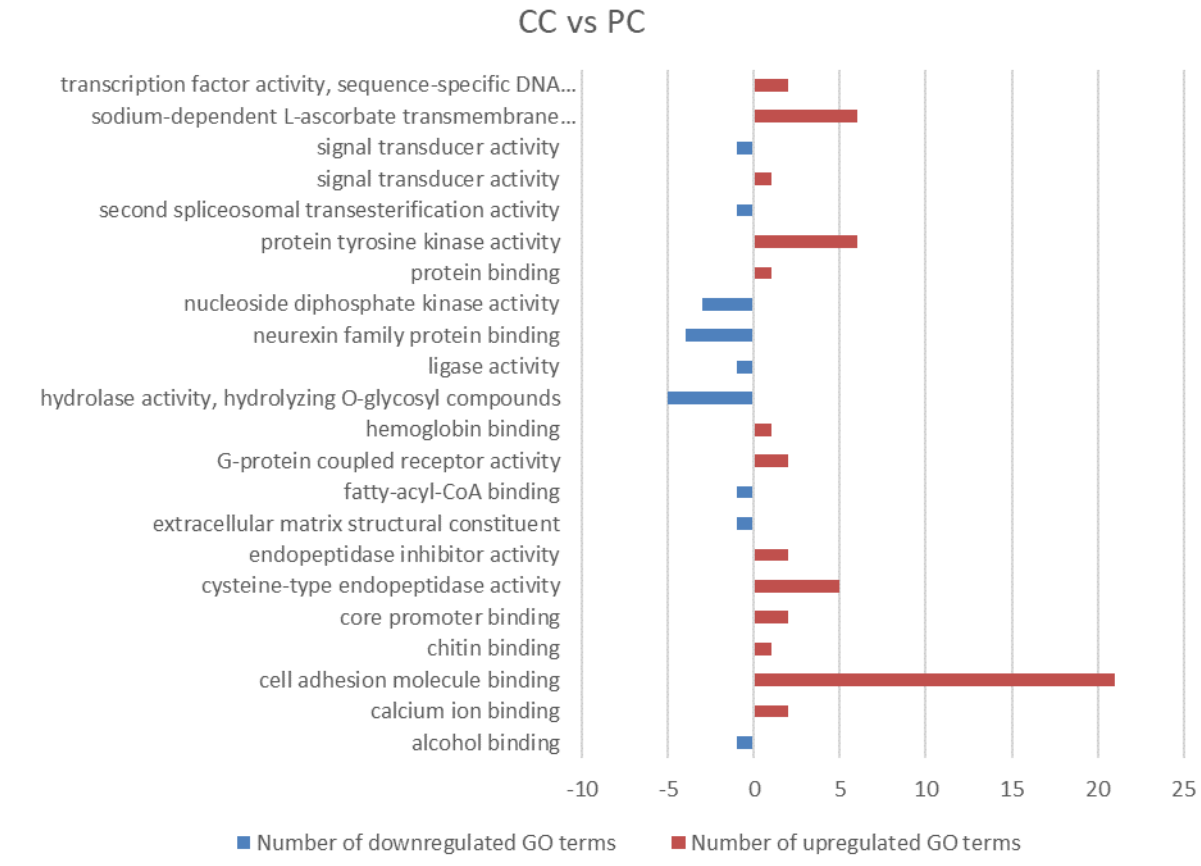
The gene enrichment in the groups related to energy metabolism appears to show that the energy metabolism in CP individuals is higher than either PC or PP worms, therefore it is likely that sudden, short term exposure is more energetically costly and that the long term exposed earthworms have developed mechanisms to mitigate this cost. However, energy metabolism was also higher in the PP group compared to PC, therefore the current exposure is still more energetically costly in this potentially adapted site population than is exposure to a control soil, suggesting no cost for any tolerance in this polluted site population.

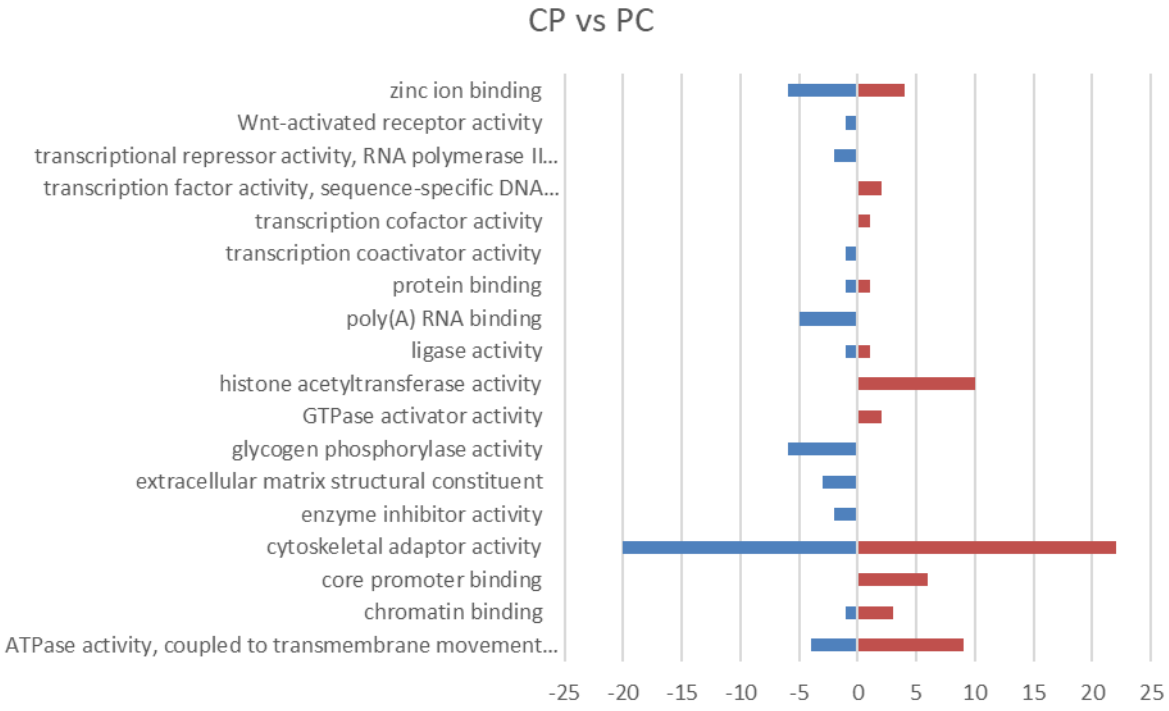
5.3.10.2.6. Cell structure related genes were differentially expressed between the groups

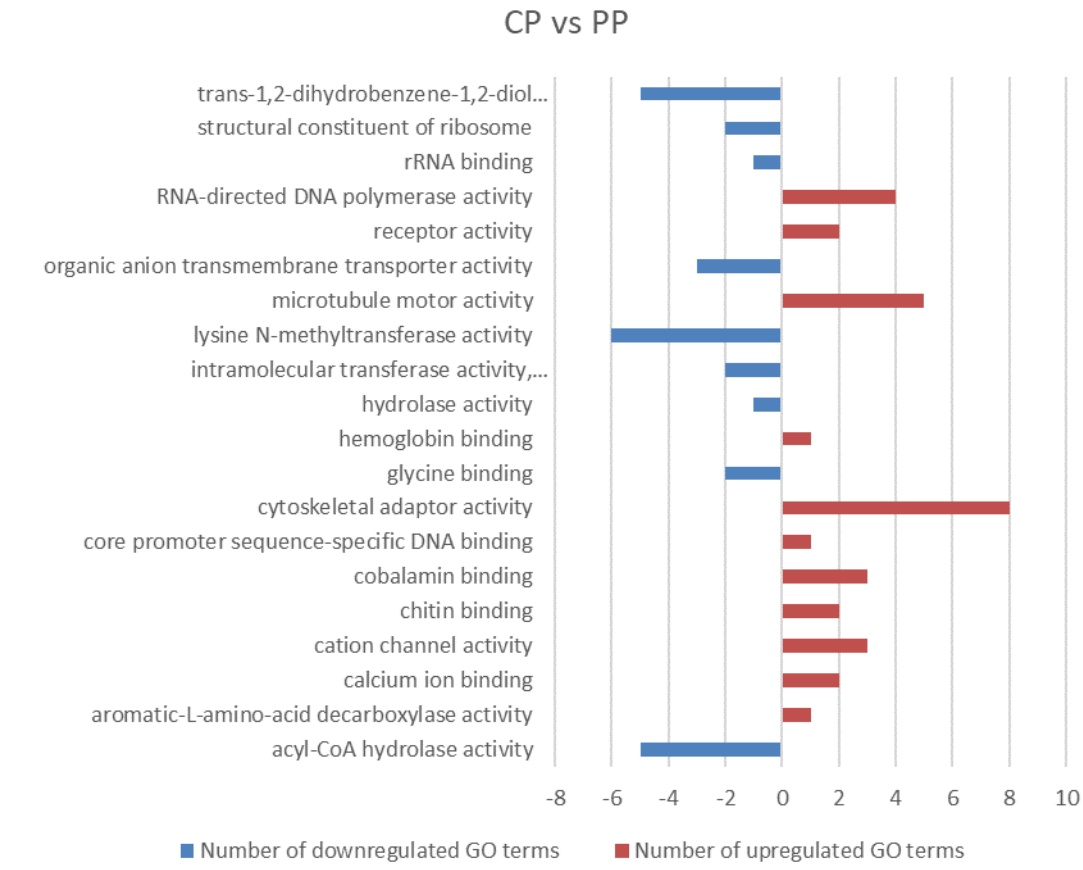
Extracellular matrix structural constituent GO terms were downregulated in the CC vs PC and CP vs PC comparisons, cytoskeletal adaptor activity was very noticeably (by 22 GO terms) upregulated in the CC vs CP comparison, simultaneously up- and downregulated in the CP vs PC comparison and upregulated to a lesser extent in the CP vs PP comparison. structural constituent of cytoskeleton was upregulated in the CC vs PP comparison and microtubule motor activity was upregulated in the CP vs PP comparison while cytoskeletal protein binding was very significantly (by 24 terms) upregulated in the PC vs PP comparison.

Genes involved in processes affecting structural components, such as cytoskeletal adaptor activity, actin binding and microtubule motor activity of the cell were strongly upregulated in the earthworms recently exposed to pollutants, implying that the exposure causes large scale alterations to the cellular structure. Interestingly, in the comparison between CP and PC groups, an almost equal amount of GO terms related to cytoskeleton was down- and upregulated, while neither the CC vs PC or the CC versus PP comparison produced such a statistically strong result.


CC vs CP
zinc ion binding
transcription factor activity, sequence-specific DNA...
transcription cofactor activity
toxin transporter activity
protein C-terminus binding
protein binding
peptidyl-prolyl cis-trans isomerase activity
nucleoside diphosphate kinase activity
monooxygenase activity
ligase activity
histone acetyltransferase activity
GTPase activator activity
fatty-acyl-CoA binding
cytoskeletal adaptor activity
core promoter binding
chromatin binding
ATPase activity, coupled to transmembrane movement...
alcohol binding
-10
-5
0
5
10
15
20
25


CC vs PC
transcription factor activity, sequence-specific DNA...
sodium-dependent L-ascorbate transmembrane...
signal transducer activity
signal transducer activity
second spliceosomal transesterification activity
protein tyrosine kinase activity
protein binding
nucleoside diphosphate kinase activity
neurexin family protein binding
ligase activity
hydrolase activity, hydrolyzing O-glycosyl compounds
hemoglobin binding
G-protein coupled receptor activity
fatty-acyl-CoA binding
extracellular matrix structural constituent
endopeptidase inhibitor activity
cysteine-type endopeptidase activity
core promoter binding
chitin binding
cell adhesion molecule binding
calcium ion binding
alcohol binding
-10
-5
0
5
10
15
20
25
Number of downregulated GO terms
Number of upregulated GO terms


CP vs PC
zinc ion binding
Wnt-activated receptor activity
transcriptional repressor activity, RNA polymerase II...
transcription factor activity, sequence-specific DNA...
transcription cofactor activity
transcription coactivator activity
protein binding
poly(A) RNA binding
ligase activity
histone acetyltransferase activity
GTPase activator activity
glycogen phosphorylase activity
extracellular matrix structural constituent
enzyme inhibitor activity
cytoskeletal adaptor activity
core promoter binding
chromatin binding
ATPase activity, coupled to transmembrane movement...
-25 -20 -15 -10 -5 0 5 10 15 20 25


CP vs PP
trans-1,2-dihydrobenzene-1,2-diol...
structural constituent of ribosome
rRNA binding
RNA-directed DNA polymerase activity
receptor activity
organic anion transmembrane transporter activity
microtubule motor activity
lysine N-methyltransferase activity
intramolecular transferase activity,...
hydrolase activity
hemoglobin binding
glycine binding
cytoskeletal adaptor activity
core promoter sequence-specific DNA binding
cobalamin binding
chitin binding
cation channel activity
calcium ion binding
aromatic-L-amino-acid decarboxylase activity
acyl-CoA hydrolase activity
-8 -6 -4 -2 0 2 4 6 8 10
Number of downregulated GO terms
Number of upregulated GO terms

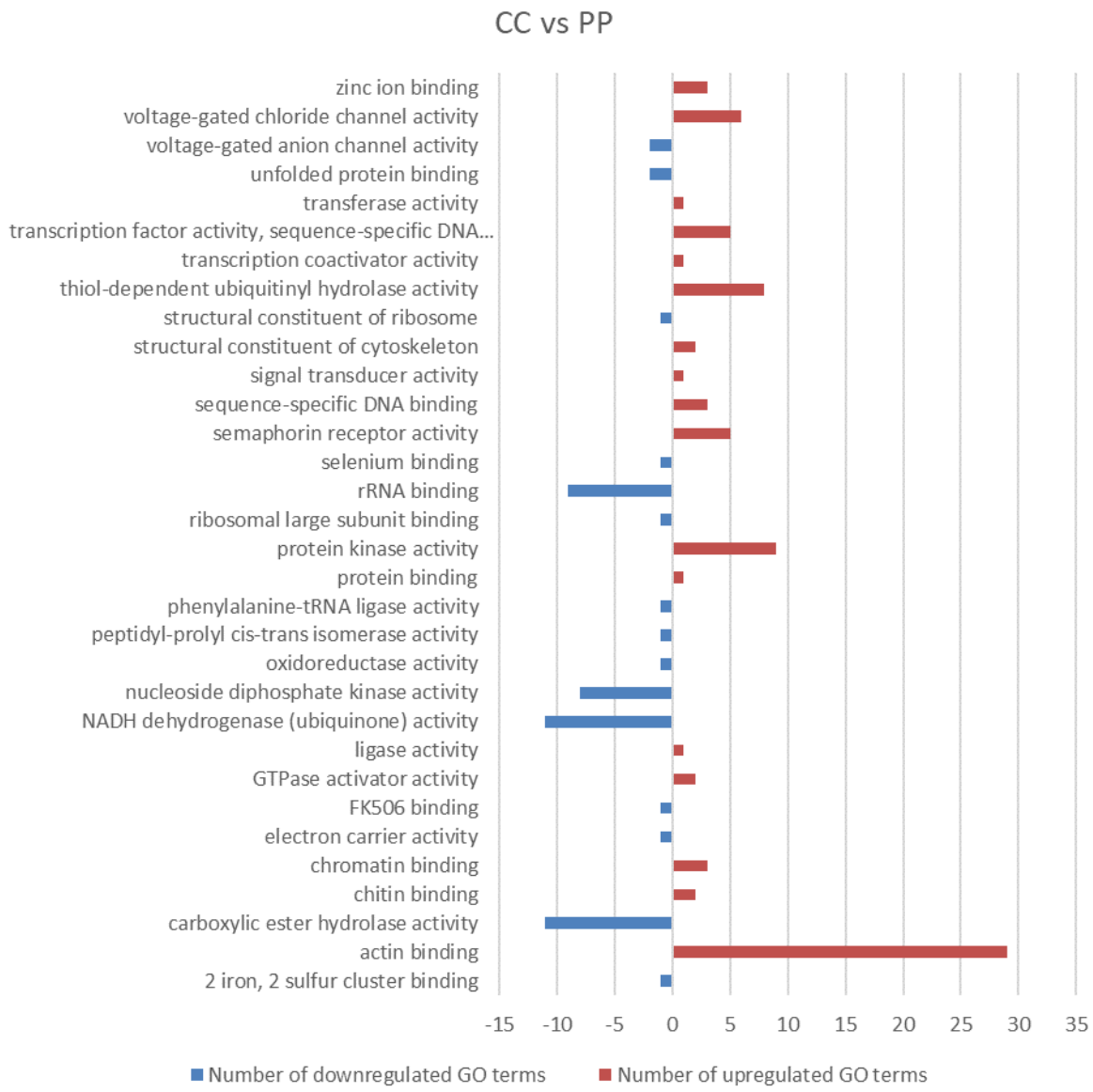


Figure 5.16. Differentially expressed molecular function GO terms in *L. rubellus*. The numbers denote the number of up (red) and down (blue) regulated GO terms in a data set comparing the gene expression as detected by RNASeq between groups *L. rubellus* collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby. The site names refer to: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil. The reference group in the comparisons is always the one coming first in the alphabet (e.g. for CP vs PC, CP is the reference)

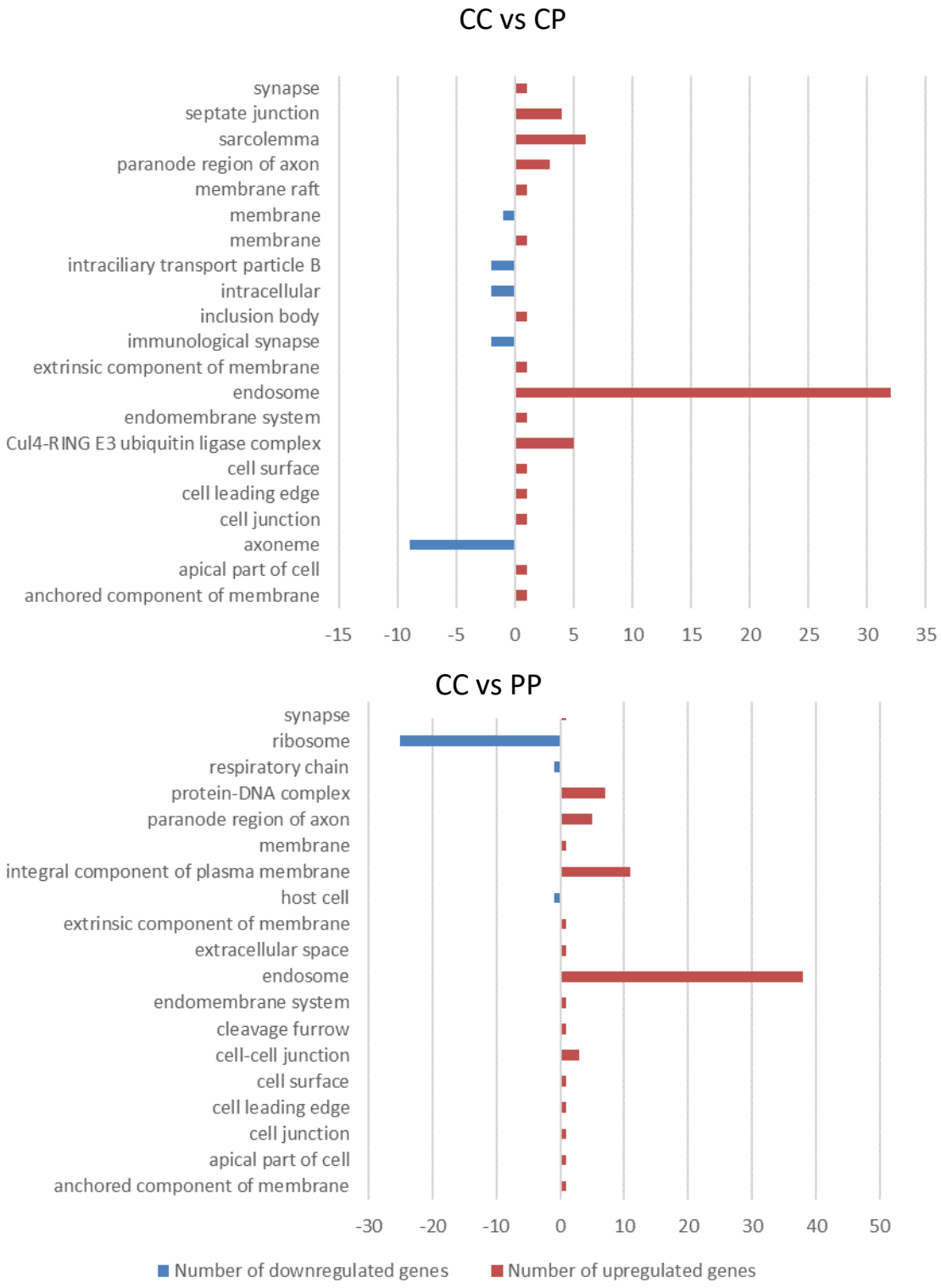
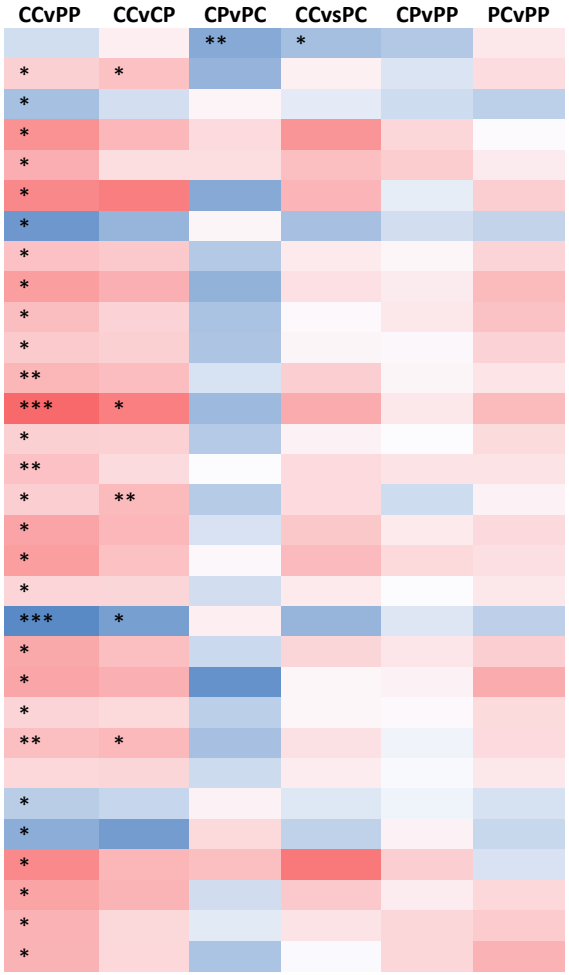


Figure 5.17. Differentially expressed cellular component GO terms in *L. rubellus*. The numbers denote the number of up (red) and down (blue) regulated GO terms in a data set comparing the gene expression as detected by RNASeq between groups *L. rubellus* collected from and transplanted between heavily polluted soils collected near the Avonmouth zinc smelter (Bristol, UK) and an uncontaminated control site nearby for 30 days. The site names refer to: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PP – Polluted worms, Polluted soil. The reference group in the comparisons is always the one coming first in the alphabet (e.g. for CP vs PC, CP is the reference)

5.3.10.3. Expression variation in genes affecting DNA methylation and chromatin states

In order to observe if there is further evidence in the gene expression data that the chromatin state of the transplanted earthworms is affected, differentially expressed genes whose sequences were orthologous to genes observed in other species to affect chromatin states were analysed between the transplant groups. When searching for the terms "DNA methyltransferase", "histone", "chromatic" and "cytosine methyltransferase", 279 amongst successfully annotated transcripts were found. Of these, 31 were significantly differentially expressed (using the adjusted *p* values) in at least one comparison between the transplant samples (See Figure 5.18). No significantly different individual genes were found in the comparisons between CP vs PP and the PC vs PP groups. CC vs PP comparison produced the most epigenetically relevant differentially expressed genes with 30 significantly different genes in this category, followed by the CC vs CP comparison with 5. The CP vs PC and CC vs PC comparisons had only a single differentially expressed gene, which was most closely orthologous to the human lysine – specific histone demethylase 1A. This pattern of differential expression in genes within the pathway suggests that most of these genes explored were more highly expressed in the currently or previously pollution exposed *L. rubellus*, implying that the exposure to a mixed heavy metal pollutant environment causes chromatin reorganisation. The only comparison carried out in this experiment where the genes involve in chromatin regulation were commonly downregulated was that between the CP and PC groups (the genes showed lower expression in PC compared to CP). This implies that current exposure is the biggest cause for epigenetic rearrangements occurring in cells (also underlined by the way most of the epigenetically important genes in the PP group were upregulated compared to PC (see Figure 5.18). These results broadly agree with the pathway analysis above, however, they also underline the benefits of different types of analysis as the CC vs PP comparison showed only a minor enrichment in chromatin-related genes.



CCvPP	CCvCP	CPvPC	CCvsPC	CPvPP	PCvPP	Protein names
		**	*			Lysine-specific histone demethylase 1A
*	*					Transcription elongation factor SPT6 (hSPT6)
*						Chromobox protein homolog 3 (Heterochromatin protein 1 homolog gamma)
*						SWI/SNF complex subunit SMARCC2 (BRG1-associated factor 170) (BAF170)
*						SWI/SNF complex subunit SMARCC2 (BRG1-associated factor 170) (BAF170)
*						Histone acetyltransferase KAT6B
*						Histone H2A.V (H2A.F/Z)
*						Probable global transcription activator SNF2L2 (ATP-dependent helicase SMARCA2) (BRG1-associated factor 190B)
*						Paired amphipathic helix protein Sin3b (Histone deacetylase complex subunit Sin3b) (Transcriptional corepressor Sin3b)
*						Transcriptional regulator ATRX (ATP-dependent helicase ATRX) (Heterochromatin protein 2)
*						Transcription elongation factor SPT6 (hSPT6) (Histone chaperone suppressor of Ty6)
**						Histone acetyltransferase KAT2A (General control of amino acid synthesis protein 5-like 2)
***	*					Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific
*						Transcription activator BRG1 (ATP-dependent helicase SMARCA4) (BRG1-associated factor 190A)
**						Chromatin-remodelling ATPase INO80 (hINO80)
*	**					FACT complex subunit SSRP1 (Facilitates chromatin transcription complex subunit SSRP1)
*						Chromatin-remodelling ATPase INO80
*						Helicase-like transcription factor (SWI/SNF-related matrix-associated actin-dependent)
*						FACT complex subunit spt16 (Facilitates chromatin transcription complex subunit SPT16) (dSPT16)
***	*					Charged multivesicular body protein 6 (Chromatin-modifying protein 6)
*						BRD4-interacting chromatin-remodelling complex-associated protein
*						E3 ubiquitin-protein ligase HUWE1 (E3Histone)
*						Histone-lysine N-methyltransferase SETD2 (Lysine N-methyltransferase 3A)
**	*					FACT complex subunit SSRP1 (Facilitates chromatin transcription complex subunit SSRP1)
						Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific
*						Charged multivesicular body protein 4b (Chromatin-modifying protein 4b) (CHMP4b)
*						Histone deacetylase complex subunit SAP18 (18 kDa Sin3-associated polypeptide)
*						Lysine-specific demethylase 7A (JmjC domain-containing histone demethylation protein 1D)
*						Protein arginine N-methyltransferase 5 (72 kDa ICln-binding protein) (Histone-arginine N-methyltransferase PRMT5)
*						Histone acetyltransferase p300 (p300 HAT) (E1A-associated protein p300)
*						AT-rich interactive domain-containing protein 4B (Histone deacetylase complex subunit SAP180)

Figure 5.18. Comparisons of differentially expressed transcripts belonging to epigenetically relevant proteins in *L. rubellus* collected from a heavily polluted (P) site near the Avonmouth zinc smelter and a control (C) site and transplanted to soils from the sites in a laboratory exposure for 30 days. The transplant codes are: CC- Control worms, Control soil; CP – Control worms, Polluted soil; PC – Polluted worms, Control soil; PP – Polluted worms, Polluted soil. The reference group in the comparisons is always the one coming first in the alphabet (e.g. for CP vs PC, CP is the reference). The red and blue colours refer to up and down regulated genes, respectively, with the deeper colours showing a higher level of each. The statistical significance values: * p≤0.05; **p≤0.01;***p≤0.001.

5.4. Discussion

Heavy metal pollution is a major source of toxicity for many animal species across the world (Spurgeon et al., 2008; Micó et al., 2006; Romaniuk et al., 2017). Exploring the long and short-term impacts of this type of stress may provide us with a better understanding of the way organisms can cope with metal exposure. Such information can help to identify the particular strategies used by earthworms to cope with short-term heavy metal stress and also perhaps give answers to evolutionary questions on how an organism adapts to a new environment in a short length of time during which genetic mutations necessary for long term evolutionary adaptations have not arisen (Gómez-Schiavon and Buchler, 2019). In this experiment, earthworms *L. rubellus* were collected from a control site and a site near the Avonmouth smelter (Bristol, UK) polluted with multiple heavy metals, including zinc, lead, arsenic copper and cadmium, all of which have been previously shown to have a detrimental impact on earthworms (Spurgeon et al., 1994; Anderson et al., 2013). These earthworms were then transplanted between the soils collected from each site in a laboratory experiment and their phenotypes, global DNA methylation and gene expression profiles as detected by RNASeq were explored.

The individuals collected from the polluted environment showed a consistently lower reproductive output. Interestingly, of the 90 day transplant groups, only the CP group gained weight, while the others had lost it, which may be a result of a hormetic effect similarly to that reported in Chapter 3.

The impacts of the multipollutant environment had a large-scale effect on the molecular mechanisms of the earthworms. At 30 days of transplantation, global DNA methylation as detected by msAFLP of the *L. rubellus* was significantly different between CC and the PP individuals and at 90 days, while not statistically significantly, the CP individuals appeared to have a DNA methylation profile that was more similar to the PP group than the CC group. It is interesting to note that the CC and PP groups separated from one another in terms of global DNA methylation distributions at 30 and 90 days, but not at 3 days of exposure. Therefore it is possible that something about the experimental conditions caused these two groups to respond differentially and that differences in DNA methylation profiles are due to this difference in response rather than long term adaptations to pollution and/or genetic differences between the populations. Studies involving immediate dissections after field sampling may address this issue in the future.

The gene expression results carried out using the 30 day transplant individuals showed that genes linked to transmembrane transport and metal ion binding were differentially expressed (mostly upregulated) due to pollution, that translation was downregulated and protein degradation upregulated as a result of heavy metal exposure and change in environment. These findings confirmed previously reported findings of responses to different heavy metals, for instance, in the oyster *Crassostrea hongkongensis* metal ion binding related proteins were upregulated as a result of long-term heavy metal adaptation (Luo et al., 2014) and the anemone *Nematostella vectensis* showed an increase in expression of transmembrane transport related genes due to mercury exposure (Elran et al., 2014).

It was also interesting to see that genes related to cellular structure were very strongly upregulated due to both long-and short-term heavy metal exposure. These findings show that pollution has very strong effects on fundamental cellular processes in *L. rubellus*. However, due to the relative under-exploration of the *L. rubellus* genome, it is possible that more specialised pathways were not identified simply because the species whose genes the transcripts were orthologous to were quite remotely related to earthworms and therefore only the more conserved, fundamental genes could be fully analysed.

A large number of genes linked to epigenetic regulations were differentially expressed between the treatment groups, particularly CC and PP, with a clear trend for upregulation of chromatin altering genes as a result of pollutant exposure, particularly in the recent transplant exposure environment. In a study on the sea anemone *N. vectensis*, multiple chromatin-related genes were differentially regulated due to copper exposure, however, in the case of the study, these were downregulated (Elran *et al.*, 2014). This may indicate a difference in single metal exposure versus a mixed exposure employed currently as well as different adaptation mechanisms employed for different metals.
DNA methylation changes and chromatin deregulation has been found to be a response to sudden environmental changes by (Gao et al., 2010; Liebl et al., 2013; Verhoeven et al., 2010). The current study shows that it possibly remains affected in *L. rubellus* even within individuals from a population that has been subject to exposure over many decades. However, as the population history of the collected earthworms is unknown, it is possible these are representatives of a more recently invaded population and the finding in the current study is not actually contradictory to previously reported (Liebl *et al.*, 2013).

The response observed, such as in metal ion trafficking and chromatin responses are potentially metabolically costly to the organisms and may result in long term phenotypic impacts, as indicated by the lower levels of reproductive output by both PC and PP earthworms. The gene expression results also showed that energy metabolism- linked genes were upregulated in the PP *L. rubellus* worms compared to PC and in CP earthworms compared to both PC and PP, underlining the likelihood that this adaptation is energetically costly to the earthworms. This observation is in accordance with findings of a previous study on the oyster *C. hongkongensis*, which showed an overexpression of energy metabolism-related proteins in response to long term heavy metal pollution (Luo *et al.*, 2014). The experiments reported in this chapter also showed the complexities of working with species living in the natural environment. *L. rubellus* comprises of two different lineages and multiple sublineages were also present. This provided the experimenters with difficulties when attempting to align the found transcripts to a reference transcriptome and resulted in a reduced number of individuals used in the gene expression experiment. Furthermore, genomic work with a group of species like earthworms is made additionally more difficult as a higher level of variability between genomes is found in this group. However, despite these complexities, exploring wild species is important for understanding real world implications of pollutants and stressors in general.

5.5. Conclusions

The extremophile population of *L. rubellus* inhabiting the soil near Avonmouth zinc and lead smelter employs multiple adaptation mechanisms, including epigenetic changes, particularly chromatin deregulation and also possibly DNA methylation changes. These were replicated to a more minor degree by earthworms collected from control soils and placed in the heavily methylated soil, therefore these adaptations are indeed the result of exposure to the heavily polluted soil. The pollutant exposure also had a major effect on the earthworms' energy metabolisms, cytoskeleton reorganisation, gene expression and translation regulation, transmembrane transport and metal ion binding gene expression.

Discussion

6.1. Introduction

In this project, I looked at the effects of prolonged exposure and epigenetic effects of pollution on earthworms, and on whether genetic background has an impact on these responses. Looking for epigenetic responses to pollution can entail two types of investigations – whether epigenetic molecular marks are affected by pollution within the exposed generation and whether exposure results in an impact on the future generations of the exposed organisms. Both were investigated in this thesis.

6.2. Key findings

Chapter 2. In this chapter, *Eisenia sp.* earthworms were exposed to sublethal concentrations of arsenic (12 mg/kg per dry weight soil), cadmium (40 mg/kg) and imidacloprid (0.2 and 1 mg/kg) throughout their development into adulthood and their growth weights and reproductive output were measured. Cadmium and both concentrations of imidacloprid significantly reduced the earthworm growth rates. Hormetic effects on total juvenile output were found to result from arsenic exposure and on cocoon hatching rates from the 0.1 mg/kg imidacloprid exposure (the latter did not, however, affect total reproduction due to a lower cocoon output). It was shown that the two genetic lineages of *Eisenia*, which have been proposed as separate species (Aira *et al.*, 2016), *E. andrei* and *E. fetida,* interbred with each other and the genetic differences associated with the lineage did not have an impact on the way the pollutants affected the exposed organisms.

Chapter 3. The experiment started in Chapter 2 was continued in an extended multigenerational exposure described in Chapter 3. The results of this continuous exposure to the three chemicals for three generations as well as for recovery populations (for one and two generations) were described. No epigenetic transgenerational effects on the phenotypes of the earthworms could be detected, but maternal effects were observed. The offspring of *Eisenia sp.* earthworms exposed to arsenic for a generation showed lower survival regardless if they were raised in control or arsenic-spiked soil but had lower growth weights only if they were exposed to the pollutant. Cadmium exposed earthworms recovered after life-time exposure for the F1 generation and showed similar phenotypic parameters to controls throughout the rest of the experiment. The 0.2 mg/kg imidacloprid exposure resulted in an overall lower reproductive output in the F1 and F2 generations, but also recovered in the F3

generation. Both concentrations of imidacloprid consistently reduced growth weights in all three generations (although to a lesser degree in F3 for 1 mg/kg and F2 for 0.2 mg/kg). Where between generation differences were seen, the continuously exposed earthworms mostly showed adaptive responses to the toxicants, the only observed parameter that showed a definite increased in sensitivity indicative of an accumulated multi-generational effect was the decreased survival of the 1 mg/kg imidacloprid exposed *Eisenia sp.*

Chapter 4. In this chapter, the growth parameters and DNA methylation patters (as detected by msAFLP) of *L. rubellus* exposed to arsenic, cadmium and fluoranthene (a polycyclic hydrocarbon) for the whole developmental period were reported. Further studies were also conducted in which the DNA methylation profiles of *L. rubellus* collected from a gradient of sites containing varying levels of heavy metal pollution were determined to investigate methylation response under field conditions. The arsenic and cadmium exposures resulted in reduction in growth, but showed no impact on DNA methylation patterns. In contrast, fluoranthene exposure affected the global DNA methylation of the exposed *L. rubellus* at the two highest concentrations used in the experiment. The highest of these two exposure levels only had an impact on growth after placement in freshly spiked soil to account for fluoranthene degradation in the middle of the experiment, at a later juvenile stage. No significant differences on DNA methylation or overall AFLP profiles were found between the earthworms collected from sites at varying levels of heavy metal pollution.

Chapter 5. In this chapter a common garden experiment was conducted in which *L. rubellus* earthworms collected from a heavily polluted soil and a control soil were transplanted between the two soil types and their global DNA methylation (as detected bey msAFLP) and gene expression (as detected by RNASeq) were measured. It was found that the continuously exposed and control groups had different DNA methylation profiles and that a very large number of genes was differentially expressed due to pollution exposure and that individuals exposed to pollution for 30 days responded differentially compared to individuals that had adapted to living in the heavily polluted site. Fundamental cellular processes, such as cell structure and cytoskeleton reorganisation, translation, transcription and transmembrane transport were affected by the metal pollution present. Many chromatin- related genes were differentially expressed due to pollution, particularly between the group adapted to pollution and recently exposed to pollution and the control group. Recent pollutant exposure had a great impact on chromatin-change related gene expression, but this impact was much greater if the individuals that had adapted to living in the heavily polluted soil. Chromatin reorganisation, therefore, likely forms a part of long-term adaptation to heavy metal pollution.

6.3. The effects of toxicants on development

Developmental exposure may be important for the establishing of epigenetically inherited traits as this type of exposure may result in changes in the developing gonads' epigenetic marks, which may be inherited in the future generations (Huypens *et al.*, 2016). Developmental exposures are also interesting for epigenetic research within a single generation experiment if the developing stem cells' epigenetic marks are affected and, therefore, the effects accrued during an early age may be exhibited in later adulthood. Epigenetic modifications may also help exposed organisms cope better with stress, as early life activation of molecular pathways may help develop resilience at an early life-history stage. This may be energetically costly, however, which is possibly why the Cd exposed *Eisenia sp.* in Chapter 2 showed reduced growth but recovered and ultimately reached a similar size to controls at the last stage of growth and also were able to achieve a similar reproductive output as unexposed earthworms.

When exposed to arsenic, the *Eisenia sp.* earthworms showed a small but quantifiable increase in growth, but, in contrast to this, the *L. rubellus* exposed to arsenic (reported in Chapter 4) showed reduced growth after developmental exposure. These results may indicate a difference in species sensitivity, which is an issue in ecotoxicological research where the species used for chemical testing need to, by necessity of the assay, be resilient enough to survive in laboratory conditions, and can also be more resilient to toxic exposures than other species that would be exposed in the wild (Kreutzweiser *et al.*, 2008). The results of the developmental exposure to cadmium in Chapter 2 showed lower effects of the metal on reproduction than reported earlier in an adult exposure (Spurgeon *et al.*, 1994), which indicates a possible slight adaptive effect of the developmental versus adult exposure.

6.4. Maternal effects of pollution

The developmental exposures carried out in Chapter 2 resulted in maternal effects on the offspring of earthworms exposed to arsenic during the F1 generation. These F1 offspring showed lower survival regardless if they were raised in arsenic-spiked or control soil and lower growth rate, but only if they were exposed to arsenic themselves. These maternal effect show the importance of multigenerational exposure experiments as, if the individuals were only exposed to arsenic for one generation, a hormetic effect would have been observed, but, after two generations, it becomes clear that there is

a trade-off and that in the long term arsenic has a negative phenotypic effect on the exposed *Eisenia sp.*. Given the finding of life-time exposure for *E. fetida*, the findings reported in Chapter 4 may show an incomplete picture on effects of arsenic, cadmium and fluoranthene on *L. rubellus*. The generation time of this species is considerably longer than *E. fetida* and *E. andrei* (Klok and Thissen, 2009). This makes multigenerational exposures with *L. rubellus* fraught with practical difficulties. The fluoranthene exposure resulted in less detrimental effects on the growth of the earthworms compared to As and Cd, but, as seen with the As effects on *Eisenia sp.* earthworms in Chapters 2 and 3, there may be a trade-off paid for by the future generation(s). Fluoranthene was also the only of the three chemicals to produce a clear concentrated-related effect on the DNA methylation of the *L. rubellus*, it is therefore possible that the DNA methylation effects caused by fluoranthene result in phenotypic effects in the next generation in this species.

6.5. Multigenerational effects of toxicants

During long term exposures, affected organisms may respond in multiple ways. For example, organism cellular mechanisms may be activated to help individuals cope with it with negligible additional energetic and toxicological damage cost of exposure. In such cases, a population that has experienced similar stress in the past may be adapted to cope with the stressor. Such adaptation, however, may place an additional energetic cost that may reduce resources available for other physiological or life-cycle parameters. The costs of adaptation may impact life-cycle traits that may ultimately reduce the viability of the exposed population. Such effects on resource allocation underlie the observed impacts after three generations of exposure to 1 mg/kg per dry weight soil imidacloprid in Chapter 3.

Generally, earthworms exposed for multiple generations during this project showed adaptive responses rather than sensitisation following life-time and multi-generational exposure to metal exposures. This may reflect the presence of adaptive traits for metal exposure given that trace-elements are ubiquitously present in the environment (Li and Thornton, 2001). The multi-generational toxicity and even sensitization seen for mortality effects under prolonged imidacloprid exposure potential reflects a specific toxicological response to this xenobiotic. Multigenerational exposures may also include transgenerational epigenetic effects on the organisms leading to accumulative impacts. The F3 mortality increase in the 1 mg/kg imidacloprid exposure (Chapter 3) may result from such a transgenerational effect for this pesticide.

6.6. Effects of toxic exposure on DNA methylation and chromatin

Heavy metals have been observed to affect DNA methylation in a large variety of species, such as *Daphnia magna* (Vandegehuchte *et al.*, 2009), humans (Virani *et al.*, 2016), maize (Erturk *et al.*, 2015), the seagrass *Zostera marina* (Greco *et al.*, 2019), rice (Oryza sativa) (Feng *et al.*, 2016), mice (Liu *et al.*, 2015) and *Drosophila melanogaster* (Guan *et al.*, 2019). Therefore, DNA methylation change is potentially a fundamental component of the response to heavy metal toxic stress present in organisms across different kingdoms of life. In Chapter 5, the *L. rubellus* living in a heavily polluted soil produced fewer offspring than the control population, indicating that this population is required to cope with the additional pollution stress at a cost to reproductive output. Within this population exposed to pollution for a long period of time, DNA methylation differences were present alongside many changes in chromatin-related gene expression. Similar differences were also seen, although to a lesser extent, when a control population of earthworms was exposed in the same site polluted soil for a short time. Therefore, it appears that DNA methylation and chromatin reorganisation are utilised as mechanisms of response to pollution in earthworms, especially after long term exposure.

The chromatin-disrupting effects of heavy metals is also considered one of the reasons why multiple heavy metals (arsenic, cadmium, chromium and nickel) are carcinogenic (Kim et al., 2015,Romaniuk et al., 2017, Reichard and Puga, 2010). This underlies the fundamental nature of the cellular response this type of toxic exposure elicits. Thus, in order to cope with the exposure to metal ions, cells activate DNA methylation and chromatin remodelling mechanisms that have a huge downstream impact on the future of the cell and potentially the organism.

It was interesting to observe that DNA methylation was affected by developmental exposure to fluoranthene, but not arsenic or cadmium in *L. rubellus* (Chapter 4). Fluoranthene is a polycyclic aromatic hydrocarbon (PAH), these chemicals are carcinogenic and can covalently bond to DNA, forming adducts that are established markers of risk of cancer. These adducts are more commonly found in methylated regions of DNA (Herbstman *et al.*, 2012). Fluoranthene has been previously observed to bind directly to DNA (Babson *et al.*, 1986). Maternal exposure to a PAH benzo[*a*]pyrene has been associated with altered DNA methylation in humans (Tang *et al.*, 2012), but, as far as the author is aware, no previous evidence of fluoranthene affecting DNA methylation has been reported.

The differences between the DNA methylation presence in the earthworms in the current study indicate a possible variation in the modes of action between heavy metals and fluoranthene, with the former perhaps affecting DNA methylation only after exposure during embryonic development which is likely the time in earthworm life when new DNA methylation patterns are naturally established,

while fluoranthene may have a more direct impact on DNA by forming adducts, which then result in immediate changes in DNA methylation patterns. It may that the differences in phenotypic responses between metal and organic xenobiotic toxicants occur because earthworms such as *L. rubellus* have been exposed to heavy metals throughout their evolutionary history. As such, response such as changes in growth and development may reflect changes in the allocation of resources to handling this stress and prevent change to fundamental processes like chromatin formation. In contrast for xenobiotics such as fluoranthene, which even though naturally occurring is not likely to be ubiquitous, changes to DNA methylation may result through mechanisms for which earthworms are poorly adapted, which likely would have further negative downstream effects. A similar process may also explain the differences between arsenic and cadmium and imidacloprid exposures in Chapter 3. The *Eisenia sp.* earthworms adapted to the heavy metal exposure (by F2 for cadmium and F3 for arsenic), but imidacloprid continued to elicit detrimental phenotypic effects throughout the three-generation long exposure.

6.7. Effects of toxic exposure on cellular functions

The results in Chapter 5 show the transcriptome wide effects of metal pollutants on cellular processes in *L. rubellus*. Pathways impacted represent in the main processes fundamental to cell functioning, such as energy metabolism, cytoskeleton reorganisation and gene expression regulation. That the effect seen corresponds to core process likely indicates a potential limitation of working with a non-model organisms, particularly ones that are distantly related to many of the animals whose gene functions have been explored to a greater extent, such as mice and humans. Mechanisms that are more specific to the earthworms to support their specific physiology and ecology are unlikely to be identified by using model organisms as a reference for genome annotation. Interestingly, even though other invertebrate organisms like *C. elegans*and *Drosophila melanogaster* were present in the database used for annotation of the earthworm transcripts, more *L. rubellus* genes aligned with human and mouse genes.

A cellular process strongly altered by metal exposure was cytoskeleton reprogramming. There are a number of other studies which have reported an effect on the cytoskeleton by different types of toxicants. Exposure to the organic neurotoxin diphenylditelluride disrupted cytoskeleton in neurons via affecting calcium signalling (Heimfarth *et al.*, 2016). Silver nanoparticles have also been observed to disrupt cytoskeleton structure by forming actin inclusions which disrupt neurites (Cooper and Spitzer, 2015) and zinc nanoparticles were observed to induce actin depolymerisation in mice macrophages (Pati *et al.*, 2016). Trace metals have been shown to have a variety of effects on the

cytoskeleton in plants (Horiunova *et al.*, 2016). Similarly, the antibacterial agent triclosan has been found to affect proteins involved in the cytoskeleton in zebrafish (Elodie *et al.*, 2017). Some of the genes altered due to heavy metal pollution in *L. rubellus* were also known to contribute to different aspects of neuron development (Chapter 5). As cytoskeleton deregulation can also be involved in neuron disruption, it is possible exposure to metal pollution may cause specific disruptions to the earthworm nervous systems, something that it established in mammalian species for known neurotoxic metals such as cadmium and lead.

6.8. Evolutionary implications of these findings

While it has already been documented that toxic exposures have an impact on evolution by having an effect on the prevalence of particular DNA mutations (Luc et al., 2016, Major et al., 2018, Weston et al., 2018), the observed effects of pollutants on the organisms' epigenomes opens up a theoretical possibility for a more direct impact pollution may have on the evolutionary adaptations that help species survive toxicity. Certain chemicals, like fluoranthene, may directly impact DNA mutations by forming DNA adducts which can then result in DNA repair machinery adding on the wrong base causing a point mutation. This potential mechanisms for mutation has parallels with the manner through which 5-methylcytosine can preferentially mutate to a thymine rather than an unmethylated cytosine molecule (Bird, 1980) . Such point changes, would, however, need to occur during meiosis for them to have an evolutionary impact.

While for genuine transgenerational inheritance to occur epigenetic marks need to be inherited meiotically, this does not mean that without this type of inheritance epigenetic modifications induced by toxic stress could not alter evolutionary processes. Long term gene silencing, for example, may result in an accumulation in genetic mutations that would otherwise be selected out of the population.

Epigenetic marks themselves can be inherited and/or be stably present in organisms over many generations (Klosin et al., 2017, Williams and Gehring, 2017, Hofmeister et al., 2017), therefore a type of epigenetic mark evolution may occur.

Further, the ability of the exposed organisms to respond via changing their epigenetic make up in itself would be subject to natural selection, presumably the types of proteins that would transduct the signal to the cell that pollution is present and the downstream cellular components that would result in the organism responding favourably when exposed to pollution over an extend exposure. Likely more specific chromatin changes would result in reduced detrimental effects while more broadscale

alterations would have multiple off-target effects (such as cancer). Therefore these more specific effects would be selected favourably. These would be more difficult to observe using global DNA methylation techniques like msAFLP, and so therefore may be missed in many analyses.

Epigenetic mechanisms have also been suggested to have a role in evolution due to their regulation of transposable elements. A model where epigenetic deregulation due to stress results in activation of transposable elements which in turn causes new mutations in the organism has been proposed as a means of adaptation to new environments by invasive species (Stapley et al., 2015;Mirouze and Paszkowski, 2011).

6.9. Future research

Organisms like earthworms may be a useful model for addressing questions relating to the role of epigenetics in evolutionary adaptation to pollutants, due to both their high levels of DNA methylation and their close relationship to soil and, thus, the susceptibility for exposure. Analysis to address such questions can be carried out using targeted sequencing and DNA methylation measurements of genes of interest in the adapted population used in this thesis and others. In Chapter 5, the role of epigenetic mechanisms in adaptation to pollution was explored. *L. rubellus* population near the Avonmouth zinc and lead smelter is one of only three species (alongside *Lumbricus castaneus* and *Lumbricus terrestris)* to have successfully adapted to living in this highly polluted environment (Spurgeon and Hopkin, 1995). It would be interesting to observe if epigenetic regulations play a role in the success of each of these known adapted species. Common garden experiments, such as used in Chapter 5, in which individuals living at polluted and clean sites undergo reciprocal transplantation followed by assessment of genome methylation (and other epigenetic mark) analysis may help elucidate how epigenetic traits contribute to such adaptation.

It would also be interesting to establish the mode of action for fluoranthene effects observed on the epigenome in Chapter 4. For example, studies could investigate the extent to which fluoranthene forms DNA adducts in earthworms and what other effect it may have on chromatin and on organism phenotypes after a more long-term exposure.

Another avenue of research could also be looking at the ecological implications of the finding relating to the impacts of metal and organics chemical following extended exposure. The studies reported in Chapters 2, 4 and 5 report some hormetic effects of exposure even over extended timescales. During such exposure individuals would accumulate the relevant pollutant. Hence, even though the exposure

may have limited effect on the earthworm directly (and could even be beneficial), it may also result in accumulation of the heavy metals in species at higher trophic levels. This may result in yet unknown ecological effects.

Whether epigenetic mechanisms directly affect evolutionary processes or not still remains largely unknown. However from the studies in Chapter 5, it is clear that such mechanisms play a role in stress response and an organism's ability to cope with toxic exposure. As most research in this field has been carried out in mammals, many environmentally important non-model organisms remain unexplored and may provide some exciting answers to questions related to both ecology and evolution.

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