Molecular Genetic Biomarkers of Reproductive Fitness in Earthworms

Submitted for the Degree of Ph.D.

by

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SUMMARY

When ecotoxicologists assess the environmental impact of pollution they must pose a fundamental question. That is, is the toxicological data produced from any experiment ecologically relevant? It has long been thought that a measure of survival of any organism is not sufficient to predict potential population effects. A more sensitive method is the measurement of sub-lethal endpoints, such as growth and reproduction. However, measurement of these parameters may not give an 'early warning' of the impact of complex pollutants within any ecosystem. Therefore, so-called biomarkers have been developed to fill this void. Biomarkers can be used at a number of organisational levels; e.g. cellular, protein or DNA, but they all have the sensitivity to act as predictive tools in ecotoxicology and risk assessment.

In the past twenty years earthworms have become model organisms in terrestrial ecotoxicology. This is mainly due to the critical role they play within the soil ecosystem in most parts of the world. Therefore the present study utilises three earthworm species (*Eisenia fetida*, *Eisenia andrei* and *Lumbricus rubellus*) to identify, characterise and validate molecular genetic biomarkers of reproduction.

To isolate potential reproductive genes a subtractive library was created from the anterior and posterior segments of *Lumbricus rubellus*. A number of potential biomarker candidate genes were identified, but a putative sperm-specific antigen warranted special attention. The gene fragment of this putative sperm antigen was identified in all three earthworm species used in the study and its potential as a biomarker is discussed.

Annetocin has previously been characterised as a member of the mammalian vasopressin/oxytocin superfamily of neuropeptides and has been shown to induce egg-laying behaviours in *Eisenia fetida*. The annetocin gene was isolated from the three earthworm species and shown to be expressed in the reproductive segments of *Eisenia fetida*. The expression levels of the annetocin gene were determined in earthworms exposed to metalliferous soils both in laboratory and semi-field exposures using

quantitative PCR. A decrease in annetocin gene expression levels correlated with a similar decrease in cocoon production rates of *Eisenia fetida* after metal exposure.

Upon exposure to the mammalian steroid hormones, 17β-oestradiol and testosterone along with the synthetic oestrogen 17α-ethynylestradiol, annetocin gene expression levels in *Eisenia andrei* were elevated in some cases, suggesting a role for oestrogens in earthworm reproduction. *Eisenia andrei* were also exposed to bisphenol A and nonylphenol in artificial soils and annetocin gene expression was determined. Although toxic to *Eisenia andrei* these two (weakly oestrogenic) compounds did not affect annetocin gene expression. These data strongly suggest that annetocin plays a critical role in earthworm reproduction and that oestrogens may modulate the expression of this gene.

The annetocin genomic structure was determined and three oestrogen-responsive elements were identified within the promoter of the gene in *Eisenia fetida*. What is more, the genomic structure of annetocin conformed to the three exon, two intron model of vasopressin/oxytocin superfamily neuropeptides.

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Measurement of Annetocin Gene Expression: a new Reproductive Biomarker in Earthworm Ecotoxicology

Ricketts, H.J., Morgan, A.J., Spurgeon, D.J., and Kille, P.

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ABBREVIATIONS

A	Adenosine
APS	Ammonium PerSulphate
ATP	Adenosine Tri-Phosphate
A_x	Absorbance at *x* nm
Blast	Basic Local Alignment Search Tool
BlastN	Basic Local Alignment Search Tool for Nucleotide sequences
BlastX	Basic Local Alignment Search Tool for nucleotide sequences translated in to amino acid sequences in all six reading frames
bp	Base Pair
BSA	Bovine Serum Albumin
C	Cytosine
Ct	Threshold Cycle
cDNA	Complimentary DeoxyriboNucleic Acid
dATP	DeoxyAdenosine Triphosphate
dCTP	DeoxyCytidine TriPhosphate
dGTP	DeoxyGuanosine TriPhosphate
DNA	DeoxyriboNucleic Acid
DNase	DeoxyriboNuclease
dNTP	DeoxyNucleoside TriPhosphate
ds	Double-Stranded
dTTP	DeoxyThymidine TriPhosphate
EA	*Eisenia andrei*
E2	17β-Oestradiol

EDTA	Ethylenediamine Tetra acetic Acid
EE2	17α-Ethynylestradiol
EF	*Eisenia fetida*
ERE	Oestrogen-Responsive Element
EtBr	Ethidium Bromide
FAM	6-Carboxyfluorescein
g	Gravity
G	Guanine
GSP	Gene-Specific Primer
HPLC	High Performance Liquid Chromatography
HEPES	N-[2-HydroxyEthyl]-Piperazine-N'-[2-EthaneSulphonic acid]
kb	Kilo Base
kDa	Kilo Dalton
LB	Luria Broth (Luria Bertani media)
LC_x	Lethal Concentration resulting in *x* % mortality
LR	*Lumbricus rubellus*
M	Molar concentration
M-MLV RT	Moloney-Murine Leukaemia Virus Reverse Transcriptase
mRNA	Messenger Ribonucleic Acid
NERC	Natural Environmental Research Council
NOEC	No Observable Effect Concentration
nt	NucleoTide
OECD	Organisation for Economic Co-operation and Development
OD	Optical Density
Oligo	Oligonucleotide

OT	Oxytocin
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNase	RiboNuclease
rpm	Revolutions per Minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
ss	Single-Stranded
SSA	Sperm-Specific Antigen
SSC	Saline Sodium Citrate Buffer
T	Thymine
TAE	Tris Acetate-EDTA Buffer
TAMRA	6-carboxy-TetrAMethyl-RhodAmine
TE	Tris-EDTA buffer
TEMED	N, N, N' N, Tetramethylenediamine
T_m	Melting Temperature
Tricine	N-Tris[Hydroxymethyl]methylglycine;N-[2-Hydroxy-1, 1-bis (hydrooxymethyl)-1, 3-propanediol
Tris	2-amino-2 (hydroxymethyl)-1,3-propanediol
U	Enzyme Units
UV	Ultra Violet light
VP	Vasopressin
w/v	Weight by Volume

"...it may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly organised creatures..."

Charles Darwin, 1881.

CHAPTER 1

GENERAL INTRODUCTION

Soil health has become of major importance in ecotoxicology during the past twenty years (Spurgeon *et al.*, 2003). Terrestrial ecotoxicologists face a number of major challenges in the assessment of the effects of environmental contaminants on soil organisms. The ecological relevance of the measured parameters is perhaps the most important consideration. Classical ecotoxicological investigations focus on the determination of lethal dose levels of the particular toxicant/pollutant in question. However, the toxic effects of such compounds will almost definitely have molecular origins so, in principle, this is the organisational level at which the earliest deleterious effects may be detected. Thus, linking molecular genetic perturbations in individual organisms to ecological-scale effects is a matter of high priority. Molecular biomarkers should ideally be directly and unequivocally linked to ecologically relevant endpoints, such as reproductive output. A major aim of the present thesis was to identify and exploit biomarkers of reproduction in the key soil macro organism, the earthworm.

1.1 EARTHWORM ECOLOGY AND DIVERSITY

Earthworms belong to the Phylum Annelida, Subphylum Clitellata [comprising two Classes: Hirudinea (the leeches) and Class: Oligochaeta (earthworms, tubificids and their taxonomic allies)]. The Oligochaeta (from the Greek *chaitē*, meaning bristle) is made up of a number of Suborders. The Suborder Tubificina contains the small aquatic worms, such as, the naids, tubificids and lumbriculids and the enchytraeids

(some of which are soil dwelling). However, earthworms occupy the Order Haplotaxida, Suborder Lumbricina, with this Suborder divided into twenty-four Families and Subfamilies. The majority of European earthworm species belong to the Lumbricidae Family.

Of the approximately 3,000 earthworm species currently identified worldwide only twenty-seven are found in the British Isles (Sims & Gerard, 1985). This is probably due to the low dispersal rate of earthworms and the fact that the British Isles has been subjected to glaciation events. Those countries south of the glaciation limit have many more species: Italy (57 species), France (180 species) and Switzerland (38 species) (Edwards & Bohlen, 1996). The classification of these and all earthworm species is complex and contentious (Sims & Gerard, 1985). It was Michaelsen in 1900 that produced the basis of the modern taxonomic criteria for the classification of earthworm species.

Many morphological traits are used for earthworm taxonomy, perhaps the most important being the position and structure of the genital organs. According to Sims & Gerard (1985) the 24 families and subfamilies of the Suborder Lumbricina are separated on many sexual characteristics including the structure of the ovary and the manner of shedding the oöcytes. The structure of the genital organs in the Lumbricidae is discussed further in *Section 1.4.*

Other morphological characters used in classification include the structures of the prostomium (an anterior-dorsal extension of the first segment forming an upper 'lip') and the calciferous gland (a swollen specialised region of the mid-oesophagus in the

Lumbricidae). The number of segments in each species is also taxonomically significant, as are the number and positional arrangements of setae. Modern taxonomic tools include metabonomics, which has been used by Bundy *et al.* (2002) to distinguish between species in the *Eisenia* complex (*Eisenia fetida* and *Eisenia andrei*, as well as the ecologically related species, *Dendrobaena veneta*) on the basis of their metabolic profiles. Mapping the number, frequency and sites of base-pair changes in the 18S rRNA (Pop *et al.*, 2002) is also being increasingly used in earthworm taxonomy. The accurate identification of earthworm species is of great importance in ecotoxicological applications, because different earthworm species may show significantly different responses to given concentrations of environmental toxicants (Spurgeon *et al.*, 2000).

Earthworms are widely distributed throughout the World, although they are only rarely encountered in deserts, areas under constant snow and ice, mountain ranges, and in areas lacking soil and vegetation. It was because of the widespread distribution of earthworms that Bouché (1977) devised three ecophysiological classes. This system took into account the differing life styles of the many species of earthworm and allowed the classification of these species by ecological parameters, thus explaining their wide global distribution. However, they are not absolute, as some of the species defined in one group may exhibit traits of another under certain conditions. These three classes are listed below:

Aneciques (or anecic species) are often large (up to 1,100 mm long) earthworms. They form permanent, deep (up to 1,000 mm), vertical burrows from which they emerge at night to forage and mate. The group may or may not be pigmented, but

generally have dark brown colouring. British examples include *Lumbricus terrestris* (Linnaeus) and *Aporrectodea longa* (Ude). Some members of this group enter a period of summer aestivation known as diapause. Bouché (1977) also referred to members of this group as 'macrophage species', because they ingest large food particles.

Endoges (or endogeic species) vary in size. They construct temporary, horizontal, branching burrows mainly in the upper 70 mm soil layer (i.e. in the organo-mineral layer); they are weakly pigmented. British examples include *Aporrectodea caliginosa* (Savigny) and *Aporrectodea rosea* (Savigny). They are also known as 'microphage species' (Bouché, 1977).

Epiges (or epigeic species) are the smallest species (typically between 10 and 30 mm in length). They are litter dwellers, i.e. they do not form burrows. They have fairly uniform, often reddish colouration. British examples include *Lumbricus rubellus* (Hoffmeister) and *Dendrodrilus rubidus* (Savigny). They are also known as 'mesophage species' (Bouché, 1977).

Three different earthworm species were chosen in this study. These species were *Eisenia fetida*, *Eisenia andrei* and *Lumbricus rubellus*. The two *Eisenia* species were chosen because of their use as recommended test organisms within OECD (1984; 2001) guidelines. They are both typically manure- or compost-inhabiting earthworms, giving rise to the concern that they may lack ecological relevance. Therefore, it was decided to also use the epigeic species, *Lumbricus rubellus*, which has been fairly widely used for both toxicity testing (Spurgeon *et al.*, 1999) and molecular genetic

work (Stürzenbaum 1997; Spurgeon, *et al.*, 1999). Further justification is the burgeoning EST database for *L. rubellus*, known as LumbriBASE (http://www.earthworms.org; Stürzenbaum *et al.*, 2003).

Earthworms constitute the dominant biomass of soil fauna, usually 10–200 g m^{-2} (Sims & Gerard, 1985). In 1881 Charles Darwin published his monograph, "*The Formation of Vegetable Mould Through the Action of Worms with Observations on Their Habits*." By 'vegetable mould' Darwin was referring to the humic content of soil and described the role that earthworms play in the incorporation of organic matter into soil. Darwin was the first naturalist to make detailed observations on the actions of earthworms, but since that time many studies have been performed to show the importance of earthworms in mechanical break down of organic matter and the subsequent mixing of this material with mineral soil (Brown *et al.*, 2003). The redistribution of nutrients through the action of earthworms increases plant growth, not least by preventing surface run-off of nutrients (Syers & Springett, 1983). Through this action earthworms increase soil microfloral activity, which in turn facilitates further enzyme activity. In general, earthworms have the ability to increase the turnover rate of nutrients within soil and hence indirectly promote plant growth. Earthworms form mucus-lined burrows of varying sizes and depths, which increases the infiltrability of the soil, thus simultaneously reducing surface water run-off and increasing moisture retention (Sims & Gerard, 1985). So, earthworms are ecosystem engineers that increase the bioavailability of nutrients within the soil both through direct (i.e. mechanical mixing) and indirect (i.e. increased microorganism activity) actions.

1.2 EARTHWORM ECOTOXICOLOGY

Ecotoxicology was first developed in the 1970s as a science to assess the impact of toxic chemicals in the environment (Truhaut, 1977). Therefore, ecotoxicology has (at least initially) used the principles of toxicology to measure 'stress' in environmentally relevant organisms. For example, it is standard practise in ecotoxicology to make experimental measurements and estimate the concentration of a particular toxic chemical that gives a 50% effect within a certain period (EC_{50}) (Van Straalen, 2003). This has enabled the regulation of many chemicals that are found within the environment, giving 'safe' levels of these chemicals. However, it has been estimated that there are over 100,000 chemicals in regular industrial use worldwide (Depledge & Fossi, 1994), so what is meant by a 'toxic chemical'? A simple definition would be that a toxic chemical is one which causes pollution. Collocott & Dobson (1974) defined pollution as a term that can be applied to "*any environmental state that is harmful or unpleasant to life*". Therefore, what do we mean when we use the terms 'toxic chemical', 'contaminant' and 'pollutant'? A contaminant can be described as a potentially harmful substance (so it may be a toxic chemical) that can become a pollutant if it is present in a high enough concentration (Dobbs & Zabel, 1996). So, under a polluted situation organisms within that environment may become stressed. However, it is important to note that 'stress' can be caused by factors other than pollutants. Van Straalen (2003), defined stress as, "*... stress is a condition evoked in an organism by one or more environmental factors that bring the organism near or over the edges of its ecological niche.*" Therefore, this definition incorporates other stress-inducing factors such as extreme temperatures, water logging, aridity, acidity, etc.

Over the past twenty years earthworms have been used increasingly in terrestrial ecotoxicology (Spurgeon *et al.*, 2003). This 'earthworm revolution' was brought about, at least in part, by the inception of the earthworm acute toxicity test (Edwards, 1983), and its subsequent ring testing and standardisation (OECD, 1984; EEC, 1985; Van Gestel *et al.*, 1989). This test has enabled the establishment of toxic effect levels of a plethora of inorganic and organic contaminants.

This section will deal with a number of studies that have been performed using earthworms exposed to a number of inorganic and organic contaminants to determine 'safe' levels. However, it will also track many of the problems confronted using the earthworm acute toxicity test and show that alternative methods (such as determination of sub-lethal consequences of contaminants) may be more environmentally meaningful.

1.2.1 HEAVY METAL TOXICITY

Metals are natural substances found in the environment in rocks, ores, soil, water and air. They normally do not provide any toxic properties as they are found at low concentrations. However, the so-called 'heavy-metals' are non-biodegradable and can therefore accumulate within ecosystems. The heavy metals can be defined as metallic elements that posses a molecular weight in excess of 50 Daltons and are capable of forming polyvalent cations (Foulkes, 2000). They are located within the d-block elements (transition elements) in the Periodic Table of Elements.

Much of the heavy metal pollution that is seen worldwide is due to human activity (EPA, 1998). These metals have been extracted and processed to produce utensils and

machines. Hence metals have been concentrated in specific locations causing adverse, 'stressful', conditions for organisms living in those anthropogenically-modified environments. Therefore, as earthworms inhabit the terrestrial environment many studies have been performed to determine the impact of excess metals on their life-cycles.

Perhaps the first question that must be addressed is whether the heavy metals found within the terrestrial environment (for example, in the vicinities of mining sites or smelting works) are actually available to the resident biota (i.e. are the metals 'bioavailable')? As heavy metals can exist in multiple oxidation states and exhibit different redox chemistries (Wilkins & Wilkins, 1997) the local environmental conditions (e.g. pH of the soil, 'age' of the pollution) are important abiotic factors influencing metal speciation and, thus, bioavailability.

Studies have shown that the metals in contaminated field soils have lower availability than those in experimentally spiked soil (Spurgeon & Hopkin, 1995; Scott-Fordsmand *et al.*, 2000). This lowered bioavailability may be due to a number of factors including, pH variations (Bengtsson *et al.*, 1986), temperature (Beyer *et al.*, 1987) and organic matter content of the soil (Spurgeon & Hopkin, 1995). Bengtsson *et al.* (1986) studied the influence of copper and cadmium on the growth of *Dendrobaena rubida* at different soil pH values. They found that at pH 6.5, growth of this earthworm was stimulated by 10 and 100 μg Cd g^{-1} dry soil, but at pH 5.5 growth was significantly reduced at both soil Cd concentrations. Therefore, a change in soil pH can affect the bioavailability of metals, and hence their toxicity to earthworms. Spurgeon & Hopkin (1995) showed that zinc was more toxic to *E. fetida* in artificial

soil than when earthworms were exposed to field soils containing similar levels of the heavy metal. They found that cocoon production was the most sensitive Zn-toxicity endpoint, whilst mortality was the least sensitive. A concentration of 32, 871 μg g^{-1} zinc was found in soil close to a lead, cadmium and zinc smelting works. When *E. fetida* were exposed to this heavily polluted soil in the laboratory no significant mortality was recorded, despite the fact that an EC_{50} (concentration of metal predicted to cause a 50% increase in mortality in 14 days) of 1,078 μg Zn g^{-1} was predicted from exposure of *E. fetida* to zinc in an artificial soil. The difference in mortality between the field and artificial soil was due to differences in the bioavailability of Zn in the two soils. The pH values of the two soils were similar, but the artificial soil had lower organic matter content than the field soil. Therefore, earthworms exposed to Zn in the artificial soil would have encountered unnatural metal species, which would have not been found in field soils (Spurgeon & Hopkin, 1995). Hence, the high recorded mortality and lower EC_{50} value in earthworms exposed to artificial soil compared with exposure to field soils. This underlines the difficulty in the extrapolation of toxicity data from the laboratory to the field.

The earthworm acute toxicity test (OECD, 1984) recommends the use of the manure earthworm, *Eisenia fetida* (or *Eisenia andrei*). This recommendation has come under some criticism over the last twenty years (Spurgeon *et al.*, 2003). This is, at least in part, due to the natural habitat in which *Eisenia fetida* are found. They are mainly found in manure and compost heaps (Sims & Gerard, 1985), which exhibit relatively constant seasonal temperatures and have high organic matter content. Therefore, toxicity tests performed using these worms (in the laboratory or field) may be difficult to relate to the 'real' situation encountered by other species exhibiting different

ecophysiological characteristics. *E. fetida* has also been shown to exhibit higher tolerance to contaminants (including heavy metals) than other earthworm species (Spurgeon *et al.*, 2000) (*Table 1.1*).

The choice of organism for toxicity testing is critical. As stated in *Section 1.1* there are three distinct ecophysiological earthworm groups. One of the criteria used in this grouping is the burrowing activity of the earthworm, i.e. an anecic earthworm will form deep, permanent burrows, whilst an epigeic species will not normally burrow to any great extent and will remain in the litter layer. Therefore, these different earthworm species may encounter varying levels of toxic chemicals. It has been shown that different earthworm species have varying degrees of sensitivity to toxic effects (Kammenga *et al.*, 1996; Spurgeon *et al.*, 2000). In the study by Spurgeon *et al.* (2000) four earthworm species were exposed to zinc in a natural loam soil. The four species (*Eisenia fetida*, *Lumbricus rubellus*, *Aporrectodea caliginosa* and *Lumbricus terrestris*) used in this study varied in their ecophysiological behaviours. It has been postulated that sensitivity to metal toxicity in earthworms is modulated by calciferous-gland activity (Spurgeon & Hopkin, 1996). According to this hypothesis, those species with high calciferous-gland activity will be less sensitive to metal toxicity. The results gained from the study by Spurgeon *et al.* (2000) are summarised in *Table 1.1*. They showed that *A. caliginosa* (a species with an inactive, non-mineralising gland) is most sensitive to Zn toxicity when survival and cocoon production were the endpoints. However, when a sublethal biomarker of toxic stress was used (neutral-red retention time, see *Section 1.3*) *L. rubellus* appeared to be the most sensitive species. There are many reasons why these differences in sensitivity may appear amongst different earthworm species. Firstly, it has been suggested that

the chemical causing the toxic effect determines sensitivity. In the study by Heimbach (1985) the toxicity of a range of pesticides to *E. fetida* and *L. terrestris* was determined and showed that for copper-oxychloride *E. fetida* had a higher LC_{50} than *L. terrestris* by a factor greater than nine. However, when these two species were exposed to aldicarb, *L. terrestris* was less sensitive than *E. fetida* by a factor of 7.8. Secondly, different earthworm species have been shown to accumulate

Table 1.1: Relative sensitivities to zinc toxicity of four ecophysiologically distinct earthworm species measured using three separate parameters.[1]

Species	14-d LC_{50} (μg Zn g^{-1})	48-d LC_{50} (μg Zn g^{-1})	EC_{50} CP[2] (μg Zn g^{-1})	EC_{50} NRR[3] (μg Zn g^{-1})
A. caliginosa	1,695	1,619	442	252
L. rubellus	1,734	1,709	599	168
L. terrestris	2,378	2,217	1,029	542
E. fetida	3,172	3,350	1,898	>2,000

[1]Data taken from Spurgeon *et al.* (2000)
[2]Cocoon production.
[3]Neutral-red Retention Time.

and eliminate heavy metals at varying rates (Spurgeon & Hopkin, 1999). The size (or more specifically, the surface-area-to-mass ratio) of an organism will also determine the uptake, and thus the effect of a particular toxic agent.

Both species- and chemical-sensitivity issues have become increasingly important in terrestrial ecotoxicology. Two of the most widely studied metals in earthworm ecotoxicology are cadmium and copper. The following examples reinforce the need

for more sensitive approaches to measuring toxic effects in earthworms due to these two metals.

Although cadmium is an extremely toxic chemical to most organisms, many studies have shown low Cd-sensitivity in the earthworm *E. fetida* when exposed in artificial soil. In fact, Spurgeon *et al.* (1994) showed that the 14-day LC_{50} for Cd in artificial soil was >300 μg g^{-1}. In a study by Van Gestel & Van Dis (1988), Cd was also shown to cause mortality in artificial soil only at the highest test concentrations (14-day LC_{50} for cadmium of >1,000 μg g^{-1}). However, when sublethal parameters are used to measure the toxicity of Cd to earthworms a different picture is seen. Cd has been shown to cause severe growth retardation in the earthworm *E. fetida* at low concentrations. Malecki *et al.* (1982) showed that there were significant decreases in growth of *E. fetida* at 50 μg Cd g^{-1} dry soil. Spurgeon *et al.* (1994) recorded a 56-day estimated NOEC for cocoon production for *E. fetida* exposed in artificial soil of 39.2 μg Cd g^{-1} dry soil. This illustrates the sensitivity of chronic endpoints when assessing toxicity effects on earthworms. Cadmium has also been shown to alter ovarian structure in the earthworm *Dendrobaena veneta*. Siekierska & Urbańska-Jasik (2002) observed that a cadmium concentration of 10 μg g^{-1} caused structural changes in the cell nuclei of young oöcytes. Therefore, Cd may affect reproduction by disrupting ovary function in earthworms.

Copper has been shown to affect cocoon production in *E. fetida* when exposed in artificial soil. Spurgeon *et al.* (1994) found a 56-day estimated NOEC for cocoon production of 32.0 μg Cu g^{-1} dry soil. Van Gestel *et al.* (1991) estimated an EC_{50} for copper on the growth of *E. andrei* of >100 μg Cu g^{-1}. However, many studies have

observed an increased reproductive output (i.e. greater number of cocoons produced per worm per week) at low concentrations of copper (e.g. 10 μg Cu g^{-1} of soil) (Van Gestel *et al.*, 1989; Spurgeon *et al.*, 1994). This is probably due to the fact that copper is an essential element for most organisms, and lower concentrations of it may be close to optimal levels required for earthworms. However, copper has been shown to decrease cocoon production and induce mortality at higher concentrations (Van Gestel *et al.*, 1989; Spurgeon *et al.*, 1994).

1.2.2 ORGANIC CHEMICAL TOXICITY

Organic compounds found in the terrestrial environment can originate from a number of different sources. Perhaps the most voluminous and varied inputs are pesticides, used to prevent crop damage by a wide variety of agricultural pests throughout the world. Another large group of compounds causing considerable concern in the terrestrial environment are the polycyclic aromatic hydrocarbons (PAHs) (Van Brummelen *et al.*, 1996). PAHs enter the soil (at least in part) via deposition from emissions to the atmosphere, through vehicular exhaust fumes (Pathirana *et al.*, 1994), wood burning (Freeman *et al.*, 1990) and a range of industrial activities.

A large volume of work has been published detailing the effects of many different pesticide groups on earthworms and this reflects the importance of pesticide use in agriculture. A major problem in applying pesticides to crops is that they inevitably enter the soil ecosystem and many persist for long periods. For example, Nash & Woolson (1967) showed that more than 50% of the initially applied DDT was present in some soils after a period greater than 15 years. Also, Boul *et al.* (1994) analysed soil samples twenty-four years after addition of DDT and found the metabolites of

DDT, DDE and DDD, were still present. Although such compounds may persist in the soil for many years there is a corresponding 'ageing effect'. This effect is the result of chemical processes, such as sorption, and results in the decreased extractability and bioavailability of organic compounds (Reid *et al.*, 2000; Johnson *et al.*, 2002). Therefore, any experiments carried out to assess the toxic impact of organic compounds (including pesticides) should take this factor into account, as the ecological relevance of such tests carried out in the laboratory might be compromised. Morrison *et al.* (2000) demonstrated that DDT, DDE, DDD and Dieldrin became increasingly less bioavailable to the earthworm *E. fetida* as the soil 'aged'. This was shown by measuring the levels of these compounds in total body extractions of the earthworms after they had been exposed to soils of various ages. Those earthworms exposed to 'freshly' spiked soils (90 days after spiking and 0 days after spiking) showed a marked increase in the uptake of all compounds (DDT, DDE, DDD and Dieldrin) when compared to those earthworms exposed to soil treated with the same compounds, but aged for 49 years. The bioaccumulation of such compounds in earthworms may be of great importance at higher levels of the food chain. That is, earthworm predators, such as birds, will also accumulate organic compounds sequestered within worm tissues and in the soil contained in the alimentary canal.

Carbamate pesticides were introduced because of their high efficiency and low persistence in the environment. They inhibit acetylcholinesterase (AchE) in the nervous system of insects and other animals (Baron, 1991). According to the World Health Organisation (1994) carbamate exposure causes disruption of detoxification processes, and particularly of the mixed function oxidase (MFO) system. A number of other studies have indicated that carbamates disrupt the biotransformation or

oxidative stress defence mechanisms in many organisms (see, Ribera *et al.*, 2001). In a study by Ribera *et al.* (2001) exposed the earthworm *E. fetida* to a range of concentrations of the insecticide, carbaryl (maximum dose was 50 $\mu g\ g^{-1}$ soil). A number of enzymes were assayed for their activity after exposure for 2, 7 and 14 days; these included glutathione-*S*-transferases (GST), which are involved in phase II biotransformation, catalase (CAT) involved in oxidative stress responses, and acetylcholinesterase (AchE) involved in neurotransmission. The results showed that oxidative stress responses were not affected by carbaryl exposure, but AchE activity was significantly reduced even at the lowest test concentration of 12 $\mu g\ g^{-1}$. Those enzymes involved in biotransformation (i.e. GST) were inhibited by carbaryl, but to a lesser extent. However, these data showed that earthworms (the non-target organism of carbaryl field applications) were affected at low concentrations of the compound. Laboratory and field data for the toxicity of carbaryl to earthworms is summarised in the review by Van Gestel (1992). These data show that earthworms are sensitive to carbaryl (LC_{50} values derived from laboratory toxicity tests range from $<4.0\ \mu g\ g^{-1}$ to 263 $\mu g\ g^{-1}$) both in the field and laboratory. In one study cited by Van Gestel (1992) greater than 50% of a field earthworm population was eliminated by application of carbaryl. Neuhauser & Callahan (1990) showed that both growth and reproduction in the earthworm *E. fetida* was significantly affected at 25 μg carbaryl g^{-1} soil.

The lipophilic polychlorinated biphenyl (PCB) compounds were produced in vast quantities during the 1930s to late 1970s (when they were banned). They were used in transformer and hydraulic fluids because of their heat resistance and electrical properties. PCBs are comprised of a carbon biphenyl with a variable number (1-10) of attached chlorines, yielding 209 possible compounds. Relatively few studies have

been conducted to test the acute or chronic toxicity of PCBs to earthworms. Fitzpatrick *et al.* (1992) exposed the earthworm species, *E. fetida* and *L. terrestris* to the commercial PCB mixture, Aroclor 1254 in a filter paper testing method. They used immunological sublethal endpoints to determine the toxicity of this PCB to earthworms. They showed that *E. fetida* was able to accumulate significantly larger quantities of PCB (4,500 μg PCB g^{-1} dry mass) than *L. terrestris* (1,150 μg PCB g^{-1} dry mass) before showing adverse effects. Fitzpatrick *et al.* (1992) concluded that immunological function in earthworms was impaired upon exposure to PCBs, but *E. fetida* was less sensitive than *L. terrestris*.

A number of studies have evaluated the effects of polychlorinated compounds on earthworms. For example, Van Gestel *et al.* (1989) measured the effect of pentachlorophenol (PCP) on the reproduction of *E. fetida* in an artificial soil over a three-week period. They found that cocoon production was significantly decreased at a PCP concentration of 100 μg g^{-1} dry soil. They also showed that cocoon fertility was affected at a PCP concentration of 32 μg g^{-1} dry soil. Therefore, they derived a no-effects level (NEL) for the effect of PCP on earthworm reproduction of 10 μg g^{-1} dry soil. Giggleman *et al.* (1998) measured the phagocytotic activity of *L. terrestris* immunoactive coelomocytes upon exposure to sub-lethal concentrations of PCP (using the filter paper contact protocol). They showed that immuno-responses in this species of earthworm were significantly affected at a PCP concentration of 15 μg/cm^2.

The use of biological mechanisms (or components of biochemical pathways; e.g. biotransformation, oxidative stress mechanisms) have allowed ecotoxicologists to study the effects of organic residues on an organism before more obvious changes are

seen (i.e. mortality). Using low levels of biological organisation to monitor toxic effects has produced biomarkers that are capable of functioning as "early warning" systems.

1.3 BIOMARKERS IN ENVIRONMENTAL MONITORING

The importance of monitoring the effects of heavy metals, organic compounds and endocrine disruptors (*Section 1.6*) has developed into the science of ecotoxicology (*Section 1.2*). The increasing number of chemicals used in industry has driven this science to develop better and more robust methods of assessing their effects on the environment and its inhabitants (including human health implications). This has lead to the development of biomarkers of toxic effects on organisms. In general a biomarker can be defined as a biological response (up or down regulation of a specific gene, for example) to a chemical or chemicals that gives a measure of exposure and, sometimes, also of toxic effect (Weeks, 1995). A list of biomarkers developed and regularly used in earthworm ecotoxicology is given in *Table 1.2.*

Biomarkers address a number of issues that have often caused problems in terrestrial ecotoxicology. Firstly, they negate, to some extent, the bioavailablity problem of chemicals in soil. This is achieved, because biomarkers will only respond to the bioactive fraction of any chemical present in the soil (Weeks, 1995; Scott-Fordsmand & Weeks, 2000), and this advantage is increased, as they will integrate the effects (antagonisms, additive, synergisms) of chemical mixtures (Weeks, 1995). Biomarkers may also aid in the extrapolation of toxicity data from the laboratory to the field (Scott-Fordsmand & Weeks, 2000), as the biological responses that are measured may be applicable under both conditions.

Although biomarkers have become increasingly used in earthworm ecotoxicology some reservations still exist about their use (Spurgeon *et al.*, 2003). These reservations were highlighted at the Third International Workshop on Earthworm Ecotoxicology (IWEE) at Åarhus, Denmark in 2001, and in the subsequent recommendations that emerged or were formulated from it. Two of these recommendations are listed below to exemplify the point (Van Gestel & Weeks, 2004):

Recommendation:

"There is a need to explore the mechanistic connections for the biomarker response to the desired endpoint. Knowledge on the mode of action of chemicals should be used to identify the targets of intoxication and assist in the development of new, sensitive and reliable biomarkers."

Recommendation:

"Biomarkers should be correlated with life-cycle parameters – more so than correlated with chemical levels. This does not neglect the need for pure compound exposure – in order to find class/compound specific markers."

Table 1.2: List of biomarkers regularly used in the assessment of pollutant impact on earthworms. This is not a complete list of biomarkers, but is used to reflect the current use of biomarkers in earthworm ecotoxicology.

Type	Measurement	Reference
Lysosomal Membrane Stability	Neutral-red Retention Time	Weeks & Svendsen 1996
Total Immune Activity	Ability to Phagocytise Rabbit Red Blood Cells	Bunn *et al.* 1996
DNA Strand Breaks	Comet Assay	Salagovic *et al.* 1996
Gamete Production	Sperm Number and Morphology	Reinecke & Reinecke 1997; Cikutovic *et al.* 1993
Antioxidant Enzymes	Peroxidase Activity	Saint-Denis *et al.* 1996
	Catalase Activity	Labrot *et al.* 1996
Detoxification Enzymes	Cytochrome P_{450} Enzymes	Eason *et al.* 1998
	Glutathione *S*-transferase (GST)	Booth & O'Halloran 2001
	Metallothioneins	Stürzenbaum *et al.* 2001
Molecular Responses	Heat Shock Family	Marino *et al.* 1999
	Cytochrome C Oxidase	Stürzenbaum 1997
	Metallothioneins	Stürzenbaum 1997

These recommendations reflect the basic needs of any potential biomarker. Firstly, a biomarker should be ecologically relevant; that is, they should correlate to specific 'higher orders' of functionality. For example, in a previous section (*Section 1.2*) much has been made of sublethal endpoints in ecotoxicology, as they are more ecologically relevant than mortality (Moriarty, 1983). Therefore, a biomarker should be statistically or, preferably, causatively/mechanistically linked to one or more of

these endpoints. An excellent example may be the counting of sperm in the earthworm *Lumbricus terrestris* after exposure to cadmium and chlordane (Cikutovic *et al.*, 1993). A decrease in sperm number in an earthworm species may be directly correlated to a reduction in reproductive output. These data can then theoretically be incorporated into mathematical life-cycle models that will predict the potential effects on population demographics. Thus, biomarkers may act as 'early warning' indicators of ecological-scale perturbations. Secondly, any biomarker response should ideally be understood at the mechanistic level if ecotoxicologists are to apply biomarker technology to a range of different species and ecosystems. This understanding of the mechanisms governing toxicity in individuals will also increase the robustness of the 'early warning' system.

It is the 'early warning' potential of biomarkers that has become of most interest to ecotoxicologists and environmental managers over the past decade. The responsibilities of environment management have at least three identifiable objectives (Depledge & Fossi, 1994):

1) To minimise threats to human health;
2) To ensure that ecosystems are self-sustaining;
3) To ensure that particular species do not decline.

These are extremely difficult objectives to meet, so any measure that gives an early sign of deleterious effects in the performance of an organism, population or ecosystem is of great benefit. An early departure from a healthy state can be picked up using a biomarker of exposure/effect. For a biomarker to have this early warning capability a dose-response relationship with a range of pollutants must be established (Scott-Fordsmand & Weeks, 2000), although the relationship may be complex and non-

linear. In fact, this point was another one of the recommendations that came out of the Third International Workshop on Earthworm Ecotoxicology (IWEE) at Åarhus, Denmark in 2001 (Van Gestel & Weeks, 2004):

Recommendation:

> *"Nonunimodal data on biomarker responses may also be good data and reinterpretation is therefore strongly recommended. If dose-response relationships do not meet expectations, the use of multivariate analysis should be considered. In addition, there is a need to consider the statistical interpretation of measured biomarkers with respect to the integration of biomarker, population and environmental variable."*

Risk management within the environment can be aided by the use of biomarkers that act as an early-warning system, but the choice of biomarker is critical. A suite of biomarkers should be utilised that relate directly to a higher life-cycle parameter. Hence, the next section addresses the processes involved in earthworm reproduction with regard to using a stage of the process at the molecular level as a biomarker in ecotoxicology.

1.4 EARTHWORM REPRODUCTION

Earthworms are hermaphrodite; they contain both male and female reproductive organs that are confined to relatively few anterior segments in most species. The reproductive organs and breeding behaviours are similar enough in most earthworm species to allow a general description of reproductive structure, function and activity to be meaningful for present purposes. The mating behaviours of earthworm

reproduction have been widely studied (Olive & Clark, 1978), but knowledge of the hormonal, molecular and physiological regulatory processes involved are rather less well understood.

Some earthworm species reproduce without mating, either by self-fertilisation or parthenogenesis (the ovum develops into a juvenile without the union with a spermatozoon) (Sims & Gerard, 1985). Those British species known, or suspected of parthenogenetic reproduction include; *Dendrodrilus rubidus*, *Eisenia fetida* and *Eiseniella tetraedra* (Sims & Gerard, 1985). However, Cluzeau *et al.* (1992) showed that isolated individual *E. fetida* posses the ability to self-fertilise rather than perform parthenogenesis. However, in *D. rubidus* the spermathecae is often absent (Sims & Gerard, 1985) making sexual reproduction and/or self-fertilisation impossible.

Self-propagation is limited and most earthworm species reproduce via sexual reproduction. Copulation begins with two mature adults coupled 'head-to-tail' with the ventro-lateral regions of the anterior segments of each animal in close contact. The positioning of the copulating pair means that there is a gross anatomical misalignment of the female and male pores. Therefore, the direct exchange of sperm via a penis is not possible. Genital setae penetrate and 'grasp' the reproductive partner to ensure close physical contact. This copulation arrangement is common to most species and allows the interchange of spermatozoa. The most obvious sign that an earthworm has reached sexual maturity is the glandular swelling of the epidermis (the clitellum) synthesises and secretes a mucoid secretion during and after copulation. Secretions from the clitellum eventually form the egg-capsule, or cocoon, within

which cross-fertilised ova are deposited and the embryo develops. Whether this gland (and others) secretes hormones that simulate copulation is unknown.

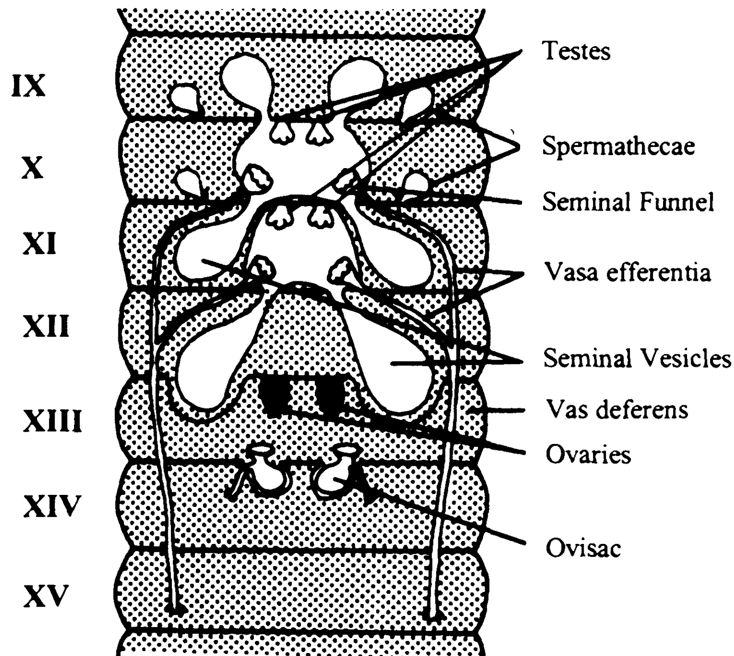
Earthworms contain storage sacs for their reproductive partner's sperm i.e. although hermaphrodite; cross-fertilisation is the norm in most species. Sperm storage sacs are known as spermathecae (*Figure 1.1*) and open in segments *xi*, *x* and *xi* (in British species). Sperm is deposited in the spermathecae of each animal during copulation utilising the mucus secreted from the clitellum as a transport medium. The external seminal groove runs from segment *xv* to the clitellum and forms a channel for sperm transportation, although the exact way this transfer takes place is not fully understood (Sims & Gerard, 1985). Copulation may last from between fifteen minutes and four hours, depending on the species. During this time mating animals do not respond readily to external stimuli, such as light and touch. Most British earthworm species copulate below ground, with the exception of *L. terrestris* that will mate on the soil surface.

The anatomical structure of the reproductive organs in the Lumbricid earthworm are shown in *Figure 1.1*. This schematic diagram shows the separation of the male and female organs, as well as other associated organs, such as the spermathecae. The paired testes (two pairs in many species) are found in segments *x* and *xi* where they are attached to the anterior septum in sperm storage structures known as seminal vesicles. There are two separate seminal vesicle sacs, each acting as a development chamber for spermatogonia. Eventually the mature spermatozoa pass into the seminal funnel where they attach ready for copulation. The sperm funnels form the openings to the vasa efferentia (which become the vasa deferentia as they move towards the

male pore openings in segment *xv*). During copulation the spermatozoa are swept along the vasa deferentia to the external environment via the action of cilia and are carried in seminal fluid via the seminal groove into the spermathecae of the opposing animal. The female genital organs are located in segments *xiii* and *xiv* (*Figure 1.1*). The ovaries (two) are attached to the anterior septum of segment *xiii* and are 'free hanging' in the coelomic cavity of that segment. Below the ovaries are the ovisacs. Oöcytes bud-off from the tips of the ovaries and enter the funnel-like structures that form the openings of the ovisacs; the oviducts open to the exterior as female pores in segment *xiv*.

After copulation the two animals separate and (external) fertilisation takes place. The clitellum secretes a mucus layer that surrounds the whole of its body. The outer surface of this mucus layer eventually hardens and forms the egg sac, or later the cocoon. The mucus band fills with albuminous liquid and is moved toward the anterior of the animal via peristaltic muscle movements. As the band passes over segment *xiv* oöcytes are discharged into the fluid. Perhaps as many as 20 oöcytes are released into one egg capsule, but it is rare for more than one to fully develop and hatch (Sims & Gerard, 1985). The band then passes over the spermathecal pores (segments *ix/x* and *x/xi*) at which point sperm (derived, of course, from the reproductive partner) is released into the mucus capsule. Therefore, fertilisation is essentially an external event, albeit within a cocoon. The mucus band continues to move forwards where it is eventually released from the anterior end of the animal and forms the cocoon.

Panel A:



Panel B:

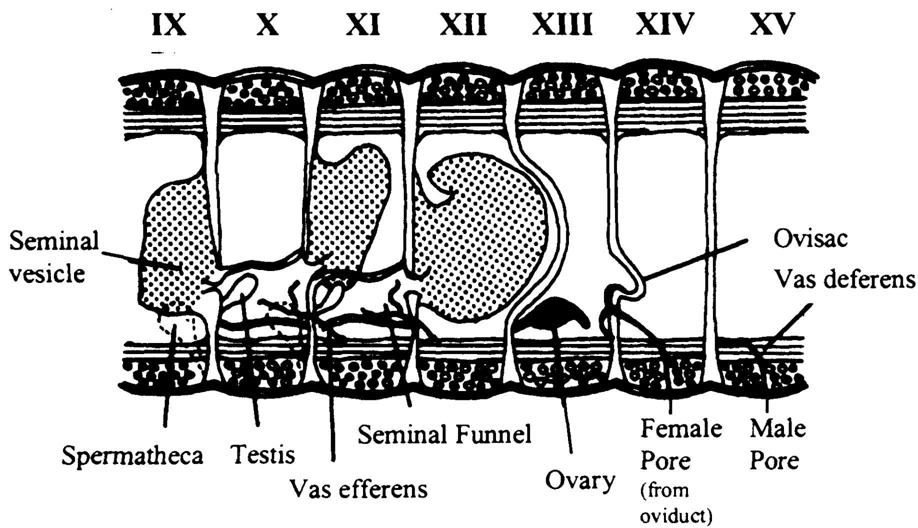


Figure 1.1: Schematic diagrams showing the reproductive organs of *Lumbricus*. Panel A: Dorsal view; **Panel B:** lateral view. Segment numbers are shown in Roman numerals. Taken from Wallwork (1983).

Cocoons are deposited in the soil at various depths depending on the species (Butt *et al.*, 2002). Embryonic development then takes place within the cocoon, which can be as long as 50 days depending on species and environmental temperature (Jensen & Holmstrup, 1997). Development can be experimentally arrested by storing cocoons at + 4 °C, thus many species in temperate climates survive winter as cocoons although this is not the sole mechanism (Holmstrup, 2003).

The gross anatomical mechanisms of sexual reproduction in earthworms are well documented and understood (Ferraguti, 1983; Olive & Clark, 1978). However, little is known about the genetic determinants and hormonal regulators of the earthworm reproductive cycle. Although many other invertebrate species control reproduction via endocrine secretions (see *Section 1.5*), it is not known whether earthworms are capable of controlling reproduction in a similar fashion. This knowledge is crucial to understanding the impact environmental pollutants have upon earthworm reproduction and hence population demographics.

1.5 INVERTEBRATE ENDOCRINOLOGY

1.5.1 GENERAL ENDOCRINOLOGY OVERVIEW

Invertebrates account for roughly 95% of the known species of animals and represent more than 30 different phyla within the animal kingdom. This diversity of body plans has resulted in distinct differences emerging between the endocrine systems of these animals (DeFur *et al.*, 1999). This section will attempt to summarise some of the key endocrine functions found in invertebrates and, more importantly, those systems employed by the earthworm. This will provide a basis for developing an

understanding of endocrine disruption in earthworms in response to chemical exposures.

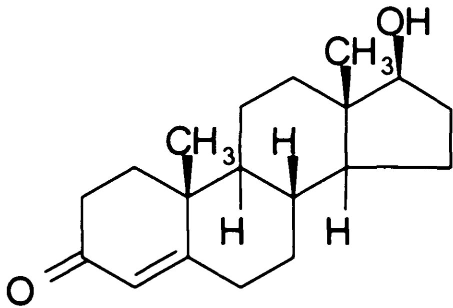
The endocrinology of mammals has been extensively studied over the past century (Turner & Bagnara, 1976). The basic principle of any endocrine response is that it proceeds via a chemical cascade (LeBlanc *et al.*, 1999) triggered by environmental or physiological signals and culminates in a specific response within the targeted cell. Thus for example, such physiological processes as growth, reproduction, development, maturation, water and ion balance are regulated by hormones in all mammals. In recent years it has become apparent that analogous physiological processes are also regulated by 'hormones' in invertebrates, as well as those processes unique to invertebrates (e.g. moulting, limb regeneration and diapause) (DeFur *et al.*, 1999).

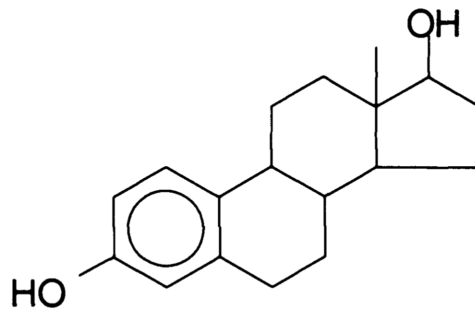
A hormone can be defined as a chemical, non-nutrient, intercellular messenger that is effective at micromolar concentrations or less (Bolander, 1994). Hormones target specific cells within the animal where they bind to receptors (either on the cell surface or intracellular) and elicit a functional response from these cells. This response is elicited via the interaction of specific molecules with the cell's DNA, which regulates the transcription of responsive genes. The structure of hormones is extremely diverse and they range from steroid hormones derived from cholesterol (e.g. testosterone), to peptide hormones (e.g. insulin) and oligosaccharides (elicitors in plants) (Bolander, 1994). Some examples of hormone structures are given in *Figure 1.2*. Hormones are transported to target organs via the blood system in vertebrates. In vertebrates hormones are secreted into the blood stream from glands such as the pituitary

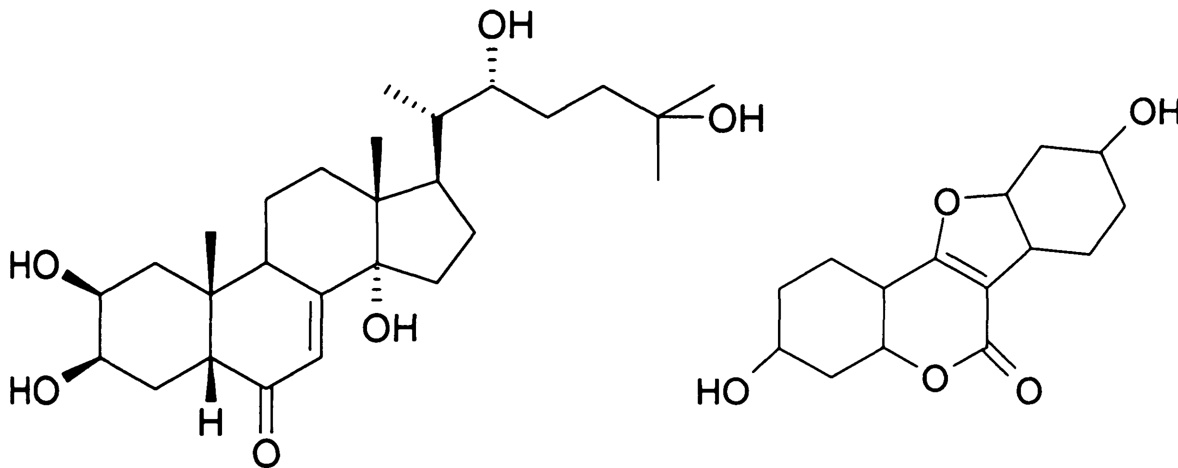
(secreting vasopressin and oxytocin, for example) and thyroid (secreting triiodothyronine [T_3] and thyroxine [T_4]) glands (Bolander, 1994). Endocrine organs are closely linked with the nervous system of both vertebrates and invertebrates (Turner & Bagnara, 1976). In fact, neurons frequently synthesise hormones in response to external stimuli (for example, auditory, visual or olfactory) (Turner & Bagnara, 1976).

Reproduction and development of secondary sex structures is controlled by endocrine secretions in all animals (Turner & Bagnara, 1976). For example, on receiving stimuli for sexual maturation, the mammalian hypothalamus secretes the peptide, gonadotropin-releasing hormone (Bolander, 1994). This hormone then stimulates the pituitary gland to secrete gonadotropins, which in turn stimulate the secretion of the steroidal hormone testosterone from the testis. This example illustrates the cascade nature of endocrine control and does have parallels in invertebrates (Turner & Bagnara, 1976).

A: Testosterone **B: 17β-oestradiol**







C: Ecdysone **D: Coumestrol**

Figure 1.2: Chemical structures of four common steroidal hormones from vertebrates, plants and invertebrates. (A) Testosterone synthesised in the testis of vertebrates. **(B)** 17β-Oestradiol from vertebrates synthesised in the ovaries via the conversion of testosterone, metabolised by the P_{450} enzyme, aromatase. **(C)** Ecdysone synthesised in the prothoracic gland of insects and also found in crustaceans. **(D)** Coumestrol from plants.

1.5.2 INVERTEBRATE ENDOCRINOLOGY

The endocrinology of insects has received extensive attention over the past century (Turner & Bagnara, 1976), with comparatively little detail of similar systems in other invertebrate species emerging. This is probably due to the need to control insect vectors of human and animal diseases, as well as agricultural pest species (LeBlanc *et al.*, 1999). Despite this discrepancy in knowledge, it is evident that a number of gross similarities exist between invertebrate and vertebrate endocrinology. For example, neurosecretory cells, producing chemical messengers (neurohormones), are found in all animal phyla (Turner & Bagnara, 1976). Also, invertebrates such as insects and molluscs appear to have endocrine gland-type structures (Turner & Bagnara, 1976) and, of course, gonads are present in invertebrates. Therefore, this section will summarise the endocrinology of some of the major invertebrate phyla, with particular focus on the Insecta, Mollusca and Annelida.

A. INSECTA

The insect endocrine system is involved in moulting, pigmentation, metamorphosis, reproduction and diapause (LeBlanc *et al.*, 1999). The insect endocrine system is comprised of neurosecretory cells found primarily in the central nervous system (CNS), three endocrine glands and the gonads (LeBlanc *et al.*, 1999). These three gland structures are: the corpora allata, comprised of neurosecretory cells situated in the head; the prothoracic glands found in the anterior thorax, and secrete ecdysteriods (see *Figure 1.2*) involved in reproduction and moulting (Bolander, 1994); and the epitracheal gland, which is also situated in the thorax of the insect and secretes a hormone that triggers moulting (LeBlanc *et al.*, 1999). The brain and various ganglia

of the CNS (e.g. the suboesophageal ganglion, which synthesises diapause hormone) also release hormones into the hemolymph (LeBlanc *et al.*, 1999).

A classical example of the effects of hormones on insects is seen during moulting and metamorphosis. As insects grow they must shed and replace their rigid exoskeletons (Bolander, 1994). Moulting (or ecdysis) and metamorphosis begin with the neurosecretory cells of the brain releasing the peptide hormone prothoracicotropic hormone (PTTH). PTTH travels via the hemolymph to the prothoracic gland, thus stimulating the release of ecdysone from this gland. Ecdysone is a steroid hormone synthesised from cholesterol, which is derived from dietary cholesterol. Ecdysone alone will produce both moulting and metamorphosis (Bolander, 1994). However, during development insect larvae must undergo a series of moults before metamorphosis. A metamorphosis-inhibiting hormone is produced at each ecdysis; this hormone is juvenile hormone (JH) (Bolander, 1994). JH is synthesised and released from the corpora allata in response to two tropic hormones from the brain (allatotropin stimulates this process, whilst allatostatin inhibits it). Therefore, metamorphosis only takes place when ecdysone is produced and JH is suppressed.

Ecdysone and JH are also present during other stages of the insect life cycle (LeBlanc *et al.*, 1999). For example, later embryonic development can be disrupted by JH, whilst ecdysone controls early moults within the egg (Riddiford, 1994). The presence or absence of JH during the moult cycle (particularly when the ecdysone levels are at a peak) determines larval pigmentation, to some extent (Nijhout & Wheeler, 1982). Reproduction in insects is also governed by ecdysone and JH (Bolander, 1994). In many female insects JH stimulates the fat body to produce vitellogenin. Vitellogenin

is then transported (via the hemolymph) to the ovaries, where it is incorporated into oöcytes. In some insect species ecdysteroids also stimulate the transcription of the vitellogenin gene in the fat body (LeBlanc *et al.*, 1999). Ecdysteroids are synthesised and released by the testis in male insects (LeBlanc *et al.*, 1999) and, in the absence of JH, they control spermatogenesis.

B. CRUSTACEA

Crustacean and insect endocrinology are substantially similar. For example, ecdysteroids have been isolated from crustaceans and shown to be identical to those ecdysteroids found in insects (Horn *et al.*, 1966). In insects the prothoracic gland releases ecdysteroids, whilst crustacea produce and secrete ecdysone from the "Y-organ". This secretion from the Y-organ is under the control of a neuropeptide inhibitor, moult-inhibiting hormone (MIH), synthesised in the "X-organ" (Bolander, 1994; Soumoff & O'Connor, 1982). Evidence for a crustacean juvenile hormone, methyl farnesoate (MF), has been presented (Laufer *et* al., 1987; Homola & Chang, 1997). MF has been implicated in the reproductive physiology of both male and female crustaceans (Laufer *et* al., 1998), whilst ecdysteroids are implicated in crustacean vitellogenesis (Young *et al.*, 1993). The endocrine system has also been shown to control pigmentation and limb regeneration in crustacea (LeBlanc *et al.*, 1999).

A further interesting point about crustacean endocrinology is the fact that vertebrate-type sex steroids have been detected and measured in malacostracan crustaceans (e.g. 17β-oestradiol, testosterone and progesterone) (Burns *et al.*, 1984; Yano, 1985; DeLoof & DeClerk, 1986). There is some evidence to suggest that these steroids act

as hormones in crustaceans. For example, Sarojini (1963) showed that testosterone stimulated the conversion of ovaries to testis in female crabs. Testosterone interferes with normal embryo development in daphnids (LeBlanc *et al.*, 1999). Yano (1985) found that progesterone administration to the shrimp, *Metapenaeus ensis*, induced ovarian maturation and spawning. If these vertebrate-type sex steroids are found to be involved in crustacean endocrine functioning, exposure to environmental endocrine disruptors (EDs) could have serious repercussions (see *Section 1.6*). As many EDs target reproduction (e.g. ethynyloestradiol) the potential for ED effects in crustacea is apparent.

C. MOLLUSCA

The molluscs are a huge, diverse invertebrate phylum that includes the bivalves (clams, oysters, and scallops), gastropods (snails, slugs, and sea slugs), and cephalopods (octopus and squid). Many of the molluscs are hermaphrodite, which complicates the view of the endocrine system, particularly the control of reproduction. However, the structure of molluscan neurosecretory cells has been well studied (Turner & Bagnara, 1976). Neurosecretory cells are found in the brains of most mollusca, whilst most cephalopods have small endocrine organs lying on the optic stalks on either side of the brain (Turner & Bagnara, 1976).

As with crustacea, it is interesting to note that the presence and biosynthesis of vertebrate-type sex steroids (e.g. progesterone and testosterone) has been shown (Wootton *et al.*, 1995). Furthermore, exposure of female gastropods to testosterone caused them to develop a penis (imposex, see *Section 1.6*) (Spooner *et al.*, 1991). However, typical invertebrate steroids (e.g. ecdysone) have only been reported in a

small number of molluscan species (LeBlanc *et al.*, 1999). The best defined of the mollusc endocrine chemical messengers are the neuropeptides, a number being involved in reproduction, including egg-laying hormone (ELH), which controls gonad maturation, egg mass production and egg-laying behaviour (LeBlanc *et al.*, 1999). Members of the vasopressin/oxytocin superfamily of neuropeptides (first isolated from vertebrates) have been isolated from freshwater snails and the octopus (Cruz *et al.*, 1987; Van Kesteren *et al.*, 1996; Reich, 1992).

D. ECHINODERMATA

The Echinodermata are marine invertebrates that include sea stars, sea cucumbers and sea urchins. They are very closely related to vertebrates, so may show similar endocrine mechanisms. The processes involved in sea star (starfish) reproduction have been most intensely studied (LeBlanc *et al.*, 1999).

An interesting observation in these invertebrate animals, and potentially very important in environmental endocrine disruption, is the fact that the sea star is capable of synthesising progesterone and testosterone (Voogt *et al.*, 1991). These two steroid hormones are of major importance in the production of other steroid hormones in vertebrates and are biologically active themselves.

E. ANNELIDA

As described in *Section 1.1* the phylum Annelida is made up of three classes of soft-bodied, segmented worms: Polychaeta (almost exclusively marine, bristle worms), Oligochaeta (freshwater, marine or terrestrial) and Hirudinea (freshwater, marine or terrestrial leeches). A large volume of work has been published on the

neuroendocrine system of the leeches (Salzet, 2001). However, the Annelida all seem to have the common trait of secreting peptide hormones from neurosecretory cells located in the CNS. These cells form ganglionic masses rather than true endocrine glands of epithelial origin (LeBlanc *et al.*, 1999). Some annelidan neuropeptides are given in *Table 1.3*.

There are three main processes that appear to be under hormonal (neurosecretory components) control in annelids namely, homeostasis/growth, regeneration and reproduction. Polychaetes exhibit a reproductive process referred to as epitokous transformation (Tombes, 1970). Most polychaetes spend the majority of the year as sexually immature animals. However, many benthic species metamorphose in order to swim to the water's surface and spawn. It has been shown that a hormone (or hormones) secreted from the cerebral ganglion controls this metamorphosis and development of the gonads (Tombes, 1970). However, very little characterisation of the hormones/neurosecretory elements involved in these processes has been done, making it difficult to describe the biochemistry and pharmacology of polychaete reproduction.

Much of the work concerned with the identification of neuroendocrine hormones has been carried out using antibodies directed against various vertebrate neuropeptides (Salzet, 2001). Although a number of hormones involved in physiological functions such as osmoregulation, growth and reproduction have been identified in this manner, little mechanistic information on these hormones is available. From Salzet's (2001) recent review it is clear that the endocrinology of osmoregulation in leeches and some polychaetes is reasonably well understood, but knowledge of the hormones involved

in the reproductive processes in these taxa (and in oligochaetes) is sparse. However, Oumi *et al.* (1994; 1996) identified a nonapeptide from the earthworm, *Eisenia fetida*, that induced egg-laying behaviours and, in some cases, egg-laying itself. This was the first example of the isolation of an intrinsic molecular regulator of earthworm reproduction. Satake *et al.* (1999) later isolated the full length cDNA encoding this peptide, and confirmed that it is homologous to the mammalian "sex hormone", oxytocin (*Chapter 4*).

Table 1.3: Neuropeptides isolated from various annelid species.[1,2]

Species	Sequence	Name/Type	Function
Theromyzon tessulatum (Leech)	ESAEAFPLE	Angiotensin I	Homeostasis
	DRVYIHPFHLLXWG	Angiotensin II	Homeostasis
	DRVYIHPF	Angiotensin III	Homeostasis
	RVYIHPF	Leech Osmoregulatory Factor (LORF)	Osmoregulation
	FMRFamide	FMRFamide	Heartbeat Control
	FLRFamide	FLRFamide	Unclear, but isolated from sex ganglia
	PLGamide	Melanocyte Inhibiting Factor (MIF)	Osmoregulation
Erpobdella octoculata (Leech)	CFIRNCPFGamide	Lysine-Conopressin	Osmoregulation
	FMRFamide	FMRFamide	Control of heart beat
Nereis diversicolor (Polychaeta)	FMRFamide	FMRFamide	Osmoregulation
	FTRFamide	FTRFamide	Osmoregulation
Nereis virens (Polychaeta)	FMRFamide	FMRFamide	Control of heart beat
	FLRFamide	FLRFamide	Unclear
Eisenia fetida (Earthworm)	CFVRNCPTGamide	Annetocin	Stimulates egg-laying behaviour

[1]Not intended as a comprehensive list.
[2]Adapted from Salzet, 2001.

1.6 ENVIRONMENTAL ENDOCRINE DISRUPTION

Environmental contaminants thought to interfere with the normal control of endocrine systems (*Section 1.5*) have been termed endocrine-disrupting chemicals (EDCs). EDCs have been causing concern amongst the scientific community since the 1930s when Dodds & Lawson (1938) showed that such chemicals as alkylphenols have oestrogenic activity. It was not until Colborn *et al.* (1996) published, *"Our Stolen Future"* that serious public debate was stimulated. Colborn *et al.* (1996) articulated the notion that chemicals found within the environment were responsible for the increasingly common reproductive and behavioural problems observed in humans and wildlife.

Currently there are approximately 20,000 man-made chemicals in use (in amounts of more than one ton/year) on the EU market. Not all of these chemicals have been tested for their potential to disrupt endocrine functioning in humans and wildlife (Pärt, 1999). Therefore, ecotoxicologists have initiated a number of testing programs (worldwide and within the EU) to identify chemicals capable of disrupting endocrine function. Moreover, ecotoxicologists have been searching for more sensitive and accurate ways of measuring the effects of these chemicals on wildlife and humans (Sumpter, 1998).

1.6.1 ENDOCRINE DISRUPTION DEFINITION

Defining endocrine disruption has proved more difficult than first thought. The endocrine systems of all animals exhibit certain functional similarities (DeFur *et al.*, 1999). Endocrine systems are extremely complex networks of hormonal cascades and pathways controlling a number of different physiological processes (e.g. reproduction,

growth and homeostasis). This complexity makes it difficult to consider a general definition of endocrine disruption. For example, a system such as moulting in arthropods is under the control of the endocrine system (Tombes, 1970). However, insecticides have been developed that inhibit chitin synthesis, thus preventing moulting and resulting in death (Ishaaya, 1992). So, although chitin-synthesis inhibitors disrupt an endocrine-regulated process (moulting), they cannot be described as endocrine disrupting chemicals (EDCs). Therefore, a more precise definition of endocrine disruption was required that was equally applicable to vertebrates and invertebrates.

At the 1996 *"European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife"*, Holmes *et al.* defined endocrine disruption and the chemicals that cause it as:

> *"An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."*

This broad definition indicates the many different mechanisms through which endocrine disruption can occur. However, only those compounds with the ability to interfere with the function of hormones can be classed as EDCs. It is also important to consider that the endocrine system controls many different aspects of animal

physiological functioning, but most endocrine disrupting work has been done on those chemicals which affect reproduction.

1.6.2 ENDOCRINE DISRUPTING COMPOUNDS: MECHANISMS AND SOURCES

Further to the definition above, an endocrine disrupting compound (EDC) can be defined as follows;

> *"An exogenous agent that can interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of the developmental processes."* (Kavlock *et al.*, 1996)

Natural steroid hormones. Steroid hormones are so-called due to the fact that they are synthesised from cholesterol (Stryer, 1997). A list of some of these hormones, their sources and mechanisms is given in *Table 1.4.* Reproductive function (i.e. sexual differentiation before birth, sexual maturation during puberty, and reproductive function in adulthood) is governed by, in simplistic terms, two steroid hormone types: androgens in males and oestrogens in females. Perhaps the most critical of these are testosterone (androgen) and 17β-oestradiol (oestrogen). In humans both sexes synthesise and secrete testosterone and 17β-oestradiol, but it is the ratio between their levels that determines functional outcome (Ganong, 1995).

Cholesterol is the precursor of the five major mammalian steroid hormones: progestagens (involved in pregnancy), glucocorticoids (maintenance of blood sugar levels), mineralocorticoids (maintenance of water and salt balance), androgens (male

sexual development), and oestrogens (female sexual development) (Stryer, 1997). Steroid hormones are lipid-soluble, and can therefore simply diffuse across the plasma membrane. Within the cell, steroid hormones have specific receptors that facilitate a functional response. There are a number of structurally distinct, functional domains in these proteins. For example, the oestrogen receptor (ER) contains four domains: the N-terminal, which is involved in the regulatory function of the protein; the mid-region, which regulates the DNA binding of the protein and has a zinc-finger motif; a hinge domain, which is poorly characterised; and the C-domain, which harbours the ligand-binding area (Weihua *et al.*, 2003). Upon ligand binding, these receptors form dimers and modulate transcription by binding to the corresponding hormone response element within the promoter/enhancer region of the target gene.

Many steroid hormones can bind to different forms of their corresponding receptors. For example, the oestrogen receptor is found in two forms, named ERα and ERβ, within different cell-types humans (Weihua *et al.*, 2003).

Natural steroid hormones within the environment have been causing concern for many years (Sumpter, 1998). The female steroid hormone 17β-oestradiol is synthesised and released from the ovaries of mammals. It is a key hormone in female reproductive functioning. However, it is excreted in the urine and faeces of mammals and is capable of becoming an exposure risk when sewage effluent is discharged into waterways (Johnson & Sumpter, 2001).

Synthetic steroid hormones. Synthetic compounds such as 17α-ethynyloestradiol (EE2) are used in pharmaceutical products such as the contraceptive pill. The anti-

cancer drug Tamoxifen prevents breast cell proliferation by acting as an antioestrogen (i.e. blocks the oestrogen receptor).

EE2 has been found in concentrations of approximately 0.5 ng L^{-1} in sewage effluents emitted into UK rivers (Johnson & Sumpter, 2001). Although this is relatively low (alkylphenols have been found at concentrations of approximately 2,000 ng L^{-1} in river water), EE2 is an extremely potent oestrogen (Thorpe *et al.*, 2003) and has caused much concern within the scientific community (Johnson & Sumpter, 2001).

During the 1950s and 1960s the synthetic oestrogen diethylstilbestrol (DES) was prescribed to women during pregnancy to prevent miscarriage, and to livestock to increase meat production (Witorsch, 2002). However, DES was found to cause many adverse reproductive health effects in offspring. These ranged from deformities of the vagina and vaginal cancer in females to undescended testes and testicular cancer in males (Stillman, 1982).

Alkylphenolic compounds. These form a group of nonionic surfactants with a worldwide production of approximately 650,000 tons/year. Nonylphenol ethoxylates are by far the most prevalent subgroup of this surfactant class (Guenther *et al.*, 2002). 4-nonylphenol (one of the many degradation products of the nonylphenol ethoxylates) has been shown to be weakly oestrogenic, stimulating the expression of the female specific vitellogenin (VTG) gene in male fish (Jobling *et al.*, 1996). As the VTG gene is under the control of the oestrogen-responsive elements (Maitre *et al.*, 1985) it can be assumed that 4-nonylphenol has the ability to mimic the action of 17β-oestradiol.

Table 1.4: Common compounds known to have endocrine disrupting activity within the environment.[1]

Compound	Class	Source	Mode of Action
17β-oestradiol	Natural Steroid Hormone	Animal waste (sewage treatment water)	Binds to oestrogen receptor and stimulates gene expression
17α-ethynyloestradiol	Synthetic Steroid Hormone	Contraceptive pill	Oestrogen antagonist
Tamoxifen	Synthetic Oestrogen	Anti-breast cancer drug	Anti-oestrogenic
Bisphenol A	Phenolic	Industrial use, plastics	Oestrogen mimic
4-Nonylphenol	Alkylphenol (non-ionic surfactant)	Industrial use	Oestrogen mimic
Tributyltin (TBT)	Organotin	Biocide in antifouling paints	Possibly inhibits cytochrome P450 aromatase
DDT and DDE	Organochlorine	Insecticide	DDD binds to oestrogen receptor
Genistein	Phytoestrogen	Plants	Oestrogen mimic

[1]Not intended as a comprehensive list.

Organochlorine compounds. These encompass a number of different chemicals, which have been shown to be oestrogenic. Polychlorinated biphenyls (PCBs) have been associated with lowered circulating testosterone levels in North Pacific porpoises, although a mode of action is difficult to establish (Subramanian *et al.*, 1987). Derivatives of the pesticide DDT (e.g. *p,p'*- DDD) have been shown to bind to the alligator oestrogen receptor (Vonier *et al.*, 1996).

Other endocrine disrupting compounds. There are many more chemicals found within the natural environment that have been shown to cause endocrine disruption. The phytoestrogens found in plants have been causatively linked to infertility in animals and stomach cancers (Kurzer & Xu, 1997; You, *et al.*, 1988). The compound bisphenol A (BPA) used in the production of polycarbonate and epoxy resins has been shown to competitively bind to the mouse oestrogen receptor β (ER β) (Matthews *et al.*, 2001).

Perhaps one of the most well known of EDCs is tributyltin (TBT), which was used as a biocide in marine antifouling paints until most European countries limited its use during the 1980s (Oehlmann *et al.*, 1996). It was observed that those dog-whelks (*Nucella lapillus*) living in a marine environment contaminated with TBT showed reproductive failure (Gibbs & Bryan, 1986). Consequently, TBT was shown to cause imposex (growth of a penis and/or vas deferens in females) in the gastropod, *Nucella lapillus* (Linnaeus) (Oehlmann *et al.*, 1991) (see below for more detail). A mechanism is not clear for the action of TBT, but it is thought that it may inhibit the cytochrome P_{450} aromatase enzyme responsible for converting testosterone to 17β-

oestradiol (Alzieu, 2000). Therefore, an increase in androgen levels (Spooner *et al.*, 1991) results in the development of imposex (Bettin *et al.*, 1996).

The heavy metal cadmium has been shown to bind to, and activate, the human oestrogen receptor α (ERα) (Stoica *et al.*, 2000).

1.6.3 ENDOCRINE DISRUPTERS AND WILDLIFE

Endocrine Disruption in Birds:

Many studies have linked exposure to the pesticides DDT (an organochlorine compound) and DDD (an analogue of DDT) with a reduction in the numbers of birds in North America and Europe (*reviewed by* Fry, 1995). Fry & Toone (1981) showed that injection of environmentally relevant DDT concentrations into the eggs of western gulls (*Larus occidentalis*) resulted in the induction of abnormal development of ovarian tissue and oviducts in male embryos. They hypothesised that DDT pollution in the waters off the southern Californian coast (and its accumulation in fish) resulted in a reduced number of males, a sex ratio of approximately four females to every male, and female-female pairing (Hunt & Hunt, 1977).

The best documented evidence for reproductive disruption in birds is exemplified by the organochlorine pesticides DDT and DDE (*see* Fry, 1995). Ratcliffe (1967) reported thinner shells in raptor eggs and hypothesised that the causative agent was organochlorine pesticide (particularly DDT). This classical study looked at the shell thickness of eggs from three birds of prey found in Britain: peregrines (*Falco peregrinus*), sparrowhawks (*Accipiter nisus*) and golden eagles (*Aquila chrysaetos*). It was found that organochlorine pesticides and their metabolites were found in much

higher concentrations in the tissues of predatory birds than in prey species (*see* Cooke, 1973).

Endocrine Disruption in Fish:

The aquatic environment acts as a sink for a huge array of chemicals released into the environment. Pesticides applied to agricultural land may leach into rivers and streams. Industries such as papermaking use vast quantities of river water during a number of processes, and return that water back into the river afterwards. As the human population increases, and populations become increasingly urbanised, the quantity of sewage (treated or untreated) released into the aquatic environment increases. It is the release of human sewage into rivers that has caused most concern over the past fifteen years, especially in the UK and other developed countries where effluent from sewage-treatment works (STWs) often contributes 50% of the flow of some rivers (but can be as high as 90% in periods of low rainfall) (Sumpter, 1998).

With this in mind, concern was raised when hermaphrodite (intersex) fish were found in the settlement lagoons of two STWs twenty-five years ago (Sumpter, 1998). Intersex is a condition where animals possess both male and female gonadal characteristics (Nolan *et al.*, 2001), and has been described in wild roach (*Rutilus rutilus*) in UK rivers (Jobling *et al.*, 1998). Purdom *et al.* (1994) published data supporting the theory that it was sewage effluent that was causing intersex in wild fish. After exposing caged male trout to river water at locations downstream of an STW for three weeks an elevated plasma vitellogenin concentration was observed (Purdom *et al.*, 1994). Induction of the oestrogen responsive egg-yolk protein vitellogenin has been widely used to determine oestrogenic activity in fish and other

animals (Sumpter & Jobling, 1995). Vitellogenin (VTG) is only expressed in female animals as a result of oestrogen induction, but male animals do contain the VTG gene. Therefore, upon exposure to environmental oestrogenic compounds VTG expression is induced in males.

Endocrine Disruption in Reptiles and Amphibians:

One of the most important studies to have been published in recent years within the EDC field was that of Guillette *et al.* (1994). Between 1980 and 1983 the alligator (*Alligator mississippiensis*) population in Lake Apopka, Florida, USA, declined from 30 juveniles/km to 4 juveniles/km (*reviewed by* Guillette *et al.*, 2000). These falls in alligator populations in this particular lake were due to a spill of pesticides (including DDT) into the water in 1980. Guillette *et al.* (1994) reported that female alligators (of six months in age) from Lake Apopka had 17β-oestradiol blood concentrations twice as high as those in females of the same age found in a 'control' lake. The Lake Apopka females also showed abnormal ovarian morphology. Male alligators (juveniles) from the contaminated Lake Apopka exhibited lowered testosterone levels (three times lower than that found in male alligators from a 'control' lake), poorly organised testes, and abnormally small phalli.

Androgens are essential for normal maturation and growth of the male reproductive system in embryonic reptiles during puberty (Guillette *et al.*, 2000). It is thought that male juvenile alligators in Lake Apopka, Florida were exposed to DDT and its derivatives during embryonic development, leading to the abnormalities seen later in life (Guillette *et al.*, 1996).

Despite the extremely important role amphibians play in a number of terrestrial and aquatic ecosystems, they are not represented very often in EDC research (Levy *et al.*, 2004). As early as 1950, Witschi & Allison showed that 17β-oestradiol treatment of *Xenopus laevis* tadpoles resulted in the development of all fertile females. Pickford & Morris (2003) showed that the pesticide, methoxychlor was capable of disrupting reproductive function in adult, female *Xenopus laevis*. Bisphenol A (BPA) has been shown to cause feminisation in *Xenopus laevis* tadpoles and increased the mRNA expression of the amphibian oestrogen receptor (Levy *et al.*, 2004).

Endocrine Disruption in Mammals:

Although ecotoxicology is used to monitor the health of the ecosystem and of its biotic constituents, the ultimate aim of the science is to predict detrimental effects on human health due to contaminants found within the environment. Therefore, the concern over the effects of EDCs on wildlife is currently extending to a concern for the possible effects of EDCs on livestock and humans.

Carlsen *et al.* (1992) published a report stating that sperm production in men throughout the world had fallen by 50% over a period of 50 years between 1940 and 1990. The authors speculated that this was as a result of exposure *in utero* to a "sea of oestrogens". This study caused huge debate within the scientific community and brought the issue of endocrine disruption to the attention of the public. However, the study was heavily criticised for the way in which the data were analysed (Juberg, 2000; Swan *et al.*, 1997). Swan *et al.* (1997) reanalysed the data taking into account many of the factors the original report neglected (e.g. age, specimen collection method, study goal and location) and confirmed a decline in sperm density in men

from the United States and Europe, but not in non-Western countries. Many investigators (reviewed by Juberg, 2000) consider that the human epidemiological data available are not sufficient to unequivocally link decreases in male sperm counts with exposure to environmental oestrogens and, further, that exposure to such low levels of oestrogens normally extant in the environment is not sufficient to cause serious health risks. Juberg (2000) proposes that this is particularly true when the level of phytoestrogens consumed by humans is considered.

Other mammals have been shown to be affected by environmental oestrogens. Facemire *et al.* (1995) showed that there was no significant difference between serum 17β-oestradiol levels in male and female Florida panthers (*Felis concolor coryi*) exposed to DDT and PCBs. The altered ratio between oestrogens and androgen levels in male panthers was hypothesised to be causing feminisation in the males.

1.8 AIMS OF THE PROJECT

Molecular genetic biomarkers have come under considerable criticism from certain factions of the ecotoxicological community over recent years and they have yet to be fully integrated into environmental monitoring and management. In part this is due to the fact that a number of molecular biomarkers presently in use have lacked the essential ecological relevance needed in ecotoxicology.

Van Straalen (2003) noted that the "testing-based" approach of ecotoxicology has benefited environmental regulation, but ecotoxicology is moving into new areas; mainly the elucidation of the underlying mechanisms behind toxic effect. Therefore, molecular genetic biomarkers will play an increasing role in the monitoring of toxic

effect at an individual level. However, for these observations to be truly useful they must be easily linked to higher levels of organisation. One way in which this can be done is to use biomarkers that are specifically-linked to key life-cycle traits in the particular organism, e.g. reproduction and/or growth. Moriarty (1983) stated that such sublethal endpoints are more ecologically relevant.

With this is mind, this thesis is focussed on the identification of potential molecular genetic biomarkers of reproduction in earthworms, and moreover, the elucidation of the mechanisms behind these biomarkers. This will be achieved through the following individual aims:

- The identification of *Lumbricus rubellus* gene transcripts involved in the reproductive processes as potential molecular genetic biomarkers
- To identify potential molecular genetic biomarkers in two other earthworm species, *Eisenia fetida* and *Eisenia andrei*
- To identify the earthworm putative sex hormone, annetocin, in three earthworm species, *Eisenia fetida*, *Eisenia andrei* and *Lumbricus rubellus*
- To map the expression pattern of the annetocin gene at a gross anatomical level in *Eisenia fetida*
- To design and produce a real-time PCR-based assay for the measurement of annetocin gene expression in these species
- To measure annetocin gene expression levels in *Eisenia fetida* and *Lumbricus rubellus* after exposure to heavy metals in soil

- To measure annetocin gene expression levels in *Eisenia andrei* after exposure to the endocrine disrupting chemicals Bisphenol A, Nonylphenol, 17α-ethynylestradiol, 17β-oestradiol and testosterone
- To identify possible modes of action for these chemicals (and other similar chemicals) in the earthworm

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND REAGENTS SUPPLY

2.1.1 REAGENTS AND SUPPLIERS

A summarised list of materials, reagents and buffers, with suppliers is given in *Table 2.1* and *Table 2.2*. All other standard laboratory reagents were supplied by Fisher Scientific Ltd. (Loughborough, Leicestershire, UK).

Table 2.1: Materials and Reagents Supply

Supplier	Reagent
Ambion (Austin, TX, USA)	RNAlater™ ROX
Appligene (Illkirch, France)	Sodium Dodecyl Sulphate (SDS)
BDH Laboratory Supplies (Poole, UK)	Glycerol
Boehringer Mannheim Supplies (Mannheim, Germany)	Tris
Clontech Laboratories Inc. (Palo Alto, Ca, USA)	PCR-Select™ cDNA Subtraction Kit
FMC BioProducts (Rockland, ME, USA)	Sybr™ Green I
Invitrogen (Paisley, UK)	DH5α Competent Cells 10 x TAE
Greiner Bio-one (Stonehouse, UK)	Disposable Sterile Universal Tubes Petri Dishes 1.5 ml Eppendorf Tubes
MWG Biotech (Erbesberg, Germany)	Custom Synthesised Primers
Promega Corporation (Southampton, UK)	dNTPs25/05/2005 6 x Loading Dye Molecular Weight DNA Markers M-MLV Reverse Transcriptase pGEM®-T Vector Cloning Kit RNasIN® T4 DNA Ligase *Taq* Polymerase Wizard® *Plus* SV MiniPrep Kit

Table 2.1 (Cont.): Materials and Reagents Supply

Supplier	Reagent
QIAGEN Ltd. (Crawley, UK)	QIAGEN DNA Purification Kit Custom Dual-labelled Oligonucleotide Probes
Sigma-Aldrich (St. Louis, USA)	Agarose Ampicillin Ethidium Bromide Isoamyl Alcohol Luria Broth Capsules LB Agar Capsules Magnesium Chloride Mineral Oil Phenol TEMED Tri-Reagent® HPLC-grade Water

Table 2.2: Solutions, Media and Reaction Buffers

Solution	Components
Agarose Gel Electrophoresis *(Section 2.2.11)*	
DNA Loading Dye (Promega)	0.03% Bromophenol Blue, 0.03% Xylene Cyanol FE, 0.4% Orange G, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8.0)
10 x TAE (Invitrogen)	40 mM Tris-Acetate, 1 mM Na_2-EDTA (pH 7.6)
Reverse Transcriptase-Polymerase Chain Reaction *(Section 2.2.15)*	
5 x M-MLV RT Reaction Buffer (Promega)	50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT
Polymerase Chain reaction *(Section 2.2.16)*	
10 x *Taq* Polymerase Reaction Buffer	50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton® x-100
Wizard® Plus SV Miniprep Kit (Promega) *(Section 2.2.20)*	
Cell Resuspension Solution	50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 μg/ml RNase A
Cell Lysis Solution	0.2 M NaOH, 1% SDS
Neutralisation Solution	4.09 M Guanidine Hydrochloride, 0.759 M Potassium Acetate, 2.12 M Glacial Acetic Acid, (adjusted to pH 4.2)
Column Wash	60 mM Potassium Acetate, 10 mM Tris-HCl (pH 7.5), 60% Ethanol

Table 2.2 (Cont.): Solutions, Media and Reaction Buffers

Solution	Components
PCR Clean Up (*Section 2.2.21*)	
Storage/Wash Solution	0.4 x SSC
Sequencing of PCR Products (*Section 2.2.22*)	
Big Dye Terminator V3.0	Proprietary Formulation
PCR-Select™ cDNA Subtraction (Clontech) (*Chapter 3, Section 3.2.2*)	
5 x First Strand Buffer	250 mM Tris-HCl (pH 8.3), 30 mM $MgCl_2$, 375 mM KCl
20 x Second Strand Buffer	500 mM KCl 50 mM Ammonium Sulphate, 25 mM $MgCl_2$, 0.75 mM β-NAD, 100 mM Tris-HCl (pH 7.5), 0.25 mg/ml BSA
10 x *Rsa*I Restriction Buffer	100 mM Bis Tris Propane-HCl (pH 7.0), 100 mM $MgCl_2$, 1 mM DTT
5 x DNA Ligation Buffer	250 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 10 mM DTT, 0.25 mg/ml BSA
4 x Hybridisation Buffer	Proprietary Formulation
Dilution Buffer	20 mM HEPES-HCl (pH 7.8), 50 mM NaCl, 0.2 mM EDTA (pH 8.0)
10 x Advantage® Klen *Taq* PCR Reaction Buffer	400mM Tricine-KOH (pH 9.2), 150 mM KOAc, 35 mM $Mg(Oac)_2$ 750 μg/ml BSA
Light-Cycler™ Real-Time Quantitative PCR Amplifications (*Section 2.2.25*)	
10 x Light-Cycler™ Buffer	500 mM Tris, 2.5 g/L BSA
Dual-Labelled Oligonucleotide Probe qPCR Amplifications (*Section 2.2.26*)	
10 x Quantitative PCR Buffer	200 mM Tris-HCl (pH 8.3), 500 mM KCl, 40 mM ROX

2.1.2 MEDIA

LB-broth (LB) and LB-agar were prepared according to the manufacturer's recommendations. The appropriate weight of powder, or designated number of capsules, was dissolved in the correct volume of distilled water. After sterilisation the medium was left to cool to below 60°C before the addition of antibiotics.

2.1.3 AUTOCLAVING CONDITIONS

Prior to the performance of any nucleic acid technique all equipment was sterilised. This was achieved by autoclaving at 120°C, 15 psi for 20 minutes. All equipment for RNA work was autoclaved twice.

2.1.4 ANTIBIOTICS

Stock solutions of antibiotics were dissolved in sterile distilled water to the required concentration and passed through a 0.22 μm Nucleopore™ filter (Millex, Millipore). The regular final concentrations of antibiotics used were, 100 μg/ml of ampicillin, and 50 μg/ml of kanamycin. Filter sterilised antibiotic solutions were stored at -20°C.

2.2 METHODS

2.2.1 EARTHWORM HUSBANDRY

All earthworm species types (*Eisenia fetida*, *Eisenia andrei* and *Lumbricus rubellus*) were stored at the following conditions; 15 ± 1 °C; 12 hours dark: 12 hours light. Earthworms were maintained in their native soil until used in exposure protocols (*Sections 2.2.2 – 5*).

In the laboratory individual animals were rinsed in distilled water to remove adhering soil particles and placed on filter paper (Whatman N°. 1) soaked in distilled water and placed in a Petri dish (9 cm diameter). Preliminary identifications were confirmed using the standard key of Gerard (1964). The animals were left on soaked filter paper for two days for depuration, with regular filter paper changes.

Following the depuration period, animals were processed for molecular biological analysis. For immediate analysis worms (or appropriate segments) were placed directly in TRI-Reagent™ (Sigma-Aldrich) and homogenised. Animals for later use were placed either directly in liquid nitrogen or in RNAlater™ (Ambion) and stored at -70°C.

2.2.2 SUB-LETHAL EXPOSURE OF EISENIA FETIDA TO A HEAVY METAL CONTAMINATED SOIL – 21 DAY EXPOSURE

Adult (clitellate) earthworms (*Eisenia fetida*), subsequently found to average ~76 mg dry weight, were sampled from a horse manure pile on a local farm (Amelia Farm, Vale of Glamorgan O.S. grid reference: ST 063 740). They were randomly assigned to treatment groups, either their native substrate under laboratory conditions (15 ± 1°C; 12 hours dark: 12 hours light) for subsequent molecular-genetic studies, or exposed to test soils for qPCR analysis and cocoon production measurements.

The two soils used for the latter experiment were the commercially available Kettering loam (Boughton Loam Ltd., UK: pH = 6.1; Loss-on-incineration = 7.0 %), and contaminated metalliferous soil from the abandoned Pb/Zn mine at Cwmystwyth, Wales, UK (O.S. grid reference: SN 805 748: pH = 6.4; Loss-on-incineration = 35 %). The metalliferous soil was air-dried over several weeks to kill any cocoons present, and then sieved (2 mm mesh) to remove these.

Food quality plastic containers (12 x 6 x 10 cm) were filled with 500 g (dry weight) of a given soil. Soils were hydrated to approximately 75 % of their water-holding capacity with de-ionised water, mixed by hand, and allowed to equilibrate for 4 days

before worms were introduced. Ground chicken manure (5 g/box) was sprinkled on the soils and mixed by hand. Worms (20 per soil type, distributed in two replicate boxes, i.e. each box contained 10 adult worms) were maintained on the soils for 20 days in a controlled environment room (15 ± 1°C; 12 hours dark: 12 hours light). Earthworms were removed from the soils at the end of the exposure period and counted. The exposure protocol was considered valid if the survival in the reference soil was 90 % or above. Cocoons were counted by wet sieving the soils (Kula & Larink, 1998).

2.2.3 EXPOSURE OF LUMBRICUS RUBELLUS TO A HEAVY METAL CONTAMINATED SOIL - 70 DAY EXPOSURE

All analyses were conducted at a series of rough grassland sites located in an area to the north east (direction of prevailing wind) of a primary Cd, Pb and Zn smelter located at Avonmouth, South-West England. Operating until December 2002, this factory was one the largest smelter of its type in the world and a major source of metal pollution (Martin & Bullock, 1994). Previous studies of soil contamination in the Avonmouth area have indicated the presence of elevated metal concentrations in soils at least 15 km downwind of the smelter (Spurgeon & Hopkin, 1996). The sites chosen for the full experiment represented a gradient of contamination ranging from severe (soil containing up to 5.0 % metal) to approaching background. However, annetocin gene expression analysis was only performed in those worms exposed to soil from two Avonmouth sites (Sites 1 and 2 in *Figure 2.1*) and the control soil (Kettering loam).

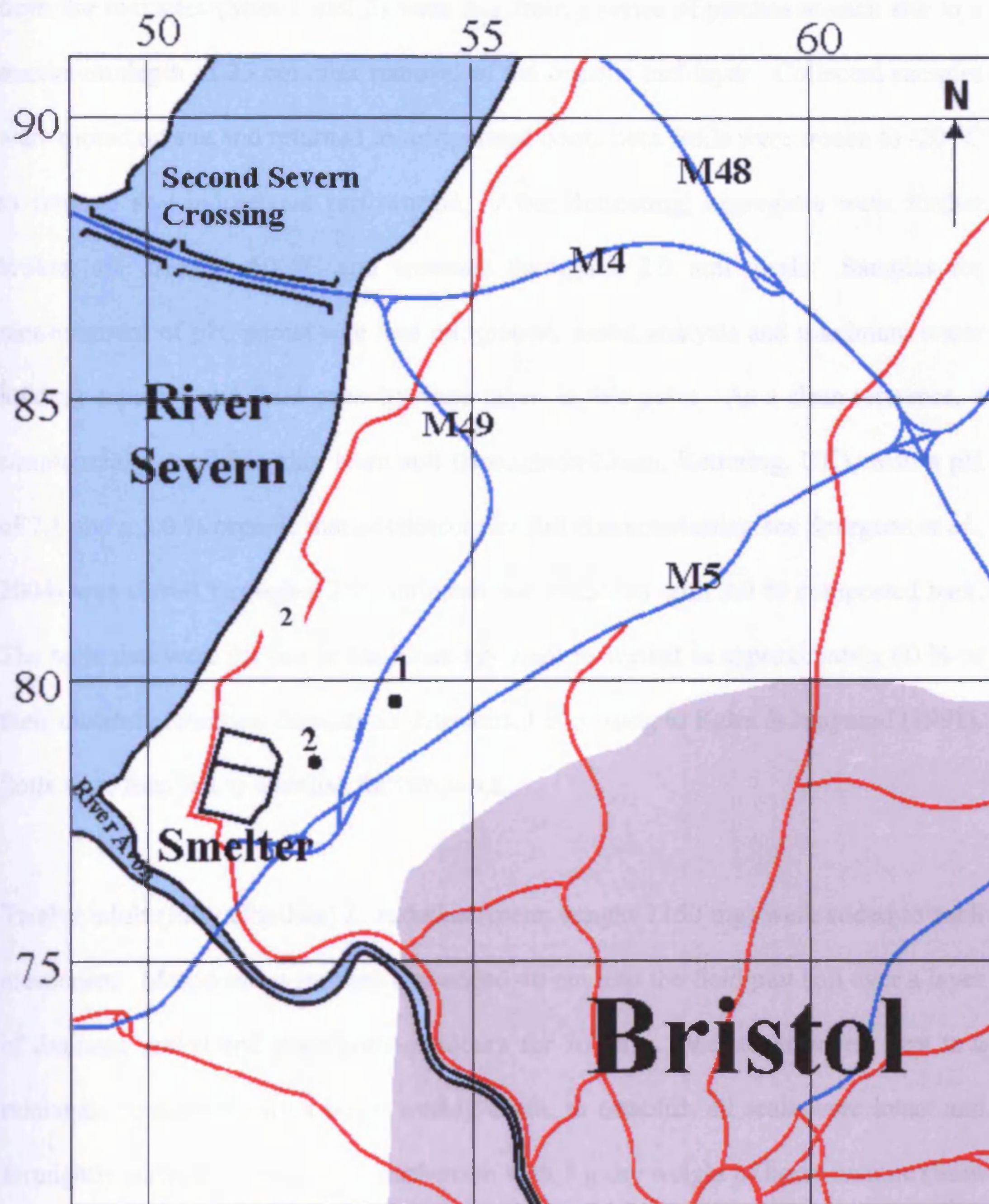


Figure 2.1: Location of the two soil sampling sites at the Avonmouth Smelting works. Soil sampling sites are indicated by the numbers 1 and 2. Site 1: furthest site from the smelting works at 3.6 km. Site 2: closest site to the smelting works at 1.0 km distance. Both sites were downwind of the smelting works.

All exposures were conducted in mesocosms after Svendsen & Weeks (1997). Soil from the two sites (Sites 1 and 2) were dug from a series of patches at each site to a maximum depth of 25 cm after removal of the organic turf layer. Collected samples were mixed on site and returned to refrigerated containers. Soils were frozen to -20 °C to remove any indigenous earthworms. After defrosting, aggregates were further broken up, dried at 60 °C and screened through a 2.0 mm mesh. Samples for measurement of pH, percentage loss on ignition, metal analysis and maximum water holding capacity and field capacity were taken at this point. As a clean reference, a commercially available clay loam soil (Broughton Loam, Kettering, UK), with a pH of 7.1 and a 5.0 % organic matter content (for full characterisation see Spurgeon *et al.*, 2004) was sieved through a 2.0 mm mesh and amended with 3.0 % composted bark. The soils that were for use in the bioassays were re-wetted to approximately 60 % of their moisture retention capacity as determined according to Kalra & Maynard (1991). Soils were then left to stabilise for two week.

Twelve adult (fully clitellate) *L. rubellus* (mean weight 1150 mg) were added to each mesocosm. Mesocosm were then embedded 40 cm into the field plot soil over a layer of drainage gravel and maintained outdoors for 70 days. Maintenance was kept to a minimum consisting only a twice weekly check to establish all seals were intact and fortnightly surface feeding of the earthworm with 5 g dry weight of horse manure (from a known source not subject to recent medication) rewetted to 80 % moisture content. Automated temperature loggers were kept on site at 0, 10 and 20 cm depth. Daily rainfall was recorded. At the end of the exposure, the individual mesocosm units were retrieved. Surviving worms were hand-sorted from the soil and placed onto moist filter paper for immediate use in physiological measurements. Soil from the mesocosms was

sieved through a 1.0 mm mesh to collect any cocoons produced during the incubation period. This number was then related to the number of worms added and surviving at termination of the experiment.

2.2.4 EARTHWORM ACUTE TOXICITY RANGE-FINDING TEST USING ARTIFICIAL SOIL – 14 DAY EXPOSURE

Eisenia andrei earthworms were exposed to test compounds in artificial soil based upon the OECD Guideline 207 (1984). The artificial soil was prepared using air dried components in the following quantities;

- 10 % sphagnum peat (finely ground)
- 20 % kaolin clay (finely powdered)
- 70 % industrial sand
- Calcium carbonate to adjust pH to 6.0 ± 0.5

The dry constituents were mixed in the correct quantities in a small electric cement mixer. Before the addition of calcium carbonate the pH of the dry soil was assessed. This was performed using 10 g of dry soil in 100 ml of a 1.0 mol/L KCL solution. This solution was thoroughly mixed and left to settle for approximately one hour or until the soil had settled into the various soil horizons. The pH of the aqueous phase of this mixture was subsequently measured using a pH meter. An appropriate weight of calcium carbonate was then added to the soil to give a pH within the correct range.

The day before the start of the test, de-ionised water was added to the soil to give a moisture content of approximately 40 % (soil was presumed to be dry before addition of water).

The chemicals used in this test were insoluble in water. Therefore, acetone was used during the application of the test substance to the artificial soil. Each test chemical was dissolved and diluted in acetone to give the correct concentration. The artificial soil (having already been moistened to 40 % of its water holding capacity) was weighed to give 500 g per replicate. This soil was placed in a metal mixing bowl and the dissolved test substance added and mixed well. The 'spiked' soils were then placed in one litre glass jars and placed in a fume cupboard for 24 hours to allow the solvent to evaporate. All replicates were then weighed and de-ionised water added to correct for weight loss due to evaporation.

Ten fully clitellate, adult *Eisenia andrei* earthworms were added to each container. The worms were washed (with de-ionised water) and weighed before being added to the soil. After fifteen minutes the containers were checked for any earthworms remaining on the soil surface. Horse manure (previously dried for 48 hours at 50 °C) was placed on the soil surface. The manure had been pre-wetted and formed into small balls weighing 5.0 g. Each container was sealed with gauze mesh and placed in a constant temperature room (20 ± 2 °C) with 24 hour light. Each container was weighed at regular intervals throughout the two week exposure period and water loss was adjusted by the addition of de-ionised water.

In addition to those vessels containing soil 'spiked' with test substances control and solvent control vessels were produced. The solvent control contained the maximum

volume of acetone used in any of the test vessels, whilst the control vessel only contained soil treated with de-ionised water.

At the end of the exposure period soils were hand sifted and all the worms were removed, counted, washed with de-ionised water and weighed. Those worms used in downstream molecular biology techniques were frozen in liquid nitrogen. Mortality was calculated in each replicate, but as only one replicate was used per concentration no statistical analysis was performed.

2.2.5 EARTHWORM REPRODUCTIVE TEST USING ARTIFICIAL SOIL – 28 DAY EXPOSURE

The OECD draft guideline from January 2000 was followed. The test organism was the earthworm, *Eisenia andrei*. Test substances were dissolved in acetone as described above (*Section 2.2.4*) and applied to 500 g of moist artificial soil. Control and Solvent Control containers were also produced as described in *Section 2.2.4*.

Only fully clitellate, adult earthworms were used during the test. The earthworms were maintained in there native media at 20 ± 2 °C prior to the test. They were then washed in de-ionised water and weighed before being added to the test soils. They were fed on 5 g/ week of dried horse manure (collected manually) sprinkled on the soil surface. Water loss from the vessels was monitored on a regular basis and corrected by the addition of de-ionised water.

After 28 days of exposure each vessel was removed from the constant temperature room. Each soil was sifted and the adult earthworms removed, washed and weighed.

Those earthworms intended for molecular analysis were frozen in liquid nitrogen immediately. Once all the worms were removed the soils were replaced in the glass jars. A further 5 g of dried horse manure was sprinkled on the soil surface (for the last time during the exposure) and then returned to the constant temperature room. The vessels remained at 20 ± 2 °C for a further 28 days. Water loss was recorded and corrected for on a regular basis.

After 56 days (from the time of the addition of the adult earthworms to the soils) the soils were wet-sieved (using two 0.5 mm mesh size sieves) (Van Gestel *et al.*, 1988) to remove cocoons and juvenile earthworms. The number of cocoons and juveniles per replicate were recorded for later statistical analysis.

2.2.6 METAL MEASUREMENT IN SOILS AND EARTHWORMS

Test soils were dried overnight at 80°C, and ground finely by hand using a pestle and mortar. Four 1 g replicates of each soil were then extracted for 2 hours at ambient temperatures in 20 ml of 1 M ammonium acetate in an orbital shaker (80 rpm). The extracts were made up to volume, diluted as necessary with distilled-deionised 'polished' water and analysed for Pb and Zn by atomic absorption spectrophotometry (Instrumentation Laboratory 457).

Eight earthworms per exposure treatment were maintained on moistened filter paper (Whatman No. 1, qualitative) for 3 days to clear gut contents. [Gut depuration is a standard procedure in studies of earthworm metal relationships, but it is not presently known whether or not gene expression levels change during this period]. Worms were immersed in liquid nitrogen and placed individually in pre-weighed glass tubes, dried

at 80°C overnight, weighed and digested in concentrated HNO_3 on a sand bath prior to atomic absorption spectrophotometric analysis for lead and zinc.

Previous studies (Corp & Morgan, 1991) have indicated that the mine site soil used does not contain appreciable concentrations of other heavy metals, such as cadmium and copper. The analysis of standard reference materials has consistently indicated that our analytical protocol yields data within approximately 7% of certified values.

2.2.7 STATISTICAL ANALYSIS

All statistical analyses were performed using the package, Minitab, Version 13.1 (Minitab Inc.). Data were statistically analysed by the Kruskal-Wallis non-parametric test; parametric tests were precluded by low 'n'-values and consequent non-normal distributions. A significant difference between groups was seen when $p < 0.05$ (equivalent to 95 % confidence).

2.2.8 TOTAL RNA ISOLATION

To minimise RNA degradation by RNAses, all RNA preparations were performed using double autoclaved (120°C at 15 psi for 20 minutes) materials. Apparatus for gel electrophoresis was soaked overnight in a 1.0% SDS solution.

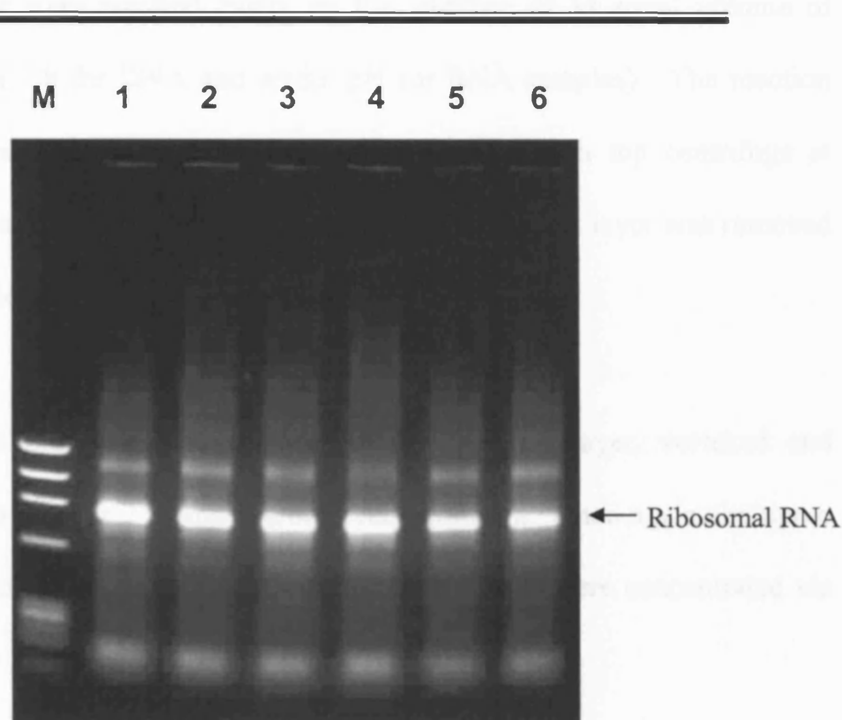


Figure 2.2: Total RNA isolated from adult, fully clitellate *Eisenia andrei* earthworms. Total RNA samples were subjected to electrophoresis on a 1 % agarose gel containing EtBr (0.4 μg/ml) and visualised under U.V. light. Lane M represents the marker DNA (*ϕ*x174*Hae*III fragments). Lanes 1 – 6, show 2.0 μg (in each well) of total RNA.

Total RNA (see *Figure 2.2*) was isolated from earthworm samples by homogenisation in TRI-reagent™ (Sigma-Aldrich Biosciences), following the manufacturer's protocol (Sigma-Aldrich Biosciences, 1995). All centrifugation steps were performed using the F-28 rotor at 12,000 x *g* and 4°C. RNA pellets were resuspended in an appropriate volume of doubled autoclaved water, and preferably stored at -80°C as an ethanol precipitate (*Section 2.2.10*), which involved the addition of a 10% volume of sodium acetate and (*Table 2.1*) and a 250% volume of 100% ethanol.

2.2.9 PHENOL-CHLOROFORM EXTRACTION FOR NUCLEIC ACID PURIFICATION

Nucleic acid samples were purified firstly by the addition of an equal volume of phenol (1:1 (v/v); pH 7.8 for DNA and acidic pH for RNA samples). The reaction mixture was vortexed and subsequently centrifuged in a bench top centrifuge at 13,000 x *g* for 2 minutes (room temperature). The upper aqueous layer was removed and transferred to a clean micro centrifuge tube.

An equal volume of chloroform was added to the aqueous layer, vortexed and centrifuged at 13,000 x *g* for 2 minutes (room temperature). Once again the upper aqueous layer was removed to a clean tube. The nucleic acids were concentrated via precipitation (*Section 2.2.10*).

2.2.10 ETHANOL PRECIPITATION

To concentrate DNA and RNA samples, a 10% volume of 3 M NaOAc (pH 6.0) (or for RNA samples acidic pH), and a 250% volume of 100% ethanol was added. After mixing by slow inversion, the samples were incubated either at -70°C for one hour or overnight at -20°C. These samples were stable at -70°C for up to a year.

After precipitation nucleic acids were recovered by centrifugation at 12, 000 x *g* for 10 minutes (at 4°C). The pelleted material was allowed to air-dry at room temperature after the removal of the supernatant. Samples were resuspended in an appropriate (5-100 μl) volume of sterile HPLC-grade water.

2.2.11 AGAROSE GEL ELECTROPHORESIS

DNA and RNA samples were routinely resolved by agarose gel electrophoresis. All gels consisted of between 1-3% (w/v) agarose, melted in 1 x TAE buffer with ethidium bromide [0.5 μg/ml]. All samples were mixed with DNA loading dye (Promega, *Table 2.2*), applied to a 60 ml agarose gel and electrophoresed at 120 mA for 30 minutes, or until adequate separation of fragments was observed. Electrophoresis took place in a Pharmacia GNA-100 submarine tank filled with 1 x TAE (*Table 2.2*) containing ethidium bromide [0.5 μg/ml]. The percentage of agarose in the gel was determined by the molecular weight of the particular nucleic acid fragment that was being analysed.

Separated nucleic acid fragments were visualised by illumination under U.V. light. DNA fragment sizes were compared to DNA markers (*Appendix A*).

2.2.12 EXTRACTION AND PURIFICATION OF DNA FROM AGAROSE GELS

DNA bands were excised from agarose gels (*Section 2.2.12*) using a sterile scalpel blade whilst under U.V. light. The DNA was purified using the QIAGEN gel extraction kit, following the manufacturer's protocol. DNA was eluted with an appropriate (10-50 μl) volume of sterile HPLC-grade water.

2.2.13 NUCLEIC ACID QUANTIFICATION

Nucleic acids absorb at a wavelength of 260 nm. Given that an absorbance of 1 O.D. is equivalent to 40 μg/ml single-stranded DNA or RNA, it is possible to quantify DNA and RNA concentrations. Furthermore, determining absorbencies at 230 nm and 280 nm provides a means of examining sample purity. Pure preparations of DNA or RNA

will have an A_{260}/A_{280} ratio of approximately 1.8 and 2.0 respectively. Any protein or phenol contaminants result in a lower A_{260}/A_{280} ratio. While a high A_{260}/A_{280} ratio is indicative of salt or ethanol contamination (Sambrook *et al.*, 1989).

Optical density readings were performed either on a Gene-Quant instrument (Pharmacia) or on a spectrophometer using DNase/RNase-free UV plastic cuvettes (Eppendorf).

2.2.14 OLIGONUCLEOTIDE PRIMER DESIGN

In order to minimise mis-priming, oligonucleotide primers were designed using the OLIGO© 1991 Primer Analysis Software (Version 4.0, Wojcieck Rychiick, National Biosciences, Plymouth, MN, USA).

Oligonucleotide primers were selected for optimal T_m, and low self-complementarity and complementarity to the second primer (if two primers were being designed), this was particularly important at the 3'-end of the oligonucleotides. This stringent assessment reduced the risk of 'primer-dimer' formation.

For 5'-exonuclease assays (*Section 2.2.26*) oligonucleotide primers and the probe itself was designed using the Primer Express™ software package (PE Applied Biosystems). For details on exact parameters used see *Section 2.2.26a.*

2.2.15 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA (approximately 1-5 μg) was used to generate a complementary DNA copy (cDNA) using a reverse transcription enzyme. The RNA sample was diluted to 19 μl

with sterile, HPLC-grade water, and heated at 70°C for 3 minutes to remove secondary RNA structure. After cooling on ice, oligo (dT_{15}) oligonucleotide primer (50 pmoles; Appendix A), 0.5 mM dNTPs, RNasIN® (40 units; Promega) and M-MLV RT (400 units; Promega), buffered with a 1 x M-MLV RT reaction buffer (Promega; *Table 2.2*), were added. After mixing the reaction mixture was incubated at 37°C for 1.5 hours. Heating at 65°C for 5 minutes terminated the reaction. The resulting cDNA (1-2 μl) was used for PCR amplifications (*Section 2.2.16*).

2.2.16 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reactions (PCRs) were performed using a PCR reaction mixture containing the target DNA (1-2 μl), 0.01 U/μl *Taq* Polymerase (Promega, where not defined), 1 μM of forward and reverse oligonucleotide primers and 0.2 mM dNTPs (final concentration of each dNTP), buffered in a 1 x *Taq* polymerase reaction buffer (Promega; *Table 2.2*) with 2.5 mM $MgCl_2$.

Analytical reaction mixtures (20 μl) were contained in either 200 μl thin-walled PCR tubes (ABgene) or 500 μl thin-walled tubes. Two PCR machines were used;

1. A non-heated lid (therefore, mineral oil (Sigma-Aldrich) was used to overlay PCR reaction mixtures in 500 μl thin-walled tubes) Perkin Elmer DNA Thermal Cycler 480.
2. A heated-lid (mineral oil not necessary) MWG-Biotech PRIMUS™

Melting, annealing and chain extension steps were based on the following cycling conditions:

95°C	for	5 minutes (initial denaturation)
95°C	for	1 minute (chain melting)
60°C*	for	1 minute (primer annealing)
72°C	for	1.5 minutes* (chain extension)
72°C	for	5 minutes (final extension)

*where primer melting temperature and amplified product length are dependent factors.

All PCR reaction products were analysed by separation on an agarose/TAE/ethidium bromide [0.5 μg/ml] gel; the percentage of agarose in the gel was dependent on the size of the PCR product. Products were visualised under U.V. light (*Section 2.2.11*).

2.2.17 CLONING OF PCR PRODUCTS

The amplified cDNA fragments were cloned in to the pGEM®-T cloning vector (Promega; *Appendix B* for vector map) according to the manufacturer's protocol (Promega, 1997). The appropriate amount of DNA was used in each case, based on the following calculation:

$$\frac{\text{50 ng vector x size (kb) of insert}}{\text{3.0 kb vector}} \times \begin{array}{c}\text{insert:vector}\\ \text{molar ratio}\\ \text{(usually 3:1)}\end{array} = \text{ng of insert}$$

Cloned inserts were transformed in to DH5α competent cells (*Section 2.2.17*).

2.2.18 TRANSFORMATION OF CLONED PRODUCTS

Ligated PCR products were transformed into DH5α competent cells (Gibco-BRL®) according to the manufacturer's protocol.

After incubation of the cells in 1.0 ml of LB or SOC media (1 hour at 37°C, 250 rpm) the bacteria were concentrated by centrifugation at 1,020 x *g* for 10 minutes. The cells were resuspended in 200 μl of the supernatant and spread on an LB-agar/ampicillin [100 μg/ml] and incubated overnight at 37°C.

2.2.19 PCR SCREENING OF TRANSFORMATION COLONIES

Cloning success was determined by random selection of colonies from the transformation plate and PCR screening for recombinants. The template was prepared by growing up an inoculant in 2 ml LB medium (containing the appropriate antibiotic) for approximately 4 hours at 37°C, at 250 rpm. A portion (1 ml) of this culture was taken and centrifuged for 2 minutes at 20, 000 x *g*.

The pelleted cells were resuspended in 100 μl of HPLC-grade water and boiled for 5 minutes. Cell debris was pelleted by centrifugation at 20, 000 x *g* for 10 minutes, and 1 μl of the supernatant was used in an analytical PCR mixture, consisting of 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of oligonucleotide primers flanking the cloning site (Appendix B), 0.2 U of *Taq* Polymerase (Promega) and buffered in 1 x *Taq* PCR buffer (Promega, *Table 2.2*). PCR amplification took place as described in *Section 2.2.16*. Positive clones were identified from the PCR amplifications by agarose gel electrophoresis (*Section 2.2.11*).

2.2.20 PREPARATION OF PLASMID DNA: WIZARD® PLUS SV MINIPREP KIT (PROMEGA)

Plasmid DNA was isolated form overnight cultures (grown in sterile 25 ml universal tubes [Greiner Bio-one]), using the Wizard® *Plus* SV MiniPrep purification system (Promega), according to the manufacturer's instructions (Promega, 1999). DNA was eluted in an appropriate volume (50-100 μl) of HPLC-grade water (Sigma-Aldrich).

2.2.21 REMOVAL OF UNINCORPORATED PCR COMPONENTS

PCR products (*Sections 2.2.16 & 2.2.19*) were cleaned for subsequent DNA sequencing (*Section 2.2.22*) using a Silent Screen 96-well plate with 3.0 μm 66 Loprodyne membranes (Griener Bio-one). An appropriate volume (150 μl) of Sepharcyl-300-HR (Sigma-Aldrich) was loaded in to each well of the Silent-Screen plate and the storage buffer (*Table 2.2)* was removed by centrifugation (3,000 x *g* for 2 minutes). The sepharcyl-300-HR was then washed with 150 μl of Wash Solution (*Table 2.2*) and centrifuged at 3,000 x *g* for 2 minutes.

A 96-well PCR plate (ABgene) was placed under the Silent-Screen plate making sure that the wells of both plates were correctly aligned. A volume (15 μl) of each PCR reaction was placed in the appropriate well of the Silent-Screen plate. PCR products were eluted by centrifugation at 3, 000 x *g* for 3 minutes.

2.2.22 SEQUENCING OF PCR PRODUCTS – ABI BIGDYE™ V2.0 TERMINATOR CYCLE SEQUENCING

PCR products or plasmid DNA was sequenced using the ABI BigDye™ V3.0 terminator sequencing kit (PE Biosystems). A final concentration of 2.4 pmoles of the

relevant PCR oligonucleotide primer was used in a PCR reaction mix of 15 μl. A concentration of 120 ng of PCR product DNA and 230 ng of plasmid DNA were used in each reaction along with a quarter of the recommended concentration of BigDye™ (*Table 2.2*).

Thermal cycling took place using the scheme detailed below on the MWG-Biotech Primus Thermal Cycler:

	95°C	for	3 minutes (chain melting)
x 25	95°C	for	15 seconds (chain melting)
	50°C	for	10 seconds (primer annealing)
	60°C	for	4 minutes (chain extension

Excess BigDye™ was removed by the addition of four times the volume of the reaction of 95% ethanol and 10% the volume of the PCR reaction of 0.25 M EDTA. The DNA was pelleted by centrifugation at 5, 000 x *g* for 30 minutes. The supernatant was removed and the pellet washed in 70% ethanol. The samples were centrifuged for a further 15 minutes at 5, 000 x *g*.

Samples were loaded on to the ABI PRISM™ 3,100 Genetic Analyzer in glass capillaries and sequence was read.

2.2.23 SEQUENCE IDENTIFICATION – DATABASE SEARCHES

DNA sequences were compared to all database entries, searching for protein similarities (in all 6 reading frames) (BlastX searches), and nucleotide similarities

(BlastN searches), using the Blast search analysis programme (Altschul *et al.*, 1997) incorporated in to the EBI on-line software suite (http://www.ebi.ac.uk/).

2.2.24 DNA SEQUENCE ANALYSIS

The computer programme BioEdit sequence alignment editor (Version 4.8.10) was used to analyse a DNA sequence using many functions, such as, open reading frame analysis, translation, and multiple alignment generation and subsequent consensus sequence generation. Multiple alignments were performed using the CLUSTAL W method (Thompson *et al.*, 1994).

The DSgene sequence alignment package was also used in the identification of specific DNA sequences using similar methods as described above.

2.2.25 LIGHT-CYCLER™ REAL-TIME QUANTITATIVE PCR AMPLIFICATIONS

Light-Cycler™ methods are PCR based technologies used for the quantification of gene expression. RNA transcripts are converted in to cDNA (*Section 2.2.15*) and gene specific oligonucleotide primer pairs are used to amplify the gene (or fragment of the gene) of interest. Fluorescent molecules that bind to DNA are used to measure the amount of cDNA present in the reaction at the end of each PCR cycle. Sybr™ Green I is a fluorescent molecule that binds to dsDNA. Sybr™ Green molecules only fluoresce when bound to dsDNA.

a) Template Isolation

Oligonucleotide primer pairs were designed using the Oligo©1991 design tool (*Section 2.2.14*) to amplify a fragment (between 200-500 bp in length) of the gene to be

quantified. Preferentially oligonucleotide primers were designed with a T_m of approximately 60°C and a G/C content of >50%. These oligonucleotides were then used to amplify the target gene from cDNA (*Section 2.2.16*). The amplified PCR product was cloned in to the pGEM®-T vector (Promega; *Section 2.2.20*). After clone sequence verification (*Sections 2.2.22, 2.2.23 & 2.2.24*) stock plasmid preparations were produced (*Section 2.2.20*).

The plasmid preparations were quantified (*Section 2.2.13*) and calibration standards were produced, in a range of 1 ng/μl to 10 fg/μl, by diluting each purified plasmid.

b) Optimisation of Quantitative PCR Amplifications

Amplifications were optimised for $MgCl_2$ concentration, Sybr™ Green I concentration, and variable annealing temperatures and extension times. Amplification reactions (5 μl total volume) consisted of 0.5 μl template cDNA (100 fg/μl of plasmid cloned DNA preparation), 1.25 μM of each oligonucleotide primer, 0.12 mM dNTPs, 1:500 dilution of Sybr™ Green I (optimisied concentration), and 1 U of *Taq* polymerase (Promega), buffered with 1 x light-cycler™ buffer supplemented with 1-4 mM $MgCl_2$ (*Table 2.2*).

The PCR reactions were performed in micro-volume capillaries and subjected to thermal cycling based upon the following parameters:

	95°C	for	10 seconds (chain melting)	at a Rate of 20°C/second
x 40	95°c	for	1second (chain melting)	at a Rate of 20°C/second
	55°C	for	3 seconds (primer annealing)	at a Rate of 20°C/second
	72°C	for	10 seconds (chain extension)	at a Rate of 5°C/second
	Single Fluorescence Measurement			
	95°C	for	1 second (melting temperature)	at a Rate of 0.1°C/second
	with continuous fluorescence measurement			

The primer annealing temperature was dependent upon the T_m of the oligonucleotide primer pairs used in the reaction, and the extension time was dependent upon the length of the fragment being amplified. The duration of the chain extension time is based upon the estimation that the light-cycler™ can amplify at a rate of 1 kb per 20 seconds.

Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from Sybr™ green molecules intercalated with ds-DNA. The chain melting analysis allowed the specificity of end products to be assessed. Optimisation was complete when exponential amplification of the target gene was obtained, with minimal primer-dimer formation.

c) Quantitative PCR Amplifications Analysis

Calibration standards (ranging in concentration from 1 ng/μl to 10 fg/μl) (*Section 2.2.25a*) were used in the quantification process, in duplicate. A standard curve was obtained and a regression line generated by plotting the cycle number required to attain a threshold (Ct value) fluorescence pertaining to the logarithmic portion of the amplification against $\log_{10}$ [molecules of target gene].

cDNA was generated from approximately 500 ng of control and test RNA samples (*Section 2.2.15*). Quantitative PCR was performed on 0.5 μl of a 1:10 dilution of each cDNA sample as template, and the optimised reaction mix. All samples quantified were analysed in triplicate, to enable statistical analysis (*Section 2.2.7*) to be performed. The number of target molecules present in each reverse-transcribed cDNA sample was quantified by computer generated regression analysis (using the Light-Cycler™ software), extrapolating each 'unknown' sample over the standard range. The resulting absolute values were normalised for differences in reverse-transcription efficiencies by using the level of a control genes expression, which is widely accepted as being invariant.

2.2.26 QUANTITATIVE PCR USING DUAL-LABELLED OLIGONUCLEOTIDE PROBES

Fluorescently labelled oligonucleotide probes (approximately 30 bp in length) can be used to quantitatively measure the number of cDNA copies there are of a particular gene in a PCR reaction. The use of sequence-specific oligonucleotide probes allows for a more sensitive assay to be developed than is possible when using a fluorescent molecule capable of binding to dsDNA (see *Section 2.2.25*).

Dual-labelled oligonucleotide probes were labelled at the 5'-end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3'-end with the universal quencher 6-carboxy-tetramethyl-rhodamine (TAMRA). The spatial proximity of the reporter dye to the universal quencher quenches the fluorescent emission. Oligonucleotide primer pairs are intended to amplify a region of approximately 150 bp, with the probe designed to hybridise within the target sequence. During amplification the hybridised probe is digested by the 5' → 3'-exonuclease activity of the *Taq* polymerase, this

releases the reporter dye and increases the spatial distance between reporter and quencher. This results in a relative increase in fluorescent signal. In all qPCR experiments described in this thesis using dual-labelled oligonucleotide probes the ABI Prism® 7700 sequence detection system was used. A laser was used to excite the fluorescent dyes and detection of emitted light was measured.

a) Oligonucleotide Primers and Dual-labelled Probe Design

The oligonucleotide primers and dual-labelled probes used in all experiments were designed using the Primer Express™ software package (PE Applied Biosystems) as described in *Section 2.2.14*. Primers were synthesised by MWG Biotech (Erbesberg, Germany) and dual-labelled probes were purchased from QIAGEN Operon (Cologne, Germany).

b) Template Preparation

i) Standard Dilution Series for Calibration

The 150 bp fragment of the gene of interest was amplified, purified, cloned and sequenced as described in *Sections 2.2.16 – 22*. Stock plasmid preparations were made of the particular gene of interest (*Section 2.2.20*). These preparations were quantified as described in *Section 2.2.13*. A standard dilution series of calibration standards were prepared by diluting each purified plasmid to a known concentration, in the range of 0.5 ng/µl to 1 fg/µl.

ii) Sample cDNA Preparation

Total RNA was used isolated from both control and exposed adult earthworms as described in *Section 2.2.8*. cDNA was generated from 2.0 µg of total RNA (*Section*

2.2.15). Each reverse-transcription reaction was then diluted 1:20 with HPLC-grade water prior to quantitative analysis.

c) Optimisation of Dual-labelled Probe Quantitative PCR Amplifications

As with any amplification procedure optimisation was required. Two variables were optimised for qPCR on the ABI Prism® 7700 sequence detection system;

(i) Primer concentrations, and

(ii) Probe concentration.

Amplification reactions prior to optimisation consisted of; 5.0 µl of template DNA (plasmid cloned DNA preparations of 0.1 ng/µl concentration), 0.9 µM concentration of each oligonucleotide primer, 200 nM dual-labelled probe, 0.2 mM dATP, dCTP, dGTP and dTTP and 1.0 µl of a 1:10 dilution of homemade *Taq* polymerase. The reactions were buffered with a 1 x qPCR Buffer (see *Table 2.2*). All qPCR reactions were performed in 25 µl volumes in 96-well PCR plates with optical lids (ABgene), and subjected to the following thermal cycling parameters using the ABI Prism® 7700 Sequence Detection System:

50°C	for	10 minutes	
95°C	for	15 seconds	x 40
60°C	for	1 minute	

Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from the dual-labelled probes. Ct values were then calculated from these fluorescence measurements.

i) Oligonucleotide Primer Concentrations

Optimisation reactions were performed as described above, but three oligonucleotide primer concentrations (final concentration in a 25 μl reaction) were tested; 50 nM, 300 nM and 900 nM. Therefore, nine different combinations of primer concentration were tested (*Table 2.3*). The reaction with the lowest Ct value and best amplification profile was taken as the optimal reaction conditions.

Table 2.3: Final primer concentrations used during optimisation of the qPCR reactions.

Final Primer Conc. (nM)	Primer Type	Final Primer Conc. (nM)	Primer Type	Final Primer Conc. (nM)	Primer Type
50	Forward	50	Forward	50	Forward
50	Reverse	300	Reverse	900	Reverse
300	Forward	300	Forward	300	Forward
50	Reverse	300	Reverse	900	Reverse
900	Forward	900	Forward	900	Forward
50	Reverse	300	Reverse	900	Reverse

ii) Dual-labelled Oligonucleotide Probe Concentration

Eight different probe concentrations were used in the probe optimisation reactions (*Table 2.4*), performed as described above in *Section 2.2.25c*, using the optimised primer concentrations. The reaction with the lowest Ct value and best amplification profile was taken as the optimal reaction conditions.

Table 2.4: Final dual-labelled probe concentrations used during optimisation of the qPCR reactions.

	Final Probe Concentration (nM)
1	25
2	50
3	75
4	100
5	125
6	150
7	175
8	200

d) Dual-labelled Probe qPCR Amplifications

Calibration standards and samples were quantified in parallel. The amplification reactions were performed using the optimised conditions specific to the gene under analysis (*Section 2.2.25c*).

A series of six standard dilutions (diluted plasmid DNA prepared as described in *Section 2.2.25bi*) were analysed in the amplification in duplicate. A standard curve was obtained and used to produce a regression line, by plotting the cycle number required to attain a threshold fluorescence pertaining to the mid logarithmic portion of the amplification against $\log_{10}$ [molecules of target gene].

The diluted cDNA samples (prepared as stated in *Section 2.2.25bii*) were used in the quantitative PCR process (5.0 µl of diluted [1:20] cDNA per 25 µl qPCR reaction volume). Each sample was only analysed a single time. Using the regression analysis described above, each sample was extrapolated and target gene levels estimated.

e) Final Primer/Probe Concentrations used in Experiment

For all quantitative PCR reactions the following primer/probe concentrations were used:

Table 2.5: Final concentrations of oligonucleotide primers and probes used in quantitative PCR.

Gene Nomenclature	Forward Primer Final Concentration (μM)	Reverse Primer Final Concentration (μM)	Dual-Labelled Probe Final Concentration (μM)
β-Actin	0.900	0.900	0.125
Annetocin	0.900	0.900	0.200

2.2.27 PRODUCTION OF MICROARRAY

Purified PCR products (*Section 2.2.21*) were diluted to 50% in DMSO and transferred to 384-well plates. The location of each gene fragment within the 384-well plate was recorded.

These PCR products were then spotted in a known format on to CMT-GAPS coated slides (Corning), using the Flexys™ High Density Arrayer (Genomic Solutions, Cambridge, UK). This was performed using the 8 x 4 solid pin head. Cy3™-labelled M13-forward oligonucleotide primer (10 pmol μl^{-1}) was also printed in the corners of each sub-array.

PCR products were immobilised on the glass slides by UV cross linking in the UV Stratalinker™ 2400 bench top transilluminator (Stratagene Ltd.). The glass slides

were then placed in a lightproof slide container and baked in an oven at 80 °C for two hours.

If the microarrays were not to be used immediately they were stored desiccated at room temperature and protected from light.

2.2.28 Prevention of Non-Specific Binding of Target DNA to Active Amine Groups

The Gamma Amino Propylsilane coating of the Corning glass slides contained reactive amine groups. If these groups were not blocked prior to DNA hybridisation non-specific DNA binding would take place. Therefore, each microarray slide was blocked by placing the slides in a 50 ml Coplin jar containing blocking solution (1% Bovine Serum Albumin [Fraction V] [Sigma-Aldrich, Poole, UK], 5 x SSC and 0.1% SDS) prewarmed to 42 °C – slides were incubated in a water bath (42 °C) for 45 minutes. The microarray slides were then dipped briefly (two seconds per dip) five times in sterile, filtered water. This was followed by a single five-second dip in room temperature isopropanol. The blocked slide was then placed in a lightproof slide box to air dry for approximately ten minutes. It was important to use the microarray slide no-longer than one hour after the blocking process, as hybridisation efficiency decreased rapidly if left for longer (Hedge *et al.*, 2000).

2.2.29 Production of Fluorescently Labelled Target cDNA Using the Cyscribe™ Kit

Fluorescently labelled cDNA was produced from total RNA utilising the Cyscribe™ labelling kit (Amersham Int. PLC., Little Chalfont, Bucks., UK). Total RNA (1 μg)

was combined with oligo-dT (1 μl) and random hexamers (1 μl) in a sterile amber microcentrifuge tube. Sterile water was added to a final volume of 11 μl and incubated at 70 °C for five minutes, then at room temperature to allow for optimal oligonucleotide primer annealing. The mixture was then placed on ice and 1 x Cyscribe™ buffer, 10 mM DTT, dUTP mix (1 μl), 1.25 mM Cy3 or Cy5 labelled dUTP and CyScript™ reverse transcriptase (1 μl) were added and mixed gently. The reaction was incubated a 42 °C for 1.5 hours.

After reverse transcription was complete any remaining RNA was degraded by the addition of 2.5 M NaOH (2 μl) and incubation at 37 °C for 15 minutes, followed by the addition of 2 M HEPES (10 μl). Degraded RNA fragments and unincorporated Cy3™/Cy5™ were removed using the AutoSeq™ columns (Amersham Pharmacia Biotech., Bucks, UK) as described in the manufacturer's protocol. Purified, labelled targets were used immediately.

The efficiency of fluor incorporation was assessed by agarose gel electrophoresis on a "slide gel" made in a plastic mould specially designed for the purpose by Mr John Harris, Cardiff School of Biosciences, Cardiff University, UK. A volume of 1 ml of 1 x TAE (no ethidium bromide) was poured in to the slide gel mould and allowed to cool and set. The sample to be analysed (1 μl) was mixed with an equal volume of 50% glycerol and loaded on to the agarose gel. DNA markers were also loaded as per *Section 2.2.11*. Electrophoresis took place at 90 V for approximately 15 minutes in a Pharmacia GNA-100 tank in 1 x TAE (with EtBr). Nucleic acid bands were visualised following scanning at the appropriate wavelength using the GeneTac™ LSIV scanner.

2.2.30 TARGET HYBRIDISATION

Cy3™ and Cy5™ labelled targets were combined in a sterile amber microcentrifuge tube and mixed. The labelled cDNAs were dried down by centrifugation in a GyroVac at 25 °C for one hour. The DNA pellet was resuspended in 6 μl of sterile water and denatured at 95 °C for two minutes before placing on ice for thirty seconds. Poly-$dA_{(30)}$ (1 mg ml^{-1}) (1.5 μl) was added to the cDNA and incubated at 75 °C for 45 minutes. Prior to hybridisation, a final volume of 110 μl was achieved through the addition of 1 x hybridisation buffer (50% formamide, 10% SSC [3M NaCl, 300 mM Sodium citrate pH 7.0] and 0.2% SDS). The labelled target was added to the microarray slide by pipetting and the slide was covered with a glass cover slip. Hybridisation took place in a static incubator at 42 °C for 18 hours.

2.2.31 SIGNAL DETECTION

Microarray slides with cDNA target hybridised were stored in the dark until they were scanned (the fluorescence would diminish in ambient light). When scanning, the Cy5™ channel was scanned first followed by the Cy3™ channel, since this fluor is slightly more photosensitive. Scanning was done in the GeneTac™ LSIV scanner (Genomic Solutions, Cambridge, UK) at the relevant wavelength according to the scanner manufacturer's recommendations.

2.2.31 IMAGENE (DISCOVERY INC.) MICROARRAY ANALYSIS

Fluorescently labelled DNA was scanned as above and the scanned image was analysed using the ImaGene (Discovery Inc.) microarray analysis package. This computer software allowed for the identification of differentially expressed genes on

the basis of the fluorescent intensity of each spot (in each of the two channels used and the two channels together).

CHAPTER 3

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED REPRODUCTIVE cDNAS FROM *LUMBRICUS RUBELLUS*: PCR-SELECT™ cDNA SUBTRACTION

3.1 INTRODUCTION

The epigeic earthworm species, *Lumbricus rubellus* has been used fairly widely for both toxicity testing and molecular genetic studies for some years (Spurgeon *et al.*, 1999; Stürzenbaum, 1997). Stürzenbaum *et al.* (2001) measured metallothionein (MT) (a metal detoxification enzyme) levels in *L. rubellus* after exposure to heavy metals (particularly cadmium). MT was selected as a biomarker in these studies as it binds heavy metals and its quantification could be related to the presence of the toxic metal ion cadmium within the earthworm's environment.

Biomarkers have been widely used in ecotoxicology (*Section 1.4*), but some reservations still exist about their use (Spurgeon *et al.*, 2004). One of the most important factors that must be addressed when using biomarkers in ecotoxicology is whether the particular mechanism/endpoint is ecologically relevant. Moriarty (1983) emphasised the need to measure sublethal endpoints (e.g. growth, reproduction), giving rise to the notion that biomarkers should be statistically or, preferably causatively/mechanistically linked to one or more of these endpoints. If biomarkers are to become fully integrated and accepted into ecotoxicological work they must meet these criteria.

Reproduction is fundamental to the survival of any population; hence any toxicant that has the ability to interfere with this process is of concern. The use of biomarkers gives ecotoxicologists the ability to look for 'early-warning' indications of ecological-scale perturbations in, for example, reproductive output of the given organism. Biomarkers can only act as early-warning indicators if the response is understood at the mechanistic level. Hence, this chapter deals with the screening of an earthworm reproductive transcriptome for potentially suitable molecular genetic biomarkers.

3.2 APPROACH AND METHOD

A cDNA subtractive library was constructed using the PCR-Select™ cDNA-Subtraction Kit (Clontech) according to the manufacturer's protocol (Clontech, 1997). All reagents used were those found in the kit, with the exception of phenol, chloroform, *iso*amyl alcohol and ethanol (see *Section 2.1.1*). Total RNA and subsequently, mRNA were isolated as described in *Sections 2.2.8* and *3.2.1*. Two pools of mRNA were isolated and designated "tester" and "driver".

3.2.1 MESSENGER RNA PURIFICATION FROM TOTAL RNA – OLIGO (DT) MAGNETIC BEADS

Isolation of messenger RNA (mRNA) from total RNA was performed using oligo (dT) linked magnetic beads. A ratio of 2 μl of oligo (dT) beads to every 1 μg of total RNA used. An appropriate volume (150-400 μl) of beads were placed in a clean, sterile eppendorf and placed in a magnetic stand (Promega). The solution was allowed to strand until cleared (approximately 30 seconds) before the supernatant was removed. The beads were washed with 250 μl of 2 x Binding Buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA), placed back in the magnetic stand and allowed to clear.

The supernatant was removed and beads resuspended in a further 250 μl of 2 x Binding Buffer.

Before adding the total RNA sample to the pre-washed magnetic beads, secondary RNA structure was removed by heating the samples at 65°C for two minutes, then placing directly on ice. The treated RNA samples were then added to the bead/2 x Binding Buffer solution and mixed thoroughly for five minutes. The samples were then placed back on the magnetic stand and allowed to clear for 30 seconds. The cleared supernatant was removed and the beads were washed with 400 μl of Washing Solution (10 mM Tris-HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA). After thorough mixing the samples were placed back on the magnetic stand and the cleared supernatant was removed. Washing was repeated twice with 300 μl of Washing Solution and once with 150 μl.

Washed mRNA samples were eluted from the oligo (dT) magnetic beads by adding an appropriate volume (10-100 μl) of Elution Solution (2 mM EDTA, pH 8.0) and heating at 75°C for two minutes. Samples were immediately placed back on the magnetic stand, allowed to clear and the supernatant (containing the eluted mRNA) was removed.

Sample integrity was analysed by agarose gel electrophoresis (*Section 2.2.11*), quantified as described in *Section 2.2.13* and stored at -70°C until needed.

3.2.2 PCR-SELECT™ cDNA SUBTRACTION KIT

a) First Strand cDNA Synthesis

Both the "tester" and "driver" poly (A^+) mRNA pools (2μg of each) were first used to synthesize single-stranded cDNA. This was achieved through the use of an oligo (dT) cDNA synthesis primer (2 μM). The reaction mixture was made up to 5.0 μl with the addition of sterile water.

A two minute incubation at 70°C and cooling on ice for two minutes was followed by the addition of 20 units of AMV-RT and a 200 μM dNTP mix. This was buffered with a 5 x First Strand synthesis buffer (Clontech, *Section 2.1; Table 2.2*) and made up to a final volume of 10 μl with sterile water. This was gently mixed by vortex and briefly centrifuged, then incubated at 42°C for 1.5 hours.

b) Second Strand cDNA Synthesis

The completed first-strand synthesis reaction was placed on ice and double-stranded cDNA was synthesized through the addition to this of 200 μM dNTPs, 24 units of DNA polymerase I, 0.8 units RNase H and 4.8 units *Escherichia coli* DNA ligase. This reaction was buffered with a 5 x Second Strand synthesis buffer (Clontech, *Section 2.1; Table 2.2*) to a final volume of 80 μl. The contains of each reaction vial were gently mixed by vortex, briefly centrifuged and incubated at 16°C for two hours. At this point 6 units of T4 DNA polymerase was added to each reaction mixture and the contents well mixed. They were then placed at 16°C for a further 30 minutes. The second-strand synthesis reaction was terminated by the addition of a 20 x EDTA/Glycogen mix (Clontech, *Section 2.1; Table 2.2*). This was followed by a phenol-chloroform extraction (*Section 2.2.9*) and ethanol precipitation (*Section*

2.2.10). The air-dried cDNA pellet was dissolved in 50 µl of HPLC-pure water. An aliquot of 6 µl was removed and stored for later analysis by agarose gel electrophoresis (*Section 2.2.11*).

c) *Rsa*I Digestion

The ds cDNAs was digested with 15 units of *Rsa*I, buffered with 1 x *Rsa*I restriction buffer (Clontech, *Section 2.1; Table 2.2*). The restriction digest mixture was incubated at 37°C for 1.5 hours, at which point a volume of 5.0 µl was removed for analysis by agarose gel electrophoresis (*Section 2.2.11*). The reaction was terminated by the addition of a 20 x EDTA/glycogen mix followed by phenol-chloroform extraction (*Section 2.2.9*) and ethanol precipitation (*Section 2.2.10*). The air-dried cDNA pellet was dissolved in 5.5 µl of HPLC-pure water.

d) Adapter Ligation

The "tester" cDNA was divided into two equal volumes and different adaptors (see *Appendix C*) ligated. This created two sub-populations, both consisting of identical cDNA fragments, but different adaptors.

Adaptor ligation was performed using 2 µl of diluted "tester" cDNA (1 µl of *Rsa*I-digested "tester" cDNA and 5 µl of sterile water) combined with 400 units of T4 DNA Ligase, 2 µM of adaptor (i.e. either adaptor 1 or adaptor 2R), buffered with 1 x Ligation Buffer (Clontech, *Section 2.1; Table 2.2*) and made up to a final volume of 10 µl with sterile water. Prior to incubation 2 µl of each of the reactions was removed and mixed. This was designated the "unsubtracted tester control".

After incubation of all reactions overnight at 16°C the ligation reaction was halted by the addition of a 20 x EDTA/glycogen mix and heating to 72°C for five minutes. The unsubtracted tester control was diluted 1:1,000 with HPLC-pure water and stored at -20°C.

e) First Hybridisation

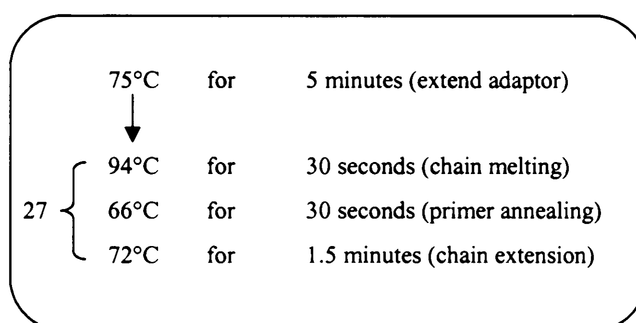
Two hybridisation reactions were set up, one containing those cDNAs with adaptor 1 ligated and one containing those with adaptor 2R ligated. Each hybridisation reaction consisted of 1.5 μl of the *Rsa*I digested driver cDNA and 1.5 μl of the adaptor ligated tester cDNA, buffered with a 1 x Hybridisation buffer (Clontech, *Section 2.1; Table 2.2*). Hybridisation was performed with 1.5 hours incubation at 98°C, followed by incubation at 68°C for 8 hours.

f) Second Hybridisation

The two samples from the first hybridisation reaction were mixed together with freshly denatured *Rsa*I digested driver cDNA. A 1 μl volume of driver cDNA was mixed with 1 x Hybridisation buffer (Clontech, *Section 2.1; Table 2.2*) and made up to 4 μl with sterile water. This mixture was heated to 98°C for 1.5 hours. The two samples from the first hybridisation were then mixed with this denatured driver cDNA. After overnight incubation at 68°C the reaction was diluted with 200 μl of dilution buffer (Clontech, *Section 2.1; Table 2.2*) and heated to 68°C for seven minutes before being stored at -20°C.

g) Primary PCR Amplification

Differentially expressed cDNAs were selectively amplified as shown in *Figure 3.4*. A 1 µl volume of diluted cDNA (prepared as in *Section 3.2.2 a & b*) was taken and combined with 200 µM dNTP mix (Clontech), 0.4 µM of Primer 1 (*Appendix C*), 1 unit of Advantage ® Klen *Taq* DNA polymerase (Clontech) and buffered with 1 x Advantage ® Klen *Taq* PCR reaction buffer (Clontech, *Section 2.1; Table 2.2*). After making up to a final volume of 25 µl with sterile water, the reaction mixture was gently vortexed and briefly centrifuged. PCR was performed in a three-step process given below:



PCR reactions were performed in the Perkin-Elmer DNA Thermal Cycler 480 and subsequently analysed by agarose gel electrophoresis (*Section 2.2.11*).

h) Secondary Nested PCR Amplification

A 1:10 dilution of the Primary PCR reaction was made and used in a nested secondary PCR amplification. A volume of 1 µl of the diluted primary PCR amplification was combined with 200 µM of dNTP mix (Clontech), 2 µM of each nested PCR primer (*Appendix C*), 1 unit of Advantage ® Klen *Taq* DNA polymerase (Clontech) and buffered with 1 x PCR reaction buffer (Clontech, *Section 2.1; Table 2.2*). After making up to a final volume of 25 µl thermal cycling was carried out as follows:

12	94°C	for	30 seconds (chain melting)
	68°C	for	30 seconds (primer annealing)
	72°C	for	1.5 minutes (chain extension)

PCR reactions were performed on the Perkin-Elmer DNA Thermal Cycler 480. Reactions were analysed by agarose gel electrophoresis (*Section 2.2.11*) and subsequently stored at -20°C.

Amplified differentially expressed cDNAs were randomly cloned into the pGEM-T® vector (Promega, *Section 2.2.17*) and transformed into DH5α competent cells (Invitrogen, *Section 2.2.18*).

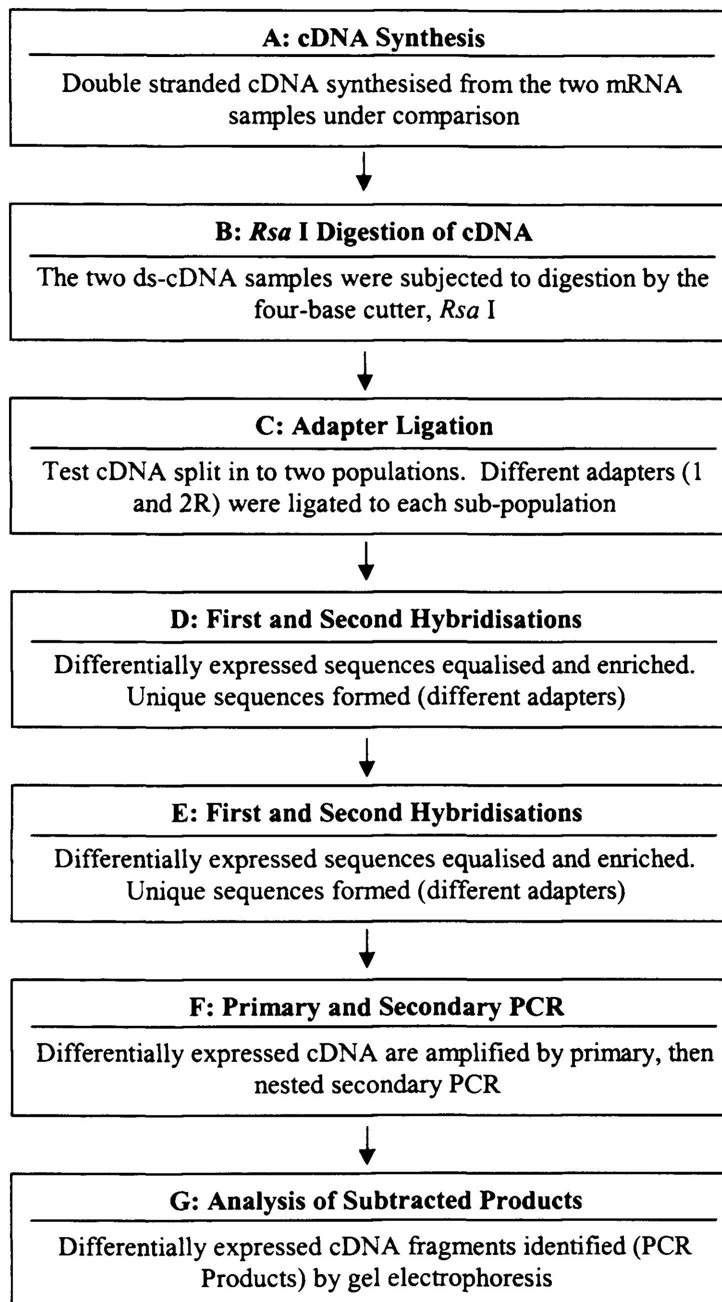


Figure 3.1: Schematic diagram showing the steps involved in the creation of the *Lumbricus rubellus* reproductive specific cDNA subtractive library.

Repro cDNA + Adaptor 1

Tail cDNA

Repro cDNA + Adaptor 2R

First Hybridization

α
β
χ
δ
ε

Second Hybridization

Mixing of samples with additional control cDNA

Formation of α, β, χ, δ, and ε type molecules, and ϕ type molecules:

ϕ

Fill in ends (creating complimentary priming sites)

α
β
χ
δ
ε
ϕ

Amplification by PCR
Priming sites situated in adaptors

α, β and χ ⟹ No amplification (missing primer sites)

ε ⟹ No amplification (formation of pan-like structures)

δ ⟹ Linear amplification

ϕ ⟹ **Exponential amplification** and

Figure 3.2: The main features of the PCR-Select™ cDNA Subtraction technique (Taken from Richards, 2001). Repro = Reproductive Tissue.

3.3 RESULTS

(A) PCR-SELECT™ cDNA SUBTRACTION

3.3.1 EARTHWORM SELECTION AND QUALITY CONTROL OF RNA

A fully clitellate adult *L. rubellus* was selected from a healthy population (Monks Wood, Cambs, UK) and depurated, as described in *Section 2.2.1.* The reproductive (segment numbers 10 – 35) and tail sections were dissected and placed directly into TRI-Reagent™ (Sigma-Aldrich) for subsequent total RNA and mRNA isolation (*Sections 2.2.8* and *3.2.1*). Quantification of total RNA by absorption spectrophotometry (*Section 2.2.13*) showed that 100 μg of RNA had been isolated from both the 'reproductive' and tail tissues, and was of high purity. As mRNA makes up approximately 2 % of total RNA, it was assumed that 2 μg of mRNA was isolated using the oligo (dT) magnetic bead mRNA isolation procedure (*Section 3.2.1*). Both RNA pools were qualitatively assessed by agarose gel electrophoresis (*Section 2.2.11; Figure 3.3*). The quality control assessments performed on both RNA pools confirmed that good quality mRNA (despite some ribosomal RNA contamination) had been isolated and was suitable for the construction of the cDNA subtractive library.

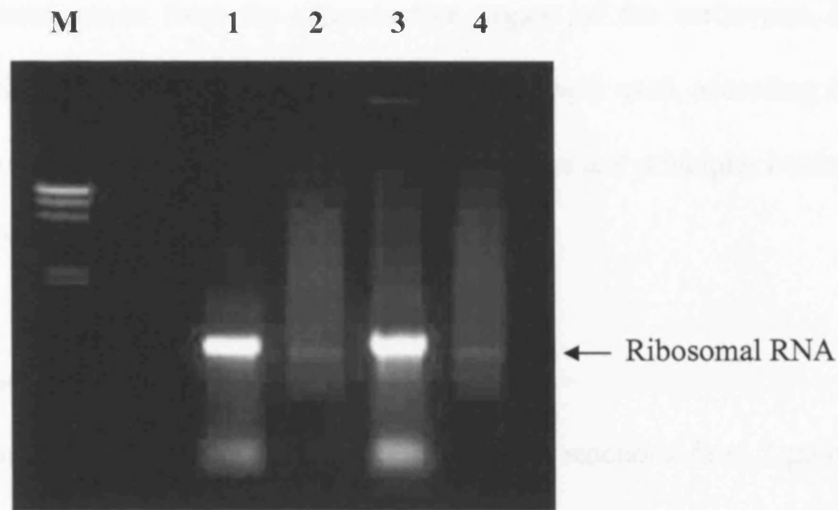


Figure 3.3: Total and mRNA Isolated from the Earthworm, *Lumbricus rubellus*. Total RNA was isolated from those body areas containing the reproductive organs (Lane 1) and from the tail area of the earthworm (Lane 3), as described in *Sections 2.2.8*. Subsequently, mRNA was isolated from these total RNA samples and used in reverse transcription reactions to yield cDNA for the production of the cDNA Subtractive library (Lane 2: mRNA from reproductive body area. Lane 4: mRNA isolated from the tail area). All RNA samples were subjected to agarose gel electrophoresis (1.0 % agarose gel [w/v]) containing ethidium bromide (0.4 μg/ml) and visualised under U.V. light.

3.3.2 PCR-SELECT™ SUBTRACTIVE LIBRARY CONSTRUCTION

To generate a subtractive library, that is a library containing cDNA fragments of differentially expressed genes from the reproductive organs of the earthworm *L. rubellus*, the PCR-Select™ cDNA Subtraction kit (Clontech) was used, according to the manufacturer's recommendations (*Section 3.2*). The process and principles behind this technique are illustrated in *Figures 3.1* and *3.2*.

a) First- and Second-Strand cDNA Synthesis

First- and second-strand cDNAs were synthesised in separate reactions from 2 μg of each mRNA population, designated 'control' and 'test' (*Section 3.2.2*). An aliquot of each of the ds-cDNAs were stored for later analysis (data not shown).

b) *Rsa* I Digestion

To create short, blunt-ended ds-cDNA fragments, which are optimal for subtraction and necessary for later adapter ligation, both ds-cDNA samples were digested with the restriction enzyme, *Rsa* I. *Rsa* I is a four-base blunt cutting enzyme and the reaction was performed as described in *Section 3.2.2*.

Analysis of the resulting *Rsa* I digested cDNA fragments were compared to the ds-undigested-cDNA species. A clear shift in the average sizes of the cDNA species was seen, indicating that the digestion of the cDNA with *Rsa* I was a success.

c) Adapter Ligation

Diluted test ds-cDNA was split in to two equal parts, and different adaptors (Adaptor 1 or Adaptor 2R, see *Appendix C*) ligated to the two aliquots, thus creating two sub-

populations with identical cDNA fragments, but differing adaptors (*Section 3.2.2*). Prior to overnight ligation a small aliquot of both sub-populations were taken and mixed. This was designated the 'unsubtracted tester control', where approximately 33% of the cDNA fragments should bear two different adaptors.

d) Primary and Secondary PCR Amplifications

Following first and second hybridisation reactions (*Sections 3.2.2 e & f*) of the 'tester' and 'driver' cDNAs to create the ds-cDNA containing different adaptors. These cDNAs were amplified by a primary and a secondary nested PCR (*Sections 3.2.2*; *Figure 3.4*). As each differentially expressed cDNA molecule contained different adaptors at either end it was possible to selectively amplify these species.

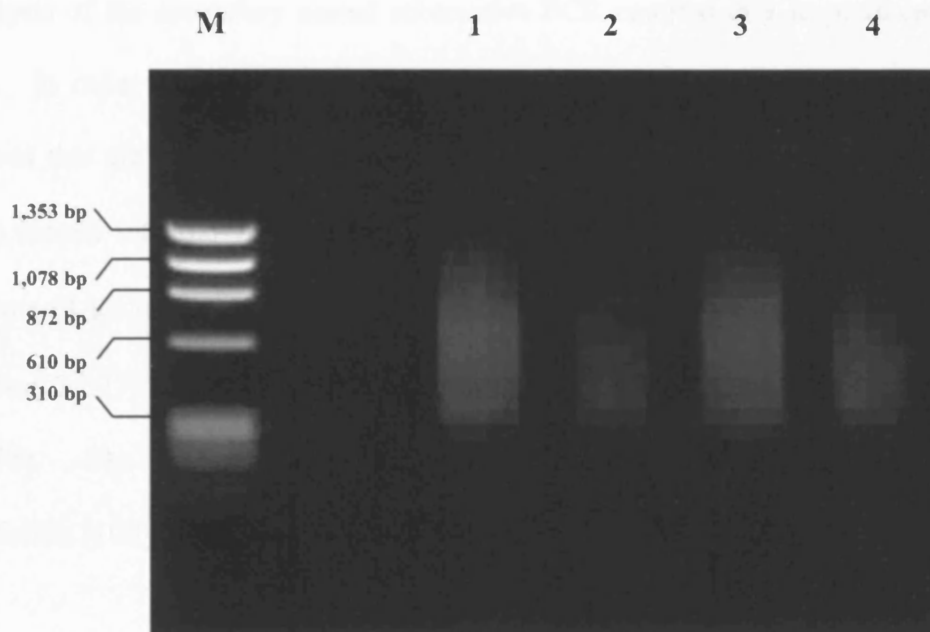


Figure 3.4: Primary and Secondary PCR Products from the *Lumbricus rubellus* reproduction-specific cDNA Subtractive Library. PCR products were subjected to agarose gel (2.0 % w/v, stained with EtBr [0.4 µg/ml]) electrophoresis and visualised under U.V. light. Lane 1: Primary PCR amplification of the 'test' sample (i.e. those containing the subtracted cDNA species). Lane 2: Secondary PCR amplification of the 'test' samples. Lane 3: Primary PCR amplification of the 'control' sample (i.e. the unsubtracted cDNA species). Lane 4: Secondary PCR amplification of the 'control' samples. Lane M: the ϕx174 marker DNA digested with *Hae* III.

3.3.3 IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PRODUCTS

a) Ligation and Transformation of Subtracted PCR Products

Analysis of the secondary nested subtractive PCR resulted in a large smear (*Figure 3.4*). In order to characterise these subtracted cDNA fragments (i.e. those cDNA species that are specific to the reproductive area of the earthworm, *L. rubellus*) they were cloned into the T/A cloning vector, pGEM®-T (Promega, *Section 2.2.17*). A volume (4 μl) of the secondary PCR reaction was ligated with pGEM®-T (Promega, *Section 2.2.17*) and subsequently transformed in to DH5α competent cells (*Section 2.2.18*). The transformed bacteria were grown on LB-agar plates containing ampicillin [100 μg/ml]; this plate was then referred to as the subtractive library plate.

b) PCR Screening of Subtractive Plate and Sequence Results

The resulting numerous colonies on the subtractive library plate were picked into 200 μl of LB-broth (containing ampicillin [100 μg/ml]) contained in the wells of a 96-well micro-titer plate (Greiner Bio-One). A PCR screen was performed on 100 μl from each well as described in *Section 2.2.19.* A volume (2.0 μl) of the resulting supernatant was taken and used in a 50 μl PCR reaction containing M13 Universal oligonucleotide primers (*Appendix C*), which flank the cloning site of the pGEM®-T cloning vector.

The PCR products were analysed on an agarose gel (*Figure 3.5*) and positive clones were identified by size. PCR using M13 Universal primers places approximately 200 bp on to the size of a PCR product, therefore any product >200 bp was considered to contain a cDNA insert.

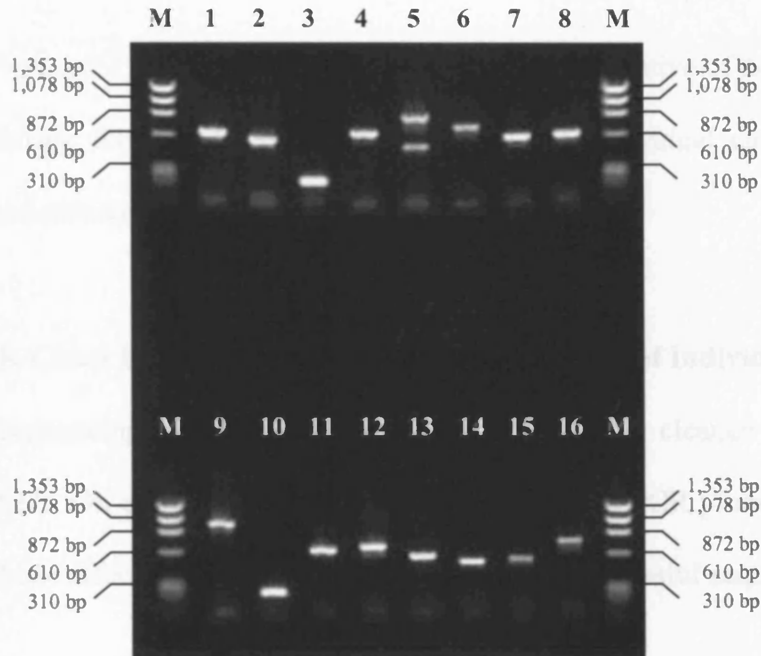


Figure 3.5: PCR analysis of Subtractive Library Cloned Fragments. PCR products were analysed on an agarose gel (2.0 % w/v, stained with EtBr [0.4 μg/ml]) and visualised under U.V. light. Lane M: the ϕx174 marker DNA digested with *Hae* III. Each product was deemed to be that of a cDNA insert if it was >200 bp in length.

Only those pGEM®-T clones that contained DNA inserts (i.e. those PCR products with a DNA size of > 200 bp) were used in the sequencing protocol.

Although *Figure 3.5* shows an illustrative set of clones indicative of the PCR products produced during this section of work. In fact, 384 individual clones were PCR amplified and visualised via electrophoresis.

c) PCR-Clean Up and Subsequent DNA Sequencing of Individual Clones

For DNA sequencing to take place the PCR products were cleaned as described in *Section 2.2.21*. On cleaning some loss in concentration of PCR product was noticed, but this did not affect the amount of DNA needed for successful sequencing (*Section 2.2.22*).

d) Analysis of Sequenced Subtractive Library Clones

All successfully sequenced pGEM®-T subtractive library clones (*Table 3.1*) were analysed using the BioEdit programme (*Section 2.2.24*). Adaptor sequences (*Appendix C*) were located within the sequenced DNA as points of reference. These adaptor sequences were removed before database searches (*Section 2.2.23*). A significant match was determined by the BLAST programme. A 'hit' of over 1.0×10^{-10} was deemed to be significant. A reduced list of successful 'hits' is shown in *Table 3.1*. The first six clones shown in *Table 3.1* were deemed to potentially be involved in reproductive events within the earthworm.

Identification	Probability	Blast Search	Anatomical Location
Tyrosine Phosphatase (*H. sapiens*)	1.0×10^{-09}	X	Whole Body (Yellow)
Sperm Specific Antigen 1 (*M. musculus*)	4.0×10^{-08}	X	Anterior (Red)
Testis Specific Lucine Zipper Protein (*M. musculus*)	4.0×10^{-13}	X	Anterior (Red)
Unknown Protein Derived from Testis (*M. musculus*)	7.0×10^{-34}	X	Anterior (Red)
Unknown Protein Derived from Testis (*M. musculus*)	2.0×10^{-27}	X	Anterior (Red)
Peritrophin-like Protein 1 (Shrimp Ovary) (*Penaeus semisulcatus*)	3.0×10^{-07}	X	Whole Body (Yellow)
H2A Histone (*D. rerio*)	2.0×10^{-25}	X	Whole Body (Yellow)
General Transcription Factor IIB (*R. norvegicus*)	5.0×10^{-24}	X	Whole Body (Yellow)
Myosin Light Chain (*E. fetida*)	1.0×10^{-32}	X	Whole Body (Yellow)
cAMP-Dependent Protein Kinase (*R. norvegicus*)	7.0×10^{-39}	X	Whole Body (Yellow)

Table 3.1: Analysis of sequenced clones from the reproductive specific cDNA subtractive library. DNA sequences were analysed by searching databases with the BlastX search tool. A good 'hit' was considered to be anything below a probability of 1.0×10^{-10}. The anatomical location of expression was determined from the microarray results detailed in *Section 3.3.5*. The first six clones listed in this table were deemed to be potentially involved in reproductive events in the earthworm. The colours indicate the artificial colour of each clone on the microarray; red = reproductive specific (anterior specific), green = tail specific (posterior) and yellow = expressed in both the anterior and posterior of the earthworm.

Sequencing was performed on 223 clones, of which 59% (132 clones) provided good quality sequence (*Section 2.2.22*). This represented the normal success rate for PCR products by the sequencing service using current procedures.

The successfully sequenced clones (132 – four of which were discounted due to PolyA tail sequence) were analysed for function (using BLAST X sequence database searches, see *Section 2.2.23*). Of these, 78 showed no significant identity to genes currently found in sequence databases, whilst 54 clones were identified as showing similarity with known genes. These clones were divided into six functional groups (*Table 3.2*).

Table 3.2: Functional Classification of Successful Sequencing of Subtracted cDNAs

Functional Group	Nº of Clones	Percentage
Ribosomal Proteins	20	37
Cell Signalling/Catalytic Activity	15	28
Transcription Factors	1	2
Reproduction	6	11
Histones	3	5
Other	9	17
Total	**54**	**100**

Of these sequences, 73 passed the necessary criteria for submission (based on sequence quality and length) to GenBank. These sequences with accession numbers are given in *Appendix G*.

(B) VALIDATION OF SUBTRACTIVE LIBRARY ENRICHMENT BY cDNA MICROARRAY ANALYSIS

The PCR-Select™ cDNA Subtraction process allows the enrichment of an RNA pool with those RNA species (i.e. gene transcripts) that are differentially expressed in two RNA pools. In this case RNA species were purified from tail and reproductive tissue from *L. rubellus*. This allowed for the enrichment of reproductive-specific gene transcripts. However, the enrichment process was monitored using DNA microarray technology through the comparison of randomly selected *L. rubellus* gene clones isolated from whole body tissue (data not published) with those gene clones isolated using the PCR-Select™ cDNA Subtraction. Total RNA from the tail and reproductive tissue of the earthworm (used to produce the subtractive library; *see Figure 3.3*) was hybridised to the microarray gene slide containing both sets of clones so that the relative expression of each set of genes could be determined. The theory underlying this approach was that clones from the subtractive library would contain more reproductive-specific genes than those randomly selected clones, hence giving an indication of enrichment in the subtractive library.

3.3.4 PRODUCTION OF cDNA MICROARRAY

For full method used to produce the cDNA microarray see *Section 2.2.27*. PCR products of the clones to be used in the microarray experiment were spotted on CMT-GAPS coated glass slides (Corning) (see *Section 2.2.27*) and immobilised by baking and U.V. cross-linking (*Section 2.2.27*). Those clones randomly selected from an *L. rubellus* whole body control cDNA library were spotted alongside those PCR products produced from the reproductive subtraction library in a known format so that each clone was identifiable. A total of 384 reproductive library clones were printed on

each of five glass slides. On the same slides 338 randomly selected clones (from *L. rubellus* whole body control library) were also spotted along with 46 "blank" PCR products. Blanks were used as hybridisation controls.

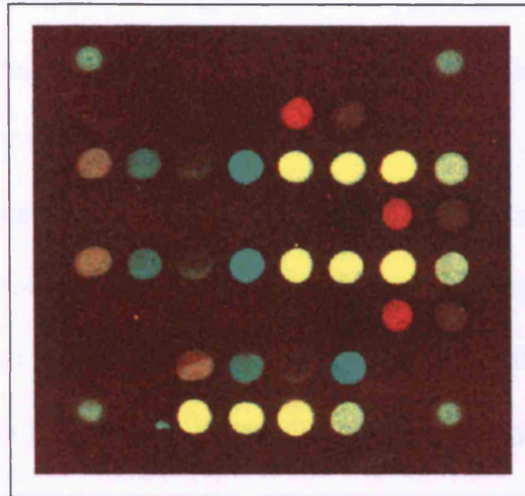
3.3.5 ANALYSIS OF cDNA MICROARRAY

Fluorescently labelled cDNA (those cDNAs originating from tail tissue were labelled with Cy5™, whilst those cDNAs originating from the reproductive tissues area were labelled with Cy3™) was produced following the protocol detailed in *Section 2.2.29* and hybridised to the PCR products immobilised on the glass slides (*Section 2.2.30*). The slides were scanned at the recommended wavelengths (*Section 2.2.31*) and analysed using the array analysis software ImaGene (Discovery Inc. - *Section 2.2.32*). An example of a subarray is given in *Figure 3.6: Panel A*. Those clones whose relative expression was higher in the reproductive tissue areas appear in red, whilst those transcripts elevated in posterior segments appear green and those transcripts found in both tissue types appear yellow.

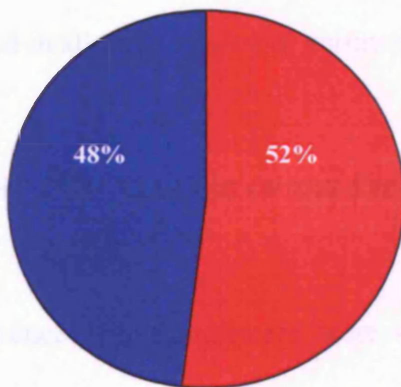
The percentage of clones from each group specific to the reproductive tissue was calculated as a proportion of those clones that generated a significant signal in one of the two channels (for there to be 95% confidence of a signal being real it must exceed the mean plus two standard deviations of that observed for the 46 blank spots of the array). Therefore, each spot giving greater intensity in the Cy3™ channel was determined as representing genes whose expression was relatively higher within the reproductive tissue.

From these manipulations (data not shown) it was found that 52% of those randomly selected gene products from a whole body *L. rubellus* control cDNA library were specific to the reproduction tissue area of the earthworm. Whilst 72% of those clones taken from the reproductive subtraction library were specific to that same tissue area (*Figure 3.6; Panel B*).

Panel A:



Panel B:



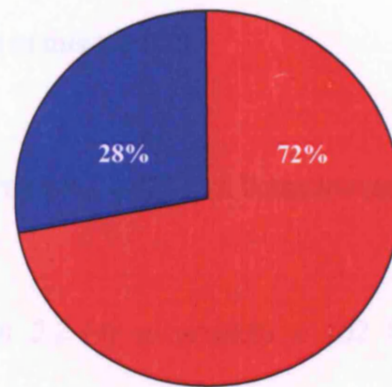


Figure 3.6: Microarray validation of the reproduction-specific subtraction. Panel A: Sub-array showing the differential expression of genes within *L. rubellus*. Green spots correspond to those genes with higher relative expression in the tail tissue; Yellow spots correspond to those genes expressed equally in the tail and reproductive tissue; and Red spots correspond to those genes with higher relative expression within the reproductive tissue areas. **Panel B:** The percentage of genes expressed in reproductive tissue areas (RED – reproductive specific) and those genes expressed only in the tail tissue and those genes expressed equally in both tissue areas (BLUE).

(C) IDENTIFICATION OF THE SPERM-SPECIFIC ANTIGEN FROM THREE EARTHWORM SPECIES

The subtraction of the reproductive areas of *L. rubellus* identified a number of genes potentially involved in reproductive processes (*Tables 3.1* and *3.2*). The *L. rubellus* clone showing sequence identity with the mouse (*M. musculus*) Sperm Specific Antigen 1 (SSA1) (*Table 3.1*) was selected for further investigation. Although this earthworm clone showed relatively low sequence identity with the mouse SSA1 on an initial BLAST X search (*Table 3.1*), it was deemed potentially important enough to warrant further investigation.

A prerequisite for a molecular genetic biomarker is that it is ubiquitous. Therefore, the first stage of identification of the mouse SSA in the earthworm was that it was found in all three species of earthworm being utilised in this research.

3.3.6 PCR CLONING OF THE SPERM SPECIFIC ANTIGEN 1 IN THREE EARTHWORM SPECIES

Sequence specific primers were designed (*Section 2.2.14*) to amplify a 102 bp fragment of the mouse SSA1 in three earthworm species (*L. rubellus*, *E. fetida* and *E. andrei*). cDNA was produced from the three species as described in *Section 2.2.15* and PCR was performed on an MWG-Biotech PRIMUS™ PCR cycler with an annealing temperature of 55 °C . Upon gel electrophoresis a PCR fragment of approximately 100 bp was isolated from each species (*Figure 3.7*). These fragments were extracted from the agarose gel and cloned into the pGEM-T (Promega) plasmid vector (*Section 2.2.12* and *2.2.17*).

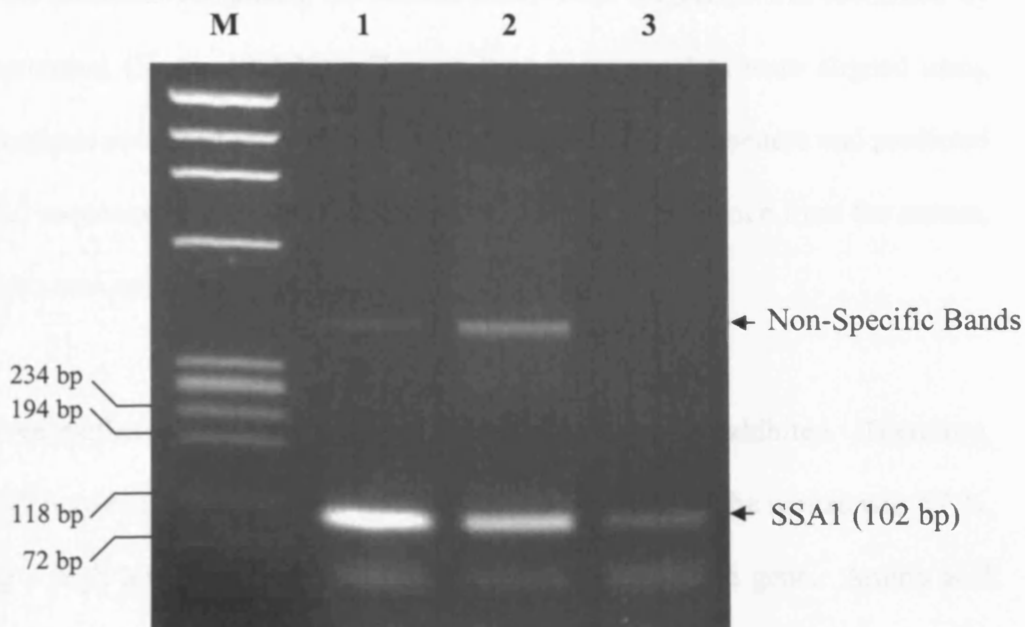


Figure 3.7: Analysis of PCR product from the amplification of the mouse Sperm Specific Antigen 1 gene fragment in three earthworm species. Samples were electrophoresed on a 2 % agarose gel containing EtBr (0.4 μg/ml) and visualised under U.V. light. Lane 1 shows the PCR product obtained from the PCR amplification of SSA1 from cDNA derived from *Eisenia fetida*. Lane 2 represents the PCR product of SSA1 obtained from *Eisenia andrei*. Lane 3 represents SSA1 from *Lumbricus rubellus*. Lane M represents DNA marker (ϕx174*Hae*III fragments).

3.3.7 SEQUENCE ANALYSIS OF THE POTENTIAL SPERM SPECIFIC ANTIGEN 1

Each of the plasmids containing the cloned SSA1 PCR fragments was identified by DNA sequencing (*Section 2.2.22*). The resulting sequence data were aligned using BioEdit analysis package (*Section 2.2.24*). The aligned DNA sequences and predicted amino acid sequences are shown in *Figure 3.8*. The SSA1 sequence from the mouse, *M. musculus* was used as a comparison.

Of the three earthworm species analysed, 100 % identity was exhibited. Therefore, the identity between the earthworm DNA sequences and that of the mouse was 57 %, indicating a high level of sequence identity in that region of the gene. Amino acid identity was similarly high, showing 61 % identity between the earthworm and the mouse.

```
                     10         20         30         40         50         60
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
M. musculus  CTACGTATTT GTGCGCTC-- -CAAGCAGGC TTTAGGACGG CGTGTGGCGT CTCCAGGCCA
              L  R  I   C  A  L     Q  A  G   F  R  T   A  C  G  V   S  R  P
L. rubellus  TACGTCTTTG TGAGATCCAA GCAAGCCTTG GGTCGT---G CATGTGGCGT GTCTCGTCCA
              Y  V  F   V  R  S  K  Q  A  L   G  R      A  C  G  V   S  R  P
E. fetida    TACGTCTTTG TGAGATCCAA GCAAGCCTTG GGTCGT---G CATGTGGCGT GTCTCGTCCA
              Y  V  F   V  R  S  K  Q  A  L   G  R      A  C  G  V   S  R  P
E. andrei    TACGTCTTTG TGAGATCCAA GCAAGCCTTG GGTCGT---G CATGTGGCGT GTCTCGTCCA
              Y  V  F   V  R  S  K  Q  A  L   G  R      A  C  G  V   S  R  P

                     70         80         90        100
             ....|....| ....|....| ....|....| ....|....|..
M. musculus  GTCATCGCCT GTTCTGTTAC CATCAAAGAA GGCTCTCAACTA
              V  I  A   C  S  V  T   I  K  E   G  S  Q  L
L. rubellus  GTGATCGCGT GTTCTGTGAC AGTCAACCGA GACTCACAGCTG
              V  I  A   C  S  V  T   V  N  R   D  S  Q  L
E. fetida    GTGATCGCGT GTTCTGTGAC AGTCAACCGA GACTCACAGCTG
              V  I  A   C  S  V  T   V  N  R   D  S  Q  L
E. andrei    GTGATCGCGT GTTCTGTGAC AGTCAACCGA GACTCACAGCTG
              V  I  A   C  S  V  T   V  N  R   D  S  Q  L
```

Figure 3.8: Nucleic acid sequence alignment and predicted amino acid sequence of the Sperm Specific Antigen 1 from three earthworm species. The three 99 bp SSA1 DNA PCR fragments were sequenced (*Section 2.2.22*) and aligned using the BioEdit sequence alignment package (*Section 2.2.24*). These sequences were compared with the SSA1 DNA sequence from the mouse (*M. musculus*) taken from Zhu & Naz (1997). Identical bases are shown in bold-face and the major area of identity is boxed.

3.4 DISCUSSION

The aim of the subtractive PCR technique was to produce a small library containing differentially expressed cDNA fragments from the reproductive areas of *L. rubellus*. The hypothesis was that genes identified as being linked to reproduction in the earthworm would potentially become important molecular genetic biomarkers in earthworm ecotoxicology.

The subtractive library was screened through the random cloning of subtracted cDNA fragments (*Section 3.3.3a*), subsequent PCR amplification of these inserts (*Section 3.3.3b; Figure 3.5*), the DNA sequencing of these inserts (*Section 3.3.3c*) and finally the identification of these DNA sequences via database searches (*Section 3.3.3d)*. Of the 132 clones successfully sequenced, only 54 were identifiable through database searches (*Table 3.2*) indicating a high abundance of potentially novel genes. Of the 54 clones identified, 11% appeared to be linked to reproduction. Many of these genes showed sequence identity with genes derived from tissues such as, testis or ovary in animals such as mice and humans (*Table 3.1*). However, a high proportion of the identified sequences appeared not to be linked to any reproductive function in any species (*Table 3.2*). This indicated that the enrichment of the subtractive library for reproduction-specific gene transcripts had not been as good as expected. Therefore, the enrichment of the library for these transcripts was assessed using a microarray approach.

A microarray was produced (*Section 3.3B*) containing those clones isolated from the subtractive library (384 clones in all, of which 223 had been sequenced) and randomly selected clones from a *L. rubellus* control (whole body) library (Chaseley *et al.*,

unpublished). This allowed the identification of those cloned transcripts that were common to the whole earthworm and those that were only found in the reproductive areas. The 'spotted' clones on the microarray slide were hybridised with total RNA either from the reproductive tissue areas or tail tissue. It was found that 72% of the clones from the subtractive library appeared to be specific to the reproductive tissue areas. In comparison, 52% of the clones from the *L. rubellus* control library were reproductive tissue-specific. This indicated that the subtractive library was enriched for clones originating from the reproductive tissue areas. This may seem to contradict the results obtained from those successfully sequenced and identified subtractive clones (*Table 3.2*). These results showed that only 11% of the clones were linked to reproduction. However, the number of unsuccessfully sequenced clones must be taken in to account and the number of novel transcripts (showing no significant homology to any known sequences when BlastX was performed) that were found. Many of these transcripts may have been specific to the reproductive tissues, indicated by the high percentage of transcripts appearing to be reproductive specific in the microarray analysis.

A possible explanation for the lower than expected enrichment of the subtractive library is the tissue areas that the total RNA was isolated from; rather than excising reproductive tissues themselves (i.e. the testis or ovaries) it was decided that the gross reproductive tissue areas should be used as more total RNA could then be isolated from a single animal. If ovaries and testis alone had been used a number of animals would have had to be used. Therefore, this may have biased the enrichment due to variations in gene expression patterns between individuals. However, this approach meant that body wall, gut lining and other ubiquitous tissues were taken as

"reproduction-specific tissue". As a consequence high levels of the ubiquitously expressed gene transcripts were present in both tissue types in comparison to the very low levels of reproductive-specific gene transcripts.

Although enrichment of the subtractive library was not as great as expected and that DNA sequencing and identification was lower than required, a number of interesting gene fragments were isolated. The identification of a potential sperm-specific antigen (SSA) was particularly interesting (*Section 3.3C*). Sperm-specific antigens are implicated in the specific interaction of sperm and oöcyte during fertilisation (Naz *et al.*, 1995). They are believed to be involved in inducing the arcosome reaction, which enables a single sperm to enter the oöcyte and resulting in fertilisation (Ganong, 1995). Therefore, it is obvious that such factors are critical in the reproductive process in many animals. Although sequence identity of the earthworm SSA with other similar sequences was relatively low this may be expected. The SSA factors would be expected to be specific to the individual species. This may be important for earthworms, as reproduction/fertilisation is essentially an external process (*Section 1.4*).

As a molecular genetic biomarker in earthworm ecotoxicology an SSA factor may be useful. In the past sperm counting has been utilised as a measure of toxic exposure in earthworms (Cikutovic *et al.*, 1993). Therefore, the measurement of gene expression levels of a SSA factor could replace this time consuming and inaccurate method. This would be based upon the assumption that the gene expression level of a sperm-specific transcript would directly correlate to the number of sperm present. Mechanistically, the role of an SSA factor may be interesting during toxicant

exposure. If a particular toxicant was able to interact with one of these factors and hence prevent fertilisation taking place it could have huge consequences for the individual and the population as a whole.

Although a fragment of the SSA factor was isolated from all three earthworm species used here, it was deemed that it was more important to look at other potential biomarkers of reproduction; in particular a molecular genetic factor that may have been implicated in overall control of the reproductive process in earthworms. This factor had previously been isolated from *E. fetida* (Oumi *et al.*, 1995) and had been termed annetocin because of its identity with the mammalian sex hormone, oxytocin.

CHAPTER 4

IDENTIFICATION OF THE SEX HORMONE ANNETOCIN FROM THREE EARTHWORM SPECIES

4.1 INTRODUCTION

Despite the large number of research papers detailing the general aspects of earthworm reproductive biology (*see Section 1.4*), little knowledge has been gained of the molecular basis that controls these complex mechanisms. However, in 1994 Oumi *et al.* identified a nonapeptide (annetocin) from the earthworm species *Eisenia fetida* that showed a high level of amino acid homology with the vasopressin/oxytocin (VP/OT) neuropeptide superfamily (*Table 4.2*). Annetocin stimulated pulsatory contractions of the earthworm nephridia (the protostome homologues of mammalian meso- and metanephros) (Oumi *et al.*, 1994) and stereotyped egg-laying behaviours (for example, mucous secretion from the clitellum and constriction of the body-wall muscles) (Oumi *et al.*, 1996).

In mammals, oxytocin (homologue of annetocin) is implicated in the contractility of the smooth muscle of the uterus at birth (Soloff *et al.*, 1979), milk lactation and during sperm ejaculation (Ivell *et al.*, 1987). When sexually mature earthworms were exposed to a 5 nmol concentration of mammalian oxytocin, egg-laying behaviours were noted (Oumi *et al.*, 1996). These data show the conservation of the VP/OT superfamily through evolution.

4.2 APPROACH

This section of work covers the isolation of the DNA sequences of annetocin from three earthworm species, *Eisenia fetida*, *Eisenia andrei* and *Lumbricus rubellus* (classified as epigeic species; *Section 1.1*). Although these three earthworm species are classified within the same ecophysiological class, the two *Eisenia* species are found mainly in manure and compost, whilst *L. rubellus* is found within the upper soil layers and leaf litter. These species have very different population growth strategies. *Eisenia* species are colonisers, characterised by rapid reproduction, growth and, as a result, population growth. These characteristics allow *Eisenia* species to exploit potential new habitats (e.g. dung pats) as they become available. *L. rubellus* on the other hand has lower growth and reproductive rates (Spurgeon *et al.*, 2000b).

Eisenia fetida (and the closely related *Eisenia andrei* earthworm) is used as the test organism in the OECD earthworm acute toxicity and reproduction tests (OECD, 1984; 2001). The use of these earthworm species has received criticism in the past (*see Sections 1.2* and *1.3*) due to their perceived lack of ecological significance (i.e. they inhabit manure and compost heaps, not true soil). Therefore, the more relevant species, *Lumbricus rubellus* has been used in earthworm ecotoxicology as an alternative (*Section 1.2*). So, for annetocin to be a relevant and ubiquitous molecular biomarker within terrestrial ecotoxicology it is important to isolate and characterise the gene encoding for annetocin from a number of different earthworm species.

4.3 RESULTS

4.3.1 TOTAL RNA ISOLATION AND QUALITY CONTROL

The species selected were; *Eisenia fetida* (collected from a manure heap; Amelia Farm, Vale of Glamorgan, O.S. grid reference: ST 063 740) and *Eisenia andrei* (purchased from Godmanchester Earthworms, Cambs.). *L. rubellus* were collected from a population maintained at Monks Wood, Cambridgeshire. The taxonomy of all three species was verified using the key of Sims & Gerard (1985).

In all investigations concerning annetocin and other sex dependent genes adult, fully clitellate earthworms were used. On average, adult *Eisenia fetida* and *Eisenia andrei* weighed between 300 and 400 mg (wet weight), whilst adult *Lumbricus rubellus* weighed between 600 and 700 mg (wet weight). Total RNA was isolated following the protocol set out in *Section 2.2.8*. Quantification of total RNA (*see Section 2.2.13*) was carried out to determine RNA concentration for subsequent applications and as a means of determining RNA purity. An average of 100 μg of total RNA was isolated per 100 mg of tissue used. Total RNA was qualitatively assessed by agarose gel electrophoresis (*Section 2.2.8; Figure 2.2*).

4.3.2 COMPLEMENTARY DNA SYNTHESIS AND QUALITY ASSESSMENT

Complementary DNA (cDNA) was synthesised as described in *Section 2.2.15* using 2 μg of total RNA. cDNA quality was assessed by the amplification of a 198 bp fragment of the *β*-actin gene through PCR (as described in *Section 2.2.16*) using an oligonucleotide primer pair specific to the *β*-actin gene (*Appendix C*).

The success of the PCR amplification was assessed by agarose gel electrophoresis (*Figure 4.1*) and the size of the amplified DNA fragment was estimated by comparison with molecular weight standards. The PCR products from the three earthworm species were observed to be of approximately the same molecular weight. PCR products were excised from the agarose gel after electrophoresis (described in *Section 2.2.11*) and cloned into the plasmid vector, pGEM-T (Promega) (*Section 2.2.17*) for subsequent sequence analysis (using the method described in *Sections 2.2.23* and *2.2.24*). The DNA sequences of the *β*-actin gene fragment from the three species used in this study were found to have 99 % identity (data not shown/*Appendix D*).

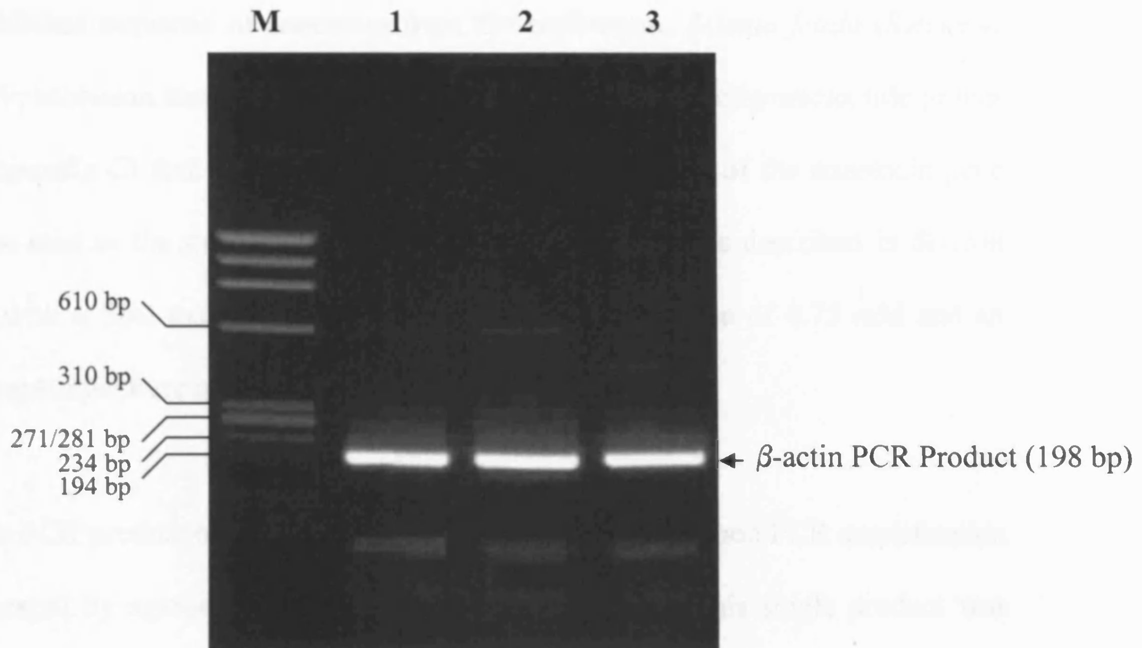


Figure 4.1: Analysis of PCR product from the amplification of a *β*-actin gene fragment in three earthworm species. Samples were electrophoresed on a 2 % agarose gel containing EtBr (0.4 μg/ml) and visualised under U.V. light. Lane 1 shows the PCR product obtained from the PCR amplification of *β*-actin from cDNA derived from *Eisenia fetida*. Lane 2 represents the PCR product of *β*-actin obtained from *Eisenia andrei*. Lane 3 represents *β*-actin from *Lumbricus rubellus*. Lane M represents DNA marker (*ϕ*x174*Hae*III fragments).

4.3.3 CHARACTERISATION OF cDNA ENCODING AN ANNETOCIN PRECURSOR IN *EISENIA FETIDA* AND *EISENIA ANDREI*

The published sequence of annetocin from the earthworm, *Eisenia fetida* (Satake *et al.*, 1999; accession number AB014478) was used to design an oligonucleotide primer pair (*Appendix C*) that would allow the specific amplification of the annetocin gene from the start to the stop codon. The PCR was carried out as described in *Section 2.2.16*, with a final magnesium chloride ($MgCl_2$) concentration of 0.75 mM and an annealing temperature of 55 °C.

A single PCR product of approximately 420 bp was obtained upon PCR amplification and assessed by agarose gel electrophoresis (*Figure 4.2*). This single product was shown not to be a PCR artefact by comparison with a negative control PCR reaction (data not shown).

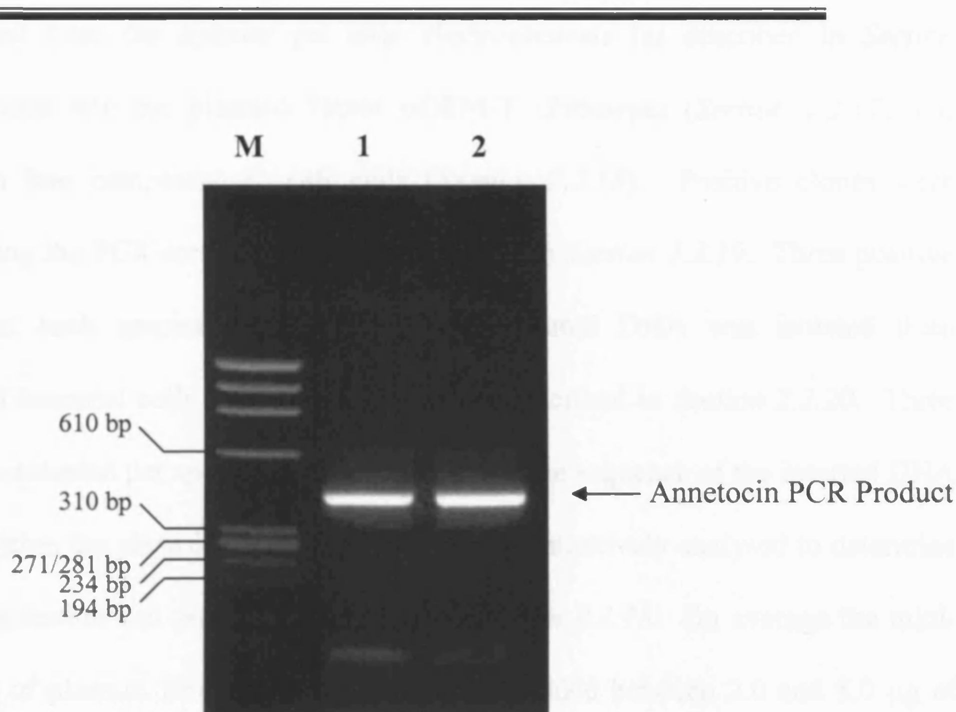


Figure 4.2: Analysis of annetocin PCR product from two earthworm species. PCR amplification products of annetocin from *Eisenia fetida* (lane 1), *Eisenia andrei* (lane 2) were run on a 2 % agarose gel containing EtBr (0.4 μg/ml). Nucleic acids were visualised under U.V. light. Lane M represents marker DNA (ϕx174*Hae*III fragments).

The PCR products from the amplification of the annetocin gene fragment (*Figure 4.2*) were excised from the agarose gel after electrophoresis (as described in *Section 2.2.11*), cloned into the plasmid vector pGEM-T (Promega) (*Section 2.2.17*) and transformed into competent *E. coli* cells (*Section 2.2.18*). Positive clones were selected using the PCR screening method described in *Section 2.2.19.* Three positive clones from each species were selected and plasmid DNA was isolated from transformed bacterial cells following the protocol described in *Section 2.2.20.* Three clones were selected per species in order to confirm the sequence of the inserted DNA fragment within the plasmid. Plasmid DNA was quantitatively analysed to determine DNA concentration and purity as described in *Section 2.2.13.* On average the mini-preparation of plasmid DNA from bacterial cells yielded between 2.0 and 5.0 μg of high purity DNA. For automated sequencing (*Section 2.2.22*) 130 ng of plasmid DNA was used.

DNA sequences of annetocin from *Eisenia fetida* and *Eisenia andrei* were analysed using the BioEdit sequence analysis program as described in *Section 2.2.24. Figure 4.3* shows the alignment of these two annetocin prohormone DNA sequences with the human (*Homo sapiens*) oxytocin gene and the octopus (*Octopus vulgaris*) cephalotocin gene (both OT-related genes).

The annetocin prohormone gene fragment from the earthworm *Eisenia fetida* was 420 bp in length, which was identical with that found by Satake *et al.* (1999) (distance between start and stop codons). Annetocin isolated from *Eisenia andrei* was found to be 408 bp in length and showed 88.89 % DNA sequence identity with that isolated from *Eisenia fetida*.

A number of important features were well conserved within the two DNA sequences (*Figure 4.3*). The DNA sequence coding for the annetocin nonapeptide itself (97 – 123 bp) showed 100 % identity between earthworm species (*Figure 4.3*). The DNA sequence coding for the dibasic protein cleavage site (127 – 132 bp) also showed identity between the two species. The fourteen cysteine residues characteristic of the neurophysin domain of OT-related peptides were also conserved in both earthworm species (*Figures 4.3* and *4.4*).

```
                    10         20         30         40         50         60         70
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   ATGGCCGGC- ---------- ---------- ---------C CCAGCCTC-- ----GCTTGC TGTCTGCTCG
O.vulgaris  ATGTCTCAAA AT-------- ----TGTTTC GCCATCGTCC AACTTTTGTT CGTGTTGTTC ACAGTATGCA
E.fetida    ATGGCTTGCA CTAAGAAGTC GGCGAACATG AAACTCAGGA AGTCATTGAC AGTAACAGCC TTCCTGCTAT
E.andrei    ATGGCTTGCA CTAAGAAGTC GGCGAACATG AAACTCAGGA AGTCATTGAC AGTAACAGCC TTCCTGCTAT

                    80         90        100        110        120        130        140
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   GCCTC---CT GGCGCTGACC TCCGCCTGCT ACATCCAGAA CTGCCCCCTG GGAGGCAAGA GG--------
O.vulgaris  GTCTCTTCAT TGCCACTACA GATGGTTGCT ATTTCCGAAA CTGTCCCATT GGTGGCAAAC GAGCGACACC
E.fetida    TTGTCAACCT GTCGCTTTCT TCCGCCTGCT TTGTTCGCAA CTGCCCGACG GGCGGGAAAC GGTCTGTTCT
E.andrei    TTGTCAACCT GTCGCTTTCT TCCGCCTGCT TTGTTCGCAA CTGCCCGACG GGCGGGAAAC GG--------

                   150        160        170        180        190        200        210
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   -GCCGCGCCG GACCTCGACG TGCGCAAGTG CCTCCCCTGC GGCCCC---- -----GGGGG CAAA------
O.vulgaris  CATGTCAGAG CAGGGATCAA ATCAAAAGTG TATGTCTTGC GGACCA---- -----AATGG TGAA------
E.fetida    ACTGAGCCCA CTGCAACCTG CCAGACAGTG TATGCCGTGC GGTGCGACTG TCGGTGGTAG GTCGGTTCTC
E.andrei    ----TCTGTT CTGCAACCTG CCAGACAGTG TTTGCCGTGC CGTGCGACAG TCGATGGTCG GTCGGTGCCT
                                           1         2

                   220        230        240        250        260        270        280
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   GGCCGCTGCT TCGGGCCCAA TATCTGCTGC GCGGAAGAGC TGGGCTGCTT CGTGGGCACC GCCGAAGCGC
O.vulgaris  GGCCAGTGTG TTGGATCCAA CATATGCTGC CACAAAGAC- --GGTTGCAT CATAGGCACA ---CTCGCAA
E.fetida    GGAGTCTGCG TCTCTGAGAA CACCTGTTGC GTCGCTCACC TCGGCTGCTT CGTCAACACT GAAGAAAGCA
E.andrei    GGGATCTGCG TCTCTGAGAA CCCCTGTTGT GTCGCTCACC TCGGCTGTTT GGTCACCATT GAAGAGAGCA
                   3                   4  5                   6

                   290        300        310        320        330        340        350
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   TGCGCTGCCA GGAGGAGAAC TACCTGCCGT CGCCCTGCCA GTCCGGCCAG AAGGCGTGCG GGAGCGGGGG
O.vulgaris  AGGAATGCAA TGAGGAAAAC GAGAGCACGA CAGCATGTTC TGTGAAAGGG GTGCCGTGTG GAACTGACGG
E.fetida    AGGTCTGTGC CCTCGAGAAC CATCTGTCGA CCCCTTGCCG CCTCCAAGGG CCCCCCTGTG GATCGGACGG
E.andrei    AGGTGTGTGC CCTCGAAAAC CATCTGTCGA CCCCTTGCCG CCTCGAAGGG CCCCCCTGTG GATCGGACGG
                  7                                 8                     9

                   360        370        380        390        400        410        420
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   CCGC------ TGCGCGGTCT TGGGCCTCTG CTGCAGCCCG GACGGCTGCC ACGCCGACCC TGCCTGCGAC
O.vulgaris  ACAAGGACGA TGTGTTGCCG ACGGTGTTTG CTGCGATGAA TCCTCCTGTT TTACAACCGA TAGATGTGAC
E.fetida    TCAAGACGTC TGCGCTGTCG AAGGAATCTG CTGCGCCGGT CACAACTGTC GTTACGACGC TCAATGC*--
E.andrei    TCAAGACGTT TGCGCTGTCG AGGGAATCTG CTGCGCCGGT CAGAGCTGTC ATCACGACGC TCAATGC*--
                        10                 11  12              13                  14

                   430        440        450        460        470        480        490
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens   GCGGAAGCCA CCTTCTCCCA GCGC*----- ---------- ---------- ---------- ----------
O.vulgaris  AGAGAGAATC ATCGCAGTAT GGCAATGCAG AAATTATTGG AAATACGTGA TGGAATTTAT TACAAGAAA*
E.fetida    ---------- ---------- ---------- ---------- ---------- ---------- ----------
E.andrei    ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

Figure 4.3: Nucleic acid sequence of annetocin isolated from *Eisenia fetida* and *Eisenia andrei*. The two earthworm DNA sequences were aligned using the BioEdit sequence alignment package (*Section 2.2.24*). Sequence homology was assessed using the human oxytocin DNA sequence (*Homo sapiens*) and the cephalotocin DNA sequence from the octopus (*Octopus vulgaris*). The DNA sequence of the biologically active nonapeptide is shown in blue and the dibasic protein cleavage site is shown in red. The DNA sequence for the fourteen conserved cysteine residues are shown in boldface and numbered 1 to 14.

4.3.4 CHARACTERISATION OF A PARTIAL cDNA FROM THREE EARTHWORM SPECIES

The partial cDNA sequence from three earthworm species (*E. fetida*, *E. andrei* and *L. rubellus*) was determined. A fragment of the annetocin gene (from the start codon to the first conserved cysteine residue within the neurophysin domain) was isolated from all three species using DNA-specific oligonucleotide primer pairs (*Appendix C*). An annealing temperature of 60 °C was used in the PCR amplification reactions.

PCR amplification products were analysed by electrophoresis on an agarose gel (2.0 %, w/v) (data not shown). From each earthworm species an amplified PCR product of approximately 180 bp was observed. *Figure 4.4* shows the nucleotide sequences and predicted amino acid sequences of the annetocin gene fragment from all three species. These sequences were aligned with the oxytocin gene sequence (*Homo sapiens*) and the cephalotocin gene sequence (*Octopus vulgaris*) using the BioEdit sequence analysis program (*Section 2.2.24*).

```
                   10         20         30         40         50         60         70
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens  ATGGCCGGC- ---------- ---------- ---------C CCAGCCTC-- ----GC---T TGCTGTCTGC
            M  A  G                                    P  S  L         A      C  C  L
O.vulgaris ATGTCTCAAA AT-------- ----TGTTTC GCCATCGTCC AACTTTTGTT CGTGTT---G TTCACAGTAT
            M  S  Q   N               C  F   A  I  V   Q  L  L  F   V  L      F  T  V
E.fetida   ATGGCTTGCA CTAAGAAGTC GGCGAACATG AAACTCAGGA AGTCATTGAC AGTAAC---A GCCTTCCTGC
            M  A  C   T  K  K  S   A  N  M   K  L  R   K  S  L  T   V  T      A  F  L
E.andrei   ATGGCTTGCA CTAAGAAGTC GGCGAACATG AAACTCAGGA AGTCATTGAC AGTAAC---A GCCTTCCTGC
            M  A  C   T  K  K  S   A  N  M   K  L  R   K  S  L  T   V  T      A  F  L
L.rubellus ATGGCTTGCA CTAAGAAGTC GTCGAGCATG AAACTCTTGA AGTTGGTTAC AGCAACCGCA GTCTTCCTGC
            M  A  C   T  K  K  S   S  S  M   K  L  L   K  L  V  T   A  T  A   V  F  L

                    80         90        100        110        120        130        140
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
H.sapiens  TCGG------ CCTC---CTG GCGCTGAC-- ---------- ---------- --CTCCGCCT GCTACATCCA
           L  G         L     L   A  L  T                             S  A   C  Y  I  Q
O.vulgaris GCAG------ TCTCTTCATT GCCACTAC-- ---------- ---------- --AGATGGTT GCTATTTCCG
           C  S         L  F  I   A  T  T                             D  G   C  Y  F  R
E.fetida   TATT------ TGTCAACCTG TCGCTTTC-- ---------- ---------- --TTCCGCCT GCTTTGTTCG
           L  F         V  N  L   S  L  S                             S  A   C  F  V  R
E.andrei   TATT------ TGTCAACCTG TCGCTTTC-- ---------- ---------- --TTCCGCCT GCTTTGTTCG
           L  F         V  N  L   S  L  S                             S  A   C  F  V  R
L.rubellus TGTTAGATTT TGTCGACCTG TCTCTTTCGA AAACTATAGT TAAACCTAAC AATGGCCGCT GTTTCGTTCG
           L  L  D  F   V  D  L   S  L  S   K  T  I  V   F  P  N   N  G  A   C  F  V  R
                                                                             1  2  3  4

              150        160        170        180        190        200
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....
H.sapiens  GAACTGCCCC CTGGGAGGCA AGAGG----- ----GCCGCG CCGGACCTCG ACGTGCGCAA GTGC
             N  C  P   L  G  G   K  R            A  A   P  D  L   D  V  R  K   C
O.vulgaris AAACTGTCCC ATTGGTGGCA AACGAGCGAC ACCCATGTCA GAGCAGGGAT CAAATCAAAA GTGT
             N  C  P   I  G  G   K  R  A  T   P  M  S   E  Q  G   S  N  Q  K   C
E.fetida   CAACTGCCCG ACGGGCGGGA AACGGTCTGT TCTACTGAGC CCACTGCAAC CTGCCAGACA GTGT
             N  C  P   T  G  G   K  R  S  V   L  L  S   P  L  Q   P  A  R  Q   C
E.andrei   CAACTGCCCG ACGGGCGGGA AACGG----- -------TCT GTTCTGCAAC CTGCCAGACA GTGT
             N  C  P   T  G  G   K  R               S   V  L  Q   P  A  R  Q   C
L.rubellus CAACTGCCCA ACGGGCGGCA AACGGTCCAT TCTGCTGATC GCGGTGCAAC CTGCCAGACA GTGT
             N  C  P   T  G  G   K  R  S  I   L  L  I   A  V  Q   P  A  R  Q   C
             5  6  7   8  9
```

Figure 4.4: Partial DNA and predicted amino acid sequence of the annetocin gene isolated from three earthworm species. DNA sequence alignment was performed using the BioEdit analysis package. The three earthworm annetocin sequences were aligned to the human oxytocin DNA sequence and the octopus cephalotocin DNA sequence. The DNA sequence encoding the biologically active nonapeptide is shown in blue (130 – 161 bp) and the nine amino acids making up this peptide are numbered 1 to 9. The dibasic protein cleavage site is shown in red (165 – 170 bp).

The annetocin gene fragment isolated from *Lumbricus rubellus* showed a number of distinct differences at the DNA level with the other two earthworm species (*Figure 4.4*). These differences were not only seen in single nucleotide changes, but areas of inserted nucleotides. The annetocin gene fragment from *Lumbricus rubellus* was 204 bp in length (showing identity of 83.63 % and 84.28 % to *Eisenia fetida* and *Eisenia andrei* respectively), compared with 171 bp of *Eisenia fetida* and 159 bp of *Eisenia andrei* (in this region the DNA identity between these two *Eisenia* species was 96.23 %).

4.3.5 PREDICTED AMINO ACID SEQUENCE OF ANNETOCIN PRECURSOR FROM *EISENIA FETIDA* AND *EISENIA ANDREI*

The DNA sequence analysis described above allowed for the prediction of the amino acid sequence of the annetocin precursor from both earthworm species (*Figure 4.5*). These two sequences were aligned with thirteen other OT-related peptides from a number of different species (representing mammals, fishes, amphibians and invertebrates).

Analysis of these amino acid sequences confirmed the high level of homology observed within the nonapeptide region itself (33 – 41 aa) and the dibasic protein cleavage site (43 – 44 aa) (*Figure 4.5*). Of the nine amino acids that make up the biologically active peptide five are conserved among all species, including the structurally important cysteine residues at positions one and six (these residues form a disulphide bond in the mature peptide). The two earthworm nonapeptide sequences shown here both contain a threonine residue at position 8 in the nonapeptide sequence, indicating that they are both OT-related peptides (OT-related peptides are characterised by a neutral amino acid at position 8).

The two earthworm sequences showed 88.89% identity at the amino acid level. The prohormone isolated from *Eisenia fetida* was 139 amino acids in length, whilst that isolated from *Eisenia andrei* was 135 amino acids in length. The fourteen cysteine residues found in the neurophysin domain of these prohormones were also highly conserved between earthworm species and other animal species.

Figure 4.5: Amino acid alignment of oxytocin-related preprohormones from fifteen different animal species. The annetocin preprohormone from the two earthworm species, *Eisenia fetida* and *Eisenia andrei* were aligned to OT-related preprohormones from a number of mammalian, amphibian and fish species. Amino acid homology was assessed using the BioEdit sequence analysis program. The nonapeptide characteristic of the OT-related peptides is shown in blue-face (residues are numbered 1 to 9), whilst the dibasic protein cleavage site is shown in red-face. Amino acid homology between all sequences is shown by an asterix and the fourteen conserved cysteine residues found in the neurophysin domain are highlighted.

```
                        10        20        30        40        50        60
               .........|.........|.........|.........|123456789|.........|.....
H. sapiens     -MAGPSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAAPD---LDVRKC
S. scrofa      -MAGPSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAVLD---LDVRKC
B. taurus      -MAGSSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAVLD---LDVRTC
O. aries       -MAGSSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAVLD---LDVRTC
R. norvegicus  -MACPSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAALD---LDMRKC
M. musculus    -MACPSLACC-LLGLLALT-S-----------------ACYIQNCPLGGKRAVLD---LDMRKC
T. rubripes    -MTGTAISVC-LLFLLSV-CS-----------------ACYISNCPIGGKRSIMD---APQRKC
N. forsteri    MMSSASIPIC-LICLFAFT-S-----------------ACFIQNCPIGGKRSVLD---TDLRKC
O. keta        -MTGAAVSVC-LLYALSV-CS-----------------ACYISNCPIGGKRSIMD---APQRKC
D. rerio       -MSGGLLSAAALLCLLSV-CS-----------------ACYISNCPIGGKRSVQD---WPIRQC
C. commersoni  -MSGSMFSVFSLLYLLSV-CS-----------------ACYISNCPIGGKRAIQD---SPSRQC
B. japonicus   -MSYTALAVT-FFGWLALS-S-----------------ACYIQNCPIGGKRSVIDF--MDVRKC
O. vulgaris    -MSQNCFAIVQLLFVLFTVCS--LF---------IATTDGCYFRNCPIGGKRATPMSEQGSNQKC
E. fetida      -MACTKKSANMKLRKSLTVTAFLLFVNLSLSS-------ACFVRNCPTGGKRSVLLSPLQPARQC
E. andrei      -MACTKKSANMKLRKSLTVTAFLLFVNLSLSS-------ACFVRNCPTGGKRSVL----QPARQC
                *                                     *  ***  ****             *

                   70        80        90       100       110       120       130
               ....|.........|.........|.........|.........|.........|.........|
H. sapiens     LPCGPGGK-----GRCFGPNICCAEELGCFVGTAEALRCQEENYLPSPCQSGQKACGS-GG-RCA
S. scrofa      LPCGPGGK-----GRCFGPSICCGDELGCFVGTAEALRCQEENYLPSPCQSGQKPCGS-EG-RCA
B. taurus      LPCGPGGK-----GRCFGPSICCGDELGCFVGTAEALRCQEENYLPSPCQSGQKPCGS-GG-RCA
O. aries       LPCGPGAK-----GRCFGPSICCGDELGCFVGTAEALRCREENYLPSPCQSGQKPCGS-GG-RCA
R. norvegicus  LPCGPGGK-----GRCFGPSICCADELGCFVGTAEALRCQEENYLPSPCQSGQKPCGS-GG-RCA
M. musculus    LPCGPGGK-----GRCFGPSICCADELGCFVGTAEALRCQEENYLPSPCQSGQKPCGS-GG-RCA
T. rubripes    MSCGPGDR-----GRCFGPGICCGESFGCLMGSPESARCAEENYLLTPCQAGGRPCGSEGG-LCA
N. forsteri    ISCGPGNR-----GRCFGPSICCGEELGCFFGTTEALRCQEENYWPSPCQSGGKPCGSEGG-RCA
O. keta        MSCGPGEQ-----GRCFGPSICCGKDVGCWMGSPETAHCMEENYLPTPCQVGGRPCGSDTV-RCA
D. rerio       MPCGPGDR-----GRCFGPSICCGEGIGCLVGSPETLRCLEEDFLPSPCEMSGKACGYEG--RCA
C. commersoni  MSCGPGDR-----GRCFGPSICCGEGLGCLLGSPETQRCLEEDFLPSPCEAGGKVCGYEG--RCA
B. japonicus   IPCGPRNK-----GHCFGPNICCGEELGCYFGTTETLRCQEENFLPSPCESGRKPCGNNGG-NCA
O. vulgaris    MSCGPNGE----GQCVGSNICCHKD-GCIIG-TLAKECNEENESTTACSVKGVPCGTDGQGRCV
E. fetida      MPCGATVGGRSVLGVCVSENTCCVAHLGCFVNTEESKVCALENHLSTPCRLQGPPCGSDGQDVCA
E. andrei      LPCRATVDGRSVPGICVSENPCCVAHLGCLVTIEESKVCALENHLSTPCRLEGPPCGSDGQDVCA
                 *            * *     **     **         *   *      *     **      *

                      140       150       160       170       180
               .........|.........|.........|.........|.........|......
H. sapiens     VLGLCCSPDGCHADPACDA-----------------EATFSQR------------
S. scrofa      AAGICCNPDGCRFDPACDP-----------------EATFSQR------------
B. taurus      AAGICCSPDGCHEDPACDP-----------------EAAFSQH------------
O. aries       AAGICCSPDGCHADPACDP-----------------EAAFSQH------------
R. norvegicus  TAGICCSPDGCRTDPACDP-----------------ESAFSER------------
M. musculus    ATGICCSPDGCRTDPACDP-----------------ESAFSER------------
T. rubripes    SSGLCCDAESCTMDQSCLSEEEGDERGSLFDGSDSGDVILKLLRLAGLTSPHQTH-
N. forsteri    APGICCSDESCSVDSTCNQ-----------------DVLFP--------------
O. keta        SPGVCCDSEGCSADQSCFAEEEGDNQIGQSEGSNSADVILRLLHLADHTPPHRVHQ
D. rerio       APGVCCDSEGCSVDQSCVDGD-AD-AAAVNQPANSPDLLLKLLHLSSHTHPSRIHQ
C. commersoni  APGVCCDSEGCSVDQSCVDGD-GD-ATAVSQPASSQDLLLKLLHLSNPAHPYRLHQ
B. japonicus   RSGICCNHESCTMDPACEQ-----------------DSVFS--------------
O. vulgaris    ADGVCCDESSCFTTDRCDREN-------------HRSMAMQKLLEIRDGIYYKK---
E. fetida      VEGICCAGHNCRYDAQC------------------------------------
E. andrei      VEGICCAGQSCHHDAQC------------------------------------
                  * **     *     *
```

Table 4.1: Amino acid identity between members of the OT neuropeptide family.

Species	OT-Related Peptide	*Eisenia fetida* (% Identity)	*Eisenia andrei* (% Identity)
Eisenia fetida	Annetocin	-	88.89
Eisenia andrei	Annetocin	88.89	-
Homo sapiens	Oxytocin	40.52	41.08
Sus scrofa	Oxytocin	42.24	40.18
Bos taurus	Oxytocin	41.38	39.29
Ovis aries	Oxytocin	42.24	41.07
Rattus norvegicus	Oxytocin	43.10	41.96
Mus musculus	Oxytocin	43.10	41.96
Takifugu rubripes	Isotocin	41.88	43.36
Neoceratodus forsteri	Mesotocin	40.17	37.17
Oncorhynchus keta	Isotocin	40.17	36.28
Danio rerio	Isotocin	41.03	39.82
Catostomus commersoni	Isotocin	36.75	38.05
Bufo japonicus	Mesotocin	38.98	39.82
Octopus vulgaris	Cephalotocin	37.40	38.66

Table 4.1 shows the amino acid identity between the preproannetocin sequence from two earthworm species and prohormone sequences of other OT-related neuropeptides from thirteen other animal species. Greatest amino acid identity was seen between annetocin from *Eisenia fetida* and oxytocin isolated from the rat (*Rattus norvegicus*) and the mouse (*Mus musculus*) (both 43.10 % identical to annetocin). Annetocin from *Eisenia andrei* showed greatest amino acid identity to isotocin from the fish, *Takifugu rubripes* (43.36 %). All other OT-related peptides showed similar levels of identity (approximately 40 %).

4.3.6 GROSS ANATOMICAL ISOLATION OF ANNETOCIN GENE EXPRESSION IN *EISENIA FETIDA*

Annetocin gene expression was determined within the gross anatomy of the adult earthworm (*Eisenia fetida*) (*Figure 4.7*). Using a qualitative PCR reaction the

annetocin gene was amplified from tissue isolated from various body locations (as described in *Section 4.2.3*). It was shown that in those earthworm tissues anterior to segment number 36 annetocin gene expression was detected (*Figure 4.7*). Qualitative evaluation of the gene expression pattern revealed maximal expression within those earthworm tissues involved in sexual reproduction (segment numbers 11-35). Relatively low expression was seen in the first ten segments, pertaining to the association of annetocin with the central nervous system (Satake *et al.*, 1999) and its possible role in osmoregulation (Omui *et al.*, 1994). No expression was seen in those tissues posterior to segment number 36 (i.e. the tail).

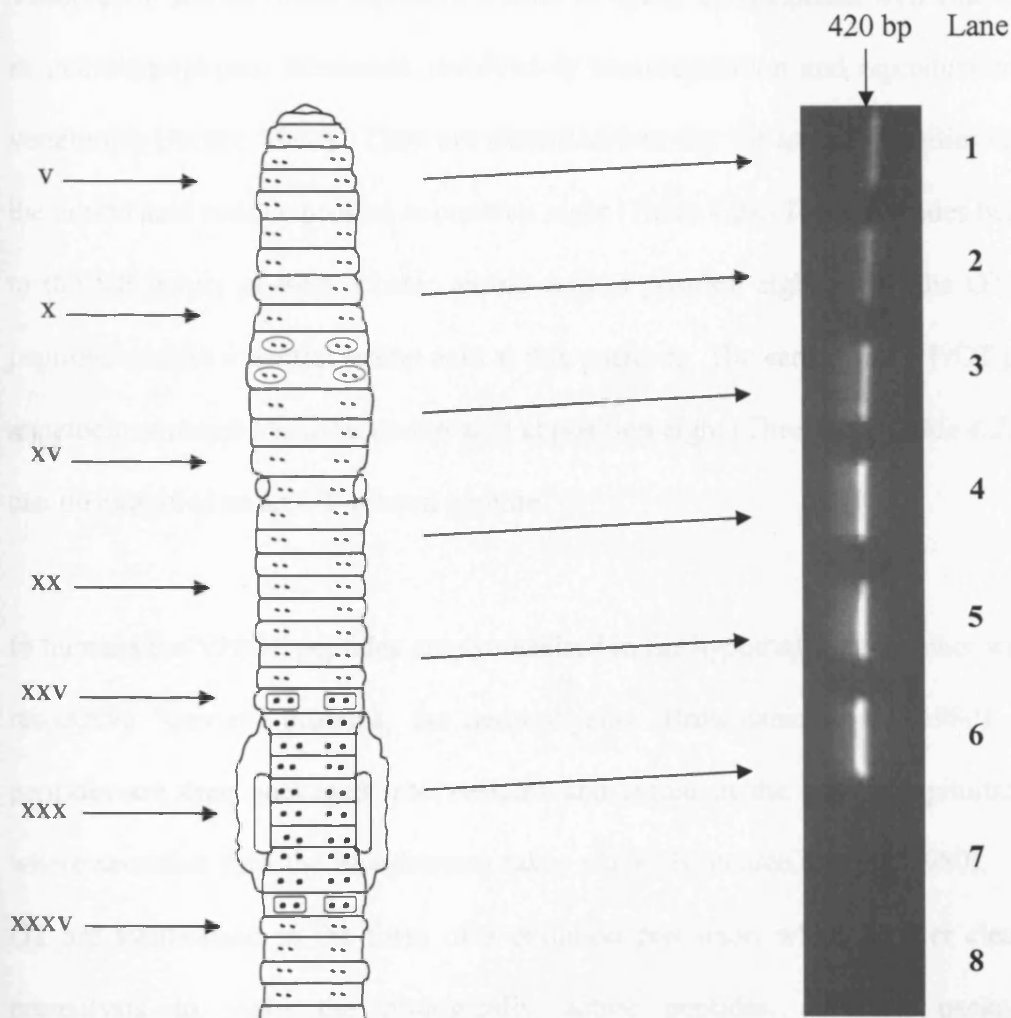


Figure 4.7: Anatomical gene expression distribution of annetocin within *Eisenia fetida*. Qualitative PCR (right) of the annetocin gene and schematic diagram representing the first 35 segments of *Eisenia fetida* (left) (adapted from Sims & Gerard, 1985). Annetocin PCR products were subjected to agarose gel (containing 0.4 µg/ml EtBr) electrophoresis and visualised under U.V. light. Molecular weight markers (Promega) (not shown) were used to determine PCR product size. Lane 1, segments i-v, containing the suboesophageal ganglia. Lane 2, segments vi-x, first spermathecal pores found in furrows ix/x/xi; seminal vesicles containing sperm spread over segments ix-xii in four pairs. Lane 3, segments xi-xv, ovaries located in segment xii/xiii with male pores found in xv. Lane 4, segments xvi-xx. Lane 5, segments xxi-xxv, spermatophores often present on segments xxi-xxiii. Lane 6, segments xxvi-xxxv, representing the clitellum region. Lane 7, first posterior portion. Lane 8, second posterior portion (Sims & Gerrard, 1985).

4.4 DISCUSSION

Vasopressin and oxytocin represent a class of cyclic nonapeptides well characterised as neurohypophysial hormones involved in osmoregulation and reproduction in all vertebrates (Acher, 1996). They are classified into the VP and OT families based on the amino acid residue present at position eight (*Table 4.2*). Those peptides belonging to the VP family possess a basic amino acid at position eight whilst the OT-related peptides contain a neutral amino acid at this position. The earthworm VP/OT peptide, annetocin contains a neutral amino acid at position eight (Threonine; *Table 4.2*), hence can be classified as an OT-related peptide.

In humans the VP/OT peptides are synthesised in the hypothalamus together with their respective "carrier" proteins, the neurophysins (Brownstein *et al.*, 1980). These peptides are then packaged into vesicles and stored in the posterior pituitary from where secretion into the bloodstream takes place (Brownstein *et al.*, 1980). VP and OT are synthesised in the form of a common precursor, which is later cleaved by proteolysis to yield the biologically active peptides. Whilst packaged in neurosecretory vesicles the hormone (nonapeptide) and neurophysin (NP) form a stable complex via noncovalent interactions (Rholam *et al.*, 1982). Once released into the blood these noncovalent bonds between hormone and NP are broken yielding mature, biologically active nonapeptide and free NP (Brewslow & Burman, 1990). It appears from the structure of annetocin that this may also be the case in the earthworm. The annetocin gene is expressed as a preprohormone containing a 5'-signal peptide, nonapeptide and a NP domain (Satake *et al.*, 1999). By comparison with known mechanisms in mammals the synthesised preprohormone would be cleaved by proteolysis to produce the active nonapeptide and NP. In fact, annetocin

contains a dibasic amino acid cleavage site immediately after the nonapeptide itself and prior to the NP domain. It is not known whether the annetocin nonapeptide interacts with its NP peptide in a similar fashion as oxytocin.

Table 4.2: Primary amino acid sequences of members of the vasopressin/oxytocin superfamily.[1]

Amino Acid Sequence										Name	Animal
Vasopressin-like Peptides											
C*	F	I	R	N	C*	P	K	G	NH_2	Lysine-Conopressin	*Lymnaea stagnalis*
C*	I	I	R	N	C*	P	R	G	NH_2	Arginine-Conopressin	*Conus striatus*
C*	L	I	T	N	C*	P	R	G	NH_2	Diuretic Hormone	*Locusta migratoria*
C*	Y	F	Q	N	C*	P	R	G	NH_2	Arginine-Vasopressin	*Homo sapiens*
Oxytocin-like Peptides											
C*	Y	F	R	N	C*	P	I	G	NH_2	Cephalotocin	*Octopus vulgaris*
C*	Y	I	S	N	C*	P	I	G	NH_2	Isotocin	*Danio rerio*
C*	F	I	Q	N	C*	P	I	G	NH_2	Mesotocin	*Neoceratodus forsteri*
C*	F	V	R	N	C*	P	T	G	NH_2	Annetocin	*Eisenia fetida*
C*	Y	I	Q	N	C*	P	L	G	NH_2	Oxytocin	*Homo sapiens*

[1] Adapted from Salzet, 2001.

To determine whether the earthworm oxytocin-homologue, annetocin, was present in three earthworm species (*E. fetida*, *E. andrei* and *L. rubellus*), a simple PCR-based method was developed. The published annetocin DNA sequence from *E. fetida* (Satake *et al.*, 1999) was used to design a number of oligonucleotide PCR primers (*Appendix C*).

The PCR products from *E. fetida* and *E. andrei* (*Figure 4.2*) were sequenced and found to be 420 bp and 408 bp in length (*Figure 4.3*). These sequences were not identical, but showed a high level of identity in biologically important areas of the

gene. The mammalian oxytocin precursor contains three features key to biological function. These features are;

1. DNA sequence encoding the oxytocin nonapeptide.
2. DNA sequence encoding the dibasic protein cleavage site.
3. DNA sequence encoding the fourteen cysteine residues of the NP domain.

Each of these key features was conserved in the annetocin precursor isolated from *E. fetida* and *E. andrei*. This indicated that these earthworm species possess the annetocin precursor that has the potential to be biologically active. This was further confirmed by the prediction of the amino acid sequences of the two earthworm annetocin precursors (*Figure 4.5*).

In terrestrial ecotoxicology molecular biomarkers have come under considerable criticism (*Section 1.2 & 1.3*). Molecular biomarkers have great potential as 'early-warning' indicators of toxic effect on individuals and populations. However, for biomarkers to achieve their potential they must have ecological relevance and be applicable to a range of different species. In this Chapter annetocin was isolated from three earthworm species. The epigeic earthworm, *L. rubellus* has a very different growth strategy than the other two species used (*Section 1.1*). The main aim of this chapter was to isolate annetocin from the three species to show that the earthworm sex hormone was a potentially strong molecular biomarker. A partial annetocin DNA sequence was isolated from *L. rubellus* (*Figure 4.4*). This partial sequence showed that *L. rubellus* possessed the area of the annetocin gene encoding for the annetocin

nonapeptide. It was deemed unnecessary to identify the full DNA sequence of annetocin from *L. rubellus*, as the nonapeptide area was the most important part for downstream applications (i.e. a dual-labelled oligonucleotide probe was designed to bind to the nonapeptide region; see *Chapters 5 & 6*).

The anatomical distribution of annetocin gene expression in *E. fetida* was assessed (*Figure 4.7*). Maximal annetocin expression was seen within those tissues containing the earthworm reproductive organs (segment numbers 15 – 30). Furthermore, there was no annetocin expression in tail tissue. This indicated that annetocin is only expressed in tissue types where reproductive processes take place.

These data strongly suggested that annetocin is involved in the reproductive processes of the earthworm. Therefore, it was hypothesised that the expression of the annetocin gene would be modulated upon exposure to soil polluted and this would be correlated with a similar change in cocoon production of those earthworms exposed. It is known that heavy metals are capable of interfering with the reproductive success of earthworms (Spurgeon *et al.*, 2000b). Therefore, the following sections deal with the effects of heavy metal pollution on earthworm reproduction and annetocin gene expression.

CHAPTER 5

DISRUPTION OF EARTHWORM REPRODUCTION BY HEAVY METALS

5.1 INTRODUCTION AND APPROACH

Heavy metals have been shown to influence the reproductive output of many earthworm species (*Section 1.2.1*). In these studies estimates of reproduction are based on changes in the rate of cocoon production, this trait being considered of high ecological relevance for population dynamics. Although this is a recognised and valid method of assessing reproduction in earthworms, it gives no insight into the mechanisms underlying the reproductive process or the manner in which the specific toxicant interacts with the reproductive mechanism.

As with all physiological processes reproduction begins with the expression of specific genes that regulate its course. In earthworms, annetocin has been identified as a key neuropeptide with a notable role in orchestrating the reproductive pathway (Oumi *et al.*, 1994; 1996; Satake *et al.*, 1999; *Chapter 4* of this Thesis). This chapter aims to provide evidence for a link between reproductive rate and the expression (activity) of annetocin. To do this, annetocin gene expression was measured in earthworms exposed to a number of different heavy metals both in the laboratory and a semi-field situation. Two exposure regimes have been utilised, using two earthworm species known to have different sensitivities to heavy metals (Spurgeon *et al.*, 2000b).

1. *Eisenia fetida* exposed to soil collected from an abandoned lead/zinc mine – exposure in the laboratory over a 21 day period.
2. *Lumbricus rubellus* exposed to soil collected from a lead/zinc smelting works – exposure in semi-field (mescosms) over a 70 day period.

In both cases, previous work (e.g. Spurgeon & Hopkin, 1995) indicted that exposure to the selected soils was likely to have an inhibitory effect on reproduction due to the high concentrations of metals present. By measuring reproduction rates and annetocin gene expression levels in these worms, it was thus planned to investigate the relationship between the two parameters.

5.2 RESULTS

(A) MEASUREMENT OF ANNETOCIN GENE EXPRESSION IN *EISENIA FETIDA*

5.2.1 METAL RESIDUE ANALYSIS – EXTENT OF METAL EXPOSURE

Lead and zinc concentrations in the two 'test' soils and earthworms exposed to them were determined as detailed in *Section 2.2.2*. Reference soil contained low levels of both the metals in comparison with the mine soil (*Table 5.1* and *Figure 5.5; Panel A*). Earthworms exposed to the reference soil contained measurable levels of both lead and zinc (*Table 5.1*); in the case of zinc the recorded values are within the physiological range of this essential metal, whilst lead values represent the normal body load (Spurgeon & Hopkin, 1996a). Exposure of *Eisenia fetida* to the metalliferous soil greatly increased the body loads of Pb and Zn. In the case of Zn, an approximately six-fold increase in tissue concentration compared with controls, was recorded; in the case of Pb, in excess of a twenty-fold increase in Pb tissue concentration was recorded compared with reference earthworms (*Table 5.1*).

Table 5.1: Metal measurements (µg/g dry weight) made in reference and metalliferous soils and *Eisenia fetida* (exposed over a 21-day period).

	Zn	Pb
Reference soil	1.8 (± 0.3)	4.30 (± 0.998)
Reference Earthworms	101 (± 10.0)	16.8 (± 3.84)
Metalliferous soil	1820 (± 112.9)	592 (± 37.6)
Metalliferous Earthworms	686 (± 209.0)	373 (± 53.7)

*Data presented as x ± SEM
*'n'(soils) = 4; 'n'(worms) = 8

5.2.2 SURVIVAL OF *EISENIA FETIDA* – METAL TOXICITY

Eisenia fetida exposed to a reference soil (Kettering loam) showed 95% survival, i.e. a value above the 90% survival limit in control soil is deemed to define a valid toxicity test according to OECD (1984) guidelines. Survival of earthworms exposed to the Pb/Zn contaminated mine soil was markedly lower, 65%. This effect of polluted field soil on *Eisenia fetida* is in line with other studies (Spurgeon & Hopkin, 1996b).

5.2.3 LIFE-CYCLE EFFECTS IN *EISENIA FETIDA* – SUBLETHAL EFFECTS

Growth and reproduction of *Eisenia fetida* were assessed to determine sublethal effects on life-cycle traits. Reproductive success was measured by removing and counting the number of cocoons produced by the earthworms at the end of the exposure period (as described in *Section 2.2.2*). This allowed the calculation of cocoon production rates (number of cocoons/worm/week) in each of the two soils used. Reproductive rates were highest in the reference soil (0.183 cocoons/worm/week), while in the metalliferous soil the rates were decreased ten-fold

(0.017 cocoons/worm/week) (see *Figure 5.5; Panel B*). Growth was also compromised by exposure to a Pb/Zn containing soil (data not shown).

5.2.4 TOTAL RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated from animals exposed to both reference and metalliferous soils as previously described (see *Section 2.2.8*). Total RNA quality was assessed by electrophoresis on a 1% agarose gel containing EtBr (0.4 μg/ml) and visualised under U.V. light (data not shown) as described in *Section 2.2.11*. A volume of total RNA was used to give 2.0 μg of RNA in each reverse transcription reaction (as previously described, *Section 2.2.15*) and cDNA quality was assessed through the PCR amplification of a fragment of the *β*-actin gene (see *Section 4.2.2*).

5.2.5 GENE EXPRESSION IN *EISENIA FETIDA* – REPRODUCTIVE DISRUPTION

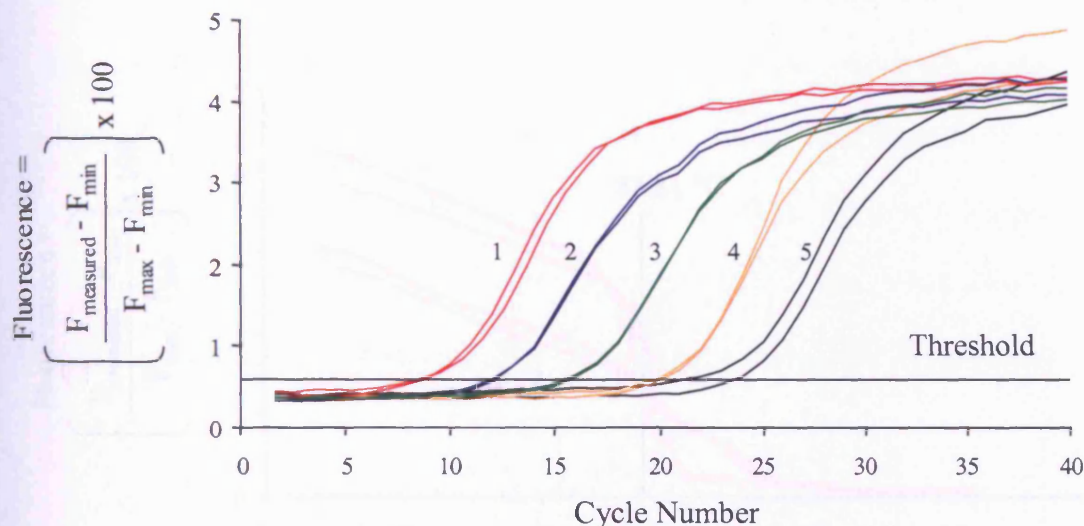
Gene expression of the earthworm sex hormone, annetocin (see *Chapter 4*) was measured using a micro volume rapid PCR method described in *Section 2.2.25*. Two genes were amplified using gene-specific oligonucleotide primer pairs so that only the gene of interest was amplified (*Figure 5.2* and *Figure 5.4*).

A 'house-keeping' gene was selected (*β*-actin) as an invariant internal control (Stürzenbaum, 1997). A 198 bp *β*-actin gene fragment was amplified by PCR and cloned into the plasmid vector, pGEM-T (Promega Ltd., Southampton, U.K.). Plasmid DNA containing the *β*-actin gene fragment was isolated from transformed *E. coli* bacterial colonies (described in *Section 2.2.20*) and used as a standard in all real-time PCR amplifications. A serial dilution (with concentrations ranging from 1,000 *ρ*g/μl to 0.1 *ρ*g/μl) of this plasmid DNA was made and real-time PCR was performed

on a LightCycler™ (Idaho Technologies Inc.) using the fluorescent dye, SYBR® Green I (*Figure 5.1; Panel A*). This dilution series was used to optimise the real-time PCR reaction for $MgCl_2$ and SYBR® Green I concentrations. In the case of the *β*-actin plasmid standard a concentration of 3.0 mM (final concentration) $MgCl_2$ was found to be optimal along with a 1:500 dilution of SYBR® Green I.

Transcript frequency was calculated by regression analysis from known standards (*Figure 5.1; Panel B*). The specificity of the PCR reaction was determined by melting point analysis (*Figure 5.2*). The PCR product obtained from the *β*-actin amplification showed a higher melting temperature than that shown by the production of oligonucleotide primer-dimers in the control amplification reaction (no DNA template added) (*Figure 5.2*). This indicated that a specific PCR product relating to the *β*-actin gene fragment was amplified. This was also confirmed by the visualisation of the PCR product via electrophoresis on an agarose gel containing ethidium bromide (*Section 2.2.11*) (data not shown).

Panel A:



Panel B:

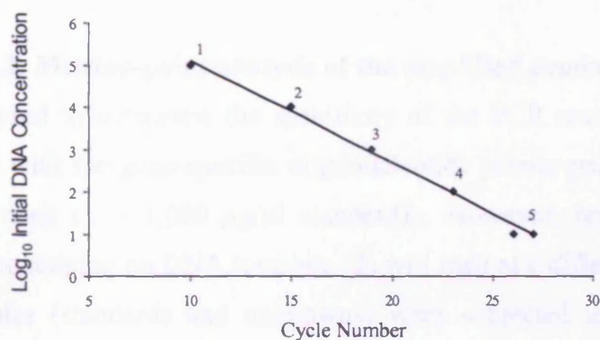


Figure 5.1: Quantitative amplification of the β-actin standard dilution series. Panel A: β-actin standard dilution series analysed in duplicate. Initial DNA concentrations range from 1-5 representing 1,000 ρg/µl to 0.1 ρg/µl. The threshold is an arbitrary point, set at a level above background and within the linear amplification range. Panel B: illustrates the regression analysis obtained from the standard dilution series, concentrations range from 1-5 representing 1,000 ρg/µl to 0.1 ρg/µl.

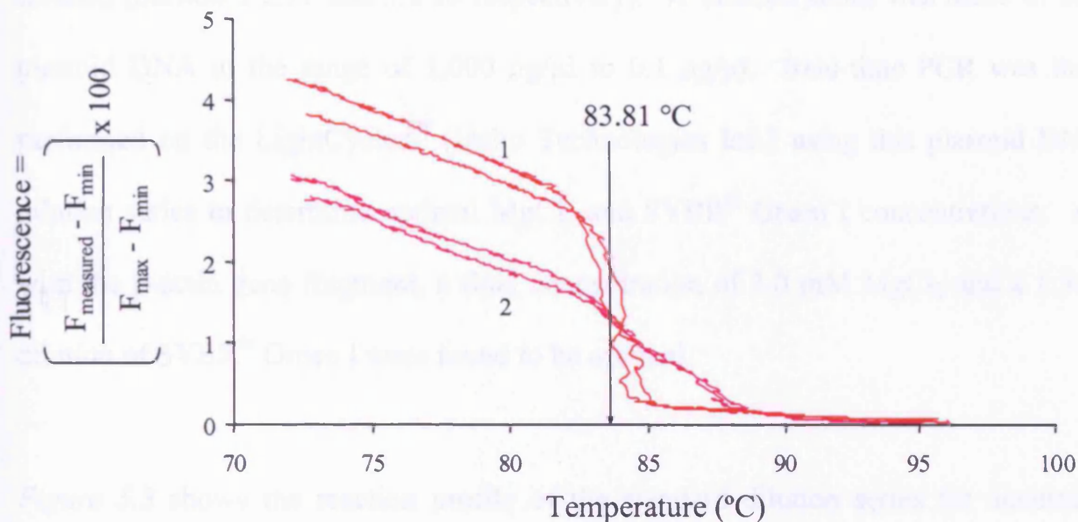


Figure 5.2: Melting-point analysis of the amplified product of β-actin. Each PCR product was analysed to determine the specificity of the PCR reaction. The specific PCR product produced with the gene-specific oligonucleotide primer pair melts at the same point for all amplifications (1 = 1,000 ρg/µl standard). However, primer-dimers formed in the PCR reaction containing no DNA template (2) will melt at a different temperature as shown above. All samples (standards and unknowns) were subjected to melting-point analysis, but for simplicity only two PCR reactions are shown here.

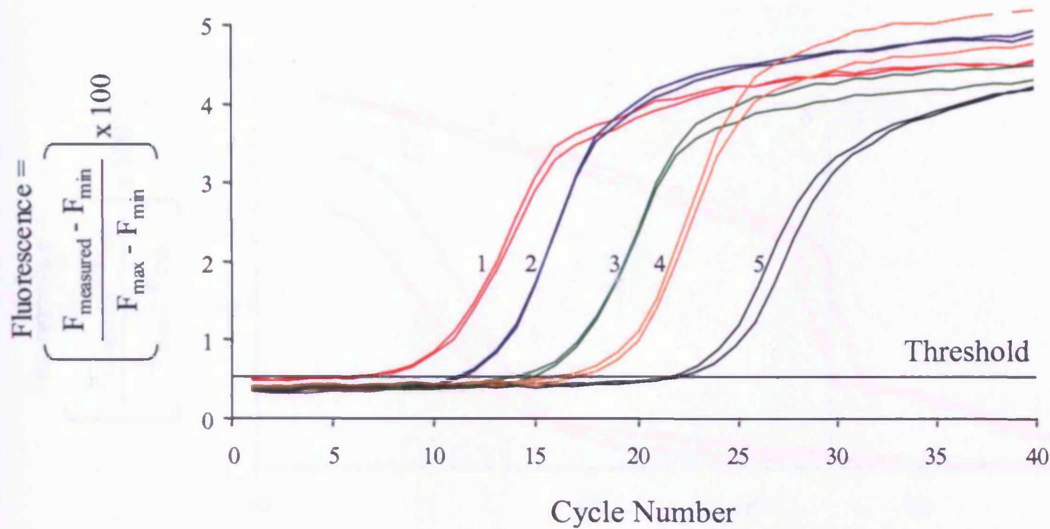
A similar process was followed with the 'test' gene, annetocin. A gene-specific oligonucleotide primer pair (*Appendix C*) was designed to amplify a 420 bp fragment of the annetocin gene from cDNA. This fragment was cloned and plasmid DNA isolated (*Section 2.2.17* and *2.2.20* respectively). A dilution series was made of this plasmid DNA in the range of 1,000 ρg/μl to 0.1 ρg/μl. Real-time PCR was then performed on the LightCycler™ (Idaho Technologies Inc.) using this plasmid DNA dilution series to determine optimal $MgCl_2$ and SYBR® Green I concentrations. As with the β-actin gene fragment, a final concentration of 3.0 mM $MgCl_2$ and a 1:500 dilution of SYBR® Green I were found to be optimal.

Figure 5.3 shows the reaction profile of the standard dilution series for annetocin (*Panel A*) and the regression analysis taken from this profile (*Panel B*). Standard amplifications were performed in duplicate along with a negative control containing no DNA. Melting-point analysis using the negative control (*Figure 5.4*) showed that a specific product was being amplified. This was also confirmed by visualisation of the PCR product on an agarose gel containing ethidium bromide (data not shown).

Separate reactions were performed on the LightCycler™ (Idaho Technologies Inc.) for β-actin and annetocin. Each cDNA sample was subjected to real-time PCR in triplicate, and transcript levels were determined using the regression analysis from each set of standard amplifications. The ratio of annetocin:β-actin gene expression gave a relative annetocin expression value (*Figure 5.5; Panel C*). Earthworms maintained on unpolluted, reference soil exhibited the highest relative annetocin expression value (2.190 ± 0.917) (*Figure 5.5; Panel C*). Earthworms exposed to the mine site soil (containing high levels of lead and zinc) experienced in excess of a

twenty-fold reduction in annetocin gene expression (0.104 ± 0.028) (*Figure 5.5; Panel C*). This lowering of annetocin gene expression was significant at $p \leq 0.05$ (Mann-Whitney 'U' test, as described in *Section 2.2.7*).

Panel A:



Panel B:

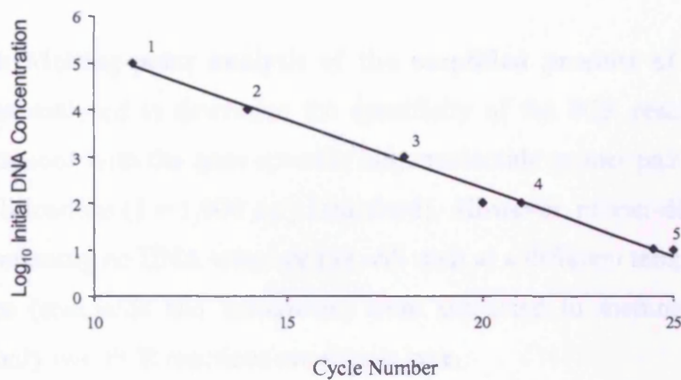


Figure 5.3: Quantitative amplification of the annetocin standard dilution series. Panel A: Annetocin standard dilution series analysed in duplicate. Initial DNA concentrations range from 1-5 representing 1,000 ρg/μl to 0.1 ρg/μl. The threshold represents the point at which DNA amplification begins. Panel B: illustrates the regression analysis obtained from the standard dilution series, concentrations range from 1-5 representing 1,000 ρg/μl to 0.1 ρg/μl.

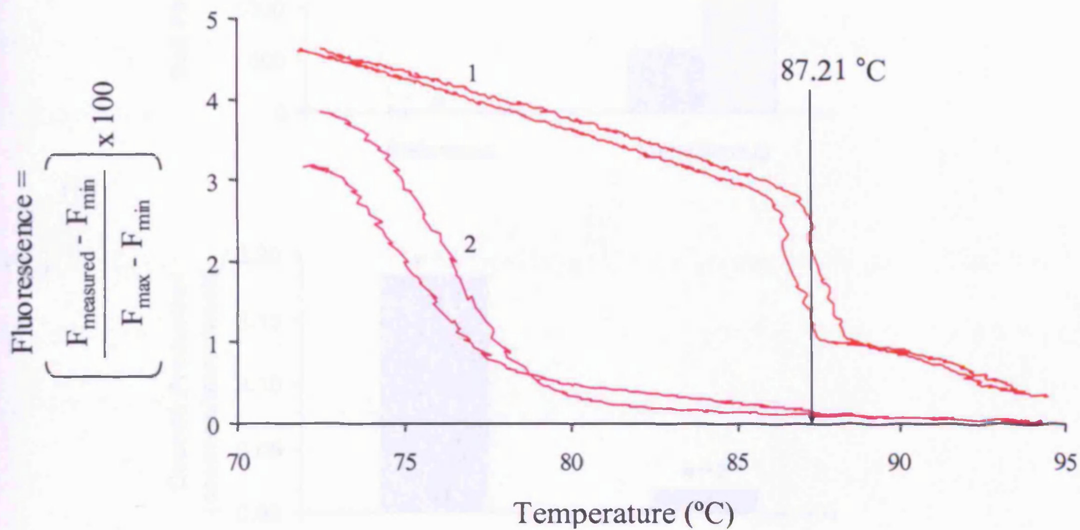


Figure 5.4: Melting-point analysis of the amplified product of annetocin. Each PCR product was analysed to determine the specificity of the PCR reaction. The specific PCR product produced with the gene-specific oligonucleotide primer pair melts at the same point for all amplifications (1 = 1,000 ρg/μl standard). However, primer-dimers formed in the PCR reaction containing no DNA template (2) will melt at a different temperature as shown above. All samples (standards and unknowns) were subjected to melting-point analysis, but for simplicity only two PCR reactions are shown here.

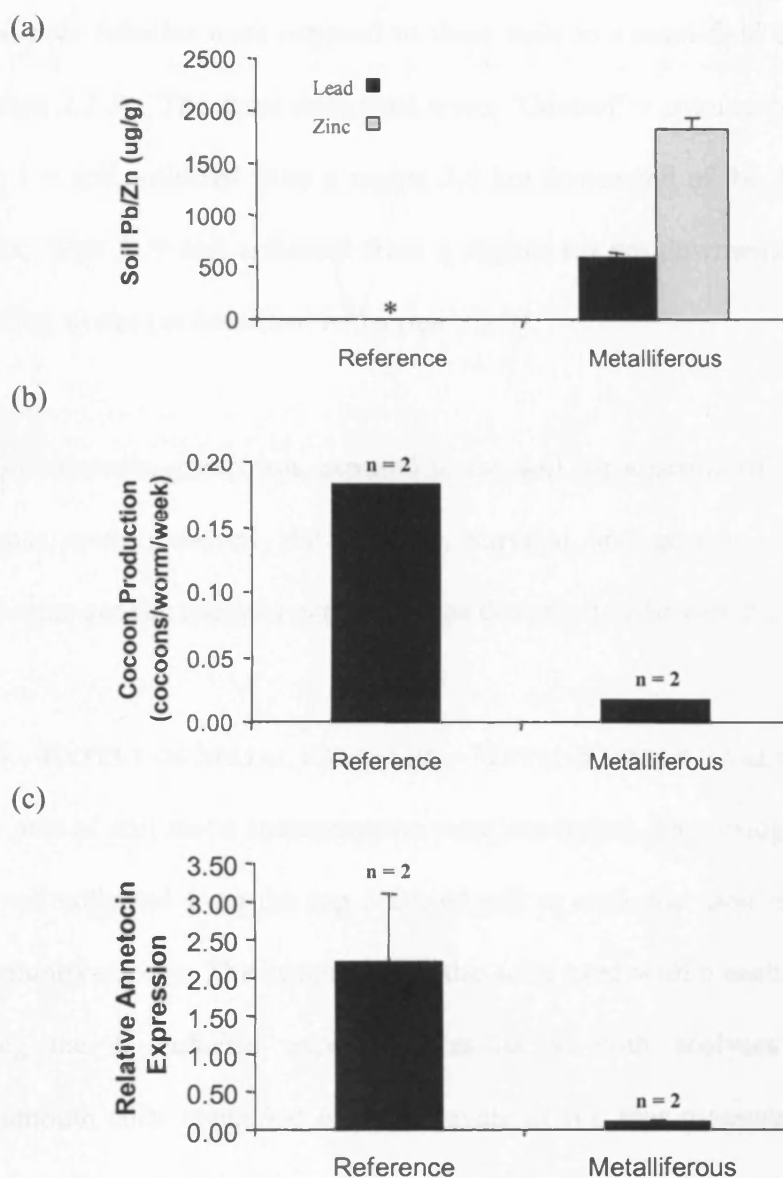


Figure 5.5: Analysis of the impact of a Pb/Zn-containing soil on the reproductive success of *Eisenia fetida*. Panel A: The 'total' lead and zinc concentrations of the two soils used in the exposure protocol (*see *Table 5.1* for reference soil values). Panel B: The cocoon production rates of the two earthworm groups were used to measure their reproductive success. Panel C: Quantitative analysis of relative annetocin gene expression (ratio of "test" gene to "internal control" gene, i.e. annetocin:β-actin) of the two earthworm groups. The experimental analyses were performed on amplifications free of primer artefacts, including all standards (in duplicate) and all "unknowns" (in triplicate). Statistical analysis was performed using the Mann-Whitney 'U' test (Minitab V. 13.1, Minitab Corporation Inc.). Error bars indicate the Standard Error of the Mean.

(B) MEASUREMENT OF ANNETOCIN GENE EXPRESSION IN *LUMBRICUS RUBELLUS*

Lumbricus rubellus were exposed to three soils in a semi-field exposure experiment (*Section 2.2.3*). The three soils used were; 'Control' = commercially available loam; 'Site 1 = soil collected from a region 3.6 km downwind of the Avonmouth smelting works; 'Site 2' = soil collected from a region 1.0 km downwind of the Avonmouth smelting works (as described in *Section 2.2.3*).

Each earthworm group was exposed to the soil for a period of 70 days, after which cocoons were removed and counted, survival and growth were determined and molecular-genetic analysis performed (as described in *Section 2.2.26*).

5.2.6 EXTENT OF METAL EXPOSURE – METAL RESIDUE ANALYSIS

Two sets of soil metal measurement were conducted. One using a pooled sample of site soil collected from the top 5 cm of soil at each site used in the population and community survey. The second using the soils used within each replicate mesocosm during the *L. rubellus* exposure. Results of both analyses indicated that the Avonmouth soils contained elevated levels of the four measured metals (cadmium, copper, lead and zinc). There was a significant linear relationship between log metal concentration and distance of site from the smelter source. Metal concentrations were highest in soils close to the factory and decreased exponentially from the source.

Table 5.2: Metal measurements ($\mu g\ g^{-1}$) made in control and metalliferous soils collected from the Avonmouth smelting works site.

	Cd	Cu	Pb	Zn
Control	0.05 ± 0.00	12.4 ± 0.7	17.2 ± 4.2	47 ± 2
Site 1	66.6 ± 1.1	326 ± 11	2,783 ± 95	4,990 ± 111
Site 2	177 ± 11	947 ± 62	5,962 ± 499	11,427 ± 855

•Standard Error of the mean (SEM) is given
•Four replicate mesocosms were used for each site

5.2.7 SURVIVAL OF ADULT *LUMBRICUS RUBELLUS* – METAL TOXICITY

Adult *L. rubellus* survival was measured in each of the three soils used after 70 days exposure (*Section 2.2.3*; *Figure 5.6*). Those earthworms maintained on 'control' soil showed a relatively low survival (68.75%). At Site 1, survival was comparable with that of the 'control' group (62.50%), whilst adult survival at Site 2 (closest to the smelting works) was significantly decreased (20.83%; $p \leq 0.05$, Mann-Whitney 'U' test).

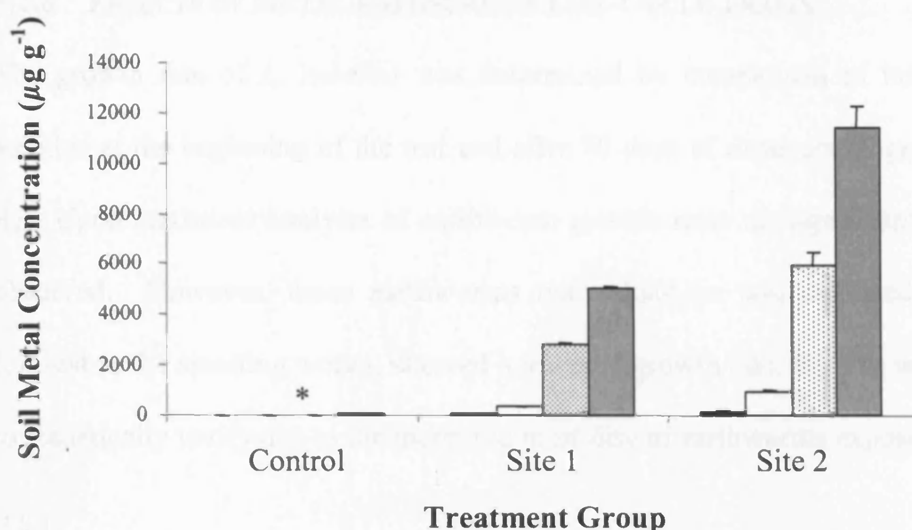


Figure 5.6: Metal residue analyses of those soils used in the exposure protocol. The 'total' Cd (black), Cu (Clear), Pb (Dotted) and Zn (Grey) concentrations of the soils used in the Avonmouth exposure experiment (*see *Table 5.2* for control soil values). Error bars indicate the Standard Error of the Mean.

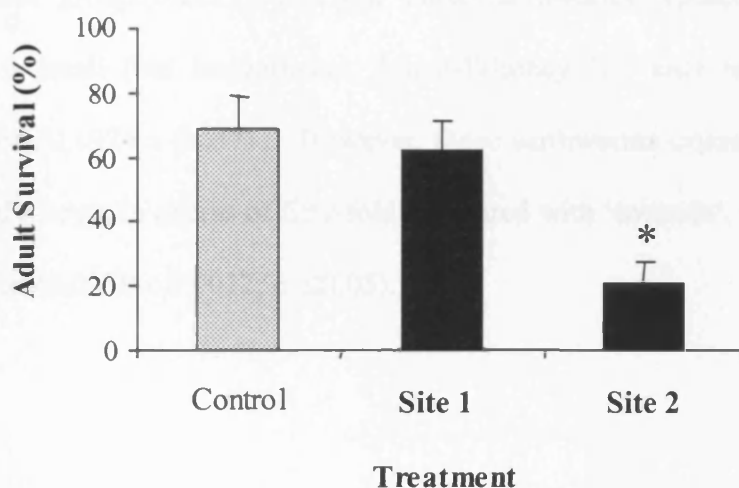


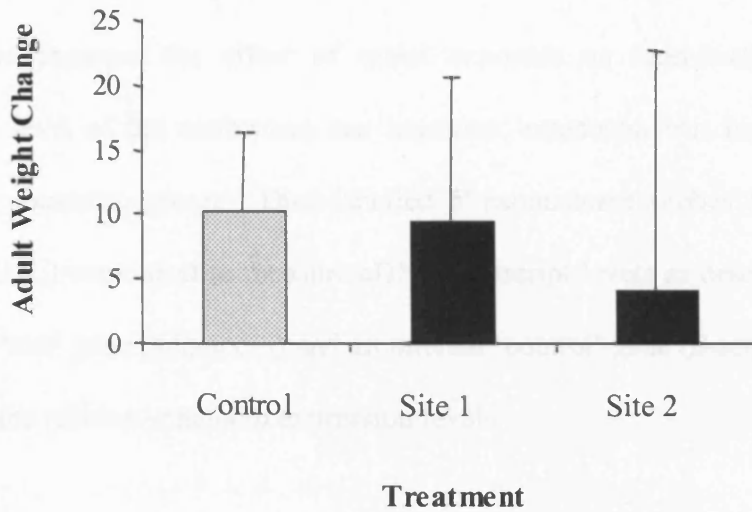
Figure 5.7: Adult *Lumbricus rubellus* survival when exposed to metal containing soils. Percentage survival of adult *Lumbricus rubellus* was determined after 70 days of exposure to the three soils. Control soil was unpolluted, commercially-available, Kettering loam, Site 1 (x km downwind of smelter) and 2 (x km downwind of smelter) were those soils collected from the vicinity of the Avonmouth smelting works. Error bars indicate the Standard Error of the Mean. (* = significantly different from control survival).

5.2.8 EFFECTS OF METAL EXPOSURE ON LIFE-CYCLE TRAITS

The growth rate of *L. rubellus* was determined by comparison of individual fresh weights at the beginning of the test and after 70 days of exposure (*Figure 5.8; Panel A*). Upon statistical analysis of earthworm growth rates no significant effects were observed. However, those earthworms maintained on soil collected from Site 2 (closest to the smelting works) showed a lowered growth rate, but this was impossible to statistically verify due to the increased mortality of earthworms exposed to this soil.

Cocoon production (number of cocoons/worm/week; based on the number of added earthworms) was determined by counting the number of cocoons produced over the exposure period in each of the unexposed/exposed populations (as described in *Section 2.2.3*) (*Figure 5.8; Panel B*). Highest cocoon production rates were seen in the 'control' group (0.2115 ± 0.0207). Those earthworms exposed to soil from Site 1 showed a small (but insignificant, Mann-Whitney 'U' test) reduction in cocoon production (0.1974 ± 0.0371). However, those earthworms exposed to soil from Site 2 showed a large, in excess of fifty-fold compared with 'controls', reduction in cocoon production (0.0038 ± 0.0022; $p \leq 0.05$).

Panel A:



Panel B:

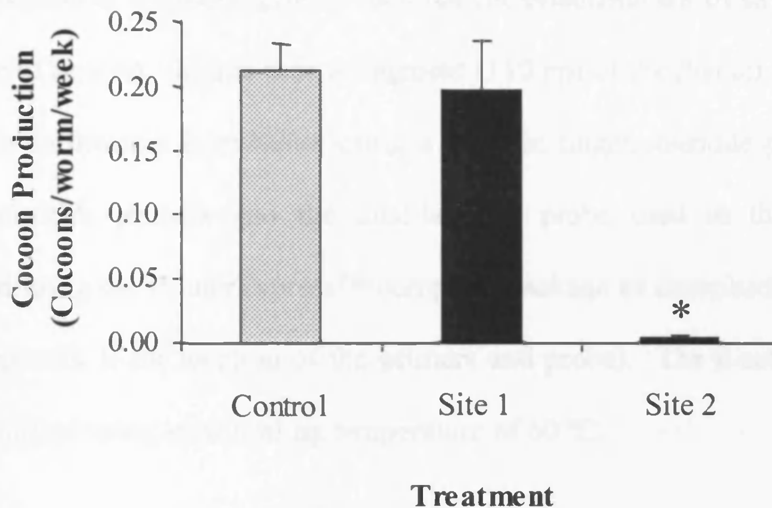


Figure 5.8: Sub-lethal effects on *Lumbricus rubellus* by metal exposure in Avonmouth soil. Panel A: the average weight change of adult *Lumbricus rubellus* over the 70 day exposure period. This was calculated using weights at the start of the test, compared to weights at the end of the test. **Panel B:** The average cocoon production rate by earthworms exposed to each of the different soils. Statistical significance is indicated by the asterix ($p \leq 0.05$; Mann-Whitney 'U' test). All test were carried out in four replicates (i.e. $n = 4$). Error bars indicate the Standard Error of the Mean.

5.2.8 REAL-TIME QUANTITATIVE PCR AMPLIFICATIONS – DUAL-LABELLED FLUORESCENT PROBES

In order to measure the effect of metal exposure on reproductive output, the expression level of the earthworm sex hormone, annetocin, was measured in each earthworm exposure group. Dual-labelled 5'-exonuclease probes (QIAGEN Ltd., Crawley, U.K.) were used to measure cDNA transcript levels as described in *Section 2.2.26.* A 'test' gene (annetocin) and an internal 'control' gene (β-actin) were used to determine the relative annetocin expression levels.

(a) β-actin "control" gene analysis

Quantification of a control gene is required for establishment of expression levels of the gene of interest. In this case a fragment (110 bp) of the β-actin gene was isolated from the earthworm *L. rubellus* using a specific oligonucleotide primer pair. The oligonucleotide primers and the dual-labelled probe used in this protocol were designed using the PrimerExpress™ computer package as described in *Section 2.2.14* (see Appendix B for location of the primers and probe). The β-actin gene fragment was amplified using an annealing temperature of 60 °C.

This fragment was cloned into the plasmid DNA vector, pGEM-T (Promega Ltd., Southampton, U.K.) and confirmed by DNA sequencing as described in *Section 2.2.17* and *Section 2.2.22* respectively. The qPCR reaction was optimised as described in *Section 2.2.26c* using 1 ng of plasmid DNA containing the target sequence. This gave the optimal final concentrations of both the oligonucleotide primers and the dual-labelled probe. For both the Actin Forward Primer (3') and the Actin Reverse Primer

(5') this was found to be 0.9 μM (final concentration). The optimal dual-labelled probe concentration for *β*-actin was found to be 0.125 μM (final concentration).

A dilution series was produced using plasmid DNA containing the cloned *β*-actin fragment and qPCR was performed as described in *Section 2.2.26d.* The amplification of the standard dilution series was validated visually (*Figure 5.9 Panel A*) and assessed for signal intensity as described in *Section 2.2.26d.* Regression analysis was performed to generate a standard curve (*Figure 5.10 Panel B*) which was utilised to determine gene expression levels within the cDNA samples.

(b) Annetocin "test" gene analysis

Annetocin transcript levels were measured in cDNA samples produced from metal exposed and control earthworms. A 107 bp fragment of the annetocin gene was amplified for the earthworm *L. rubellus* using gene specific oligonucleotide primers as described above and in *Section 2.2.26* (PCR cycling parameters were identical to those stated above). The resulting amplified PCR fragment was cloned and sequenced. Plasmid DNA containing the cloned annetocin fragment was used to optimise the qPCR amplification as described in *Section 2.2.26d.* Both annetocin primers (forward and reverse, see Appendix C) were found to be most effective at final concentrations of 0.9 μM. The annetocin dual-labelled oligonucleotide probe was found to hybridise most efficiently to its target sequence at a final concentration of 0.2 μM.

A dilution series was produced using plasmid DNA containing the cloned annetocin fragment and qPCR was performed as described in *Section 2.2.26d.* The amplification of the standard dilution series was validated visually (*Figure 5.10 Panel A*) and

assessed for signal intensity. Regression analysis was performed to generate a standard curve (*Figure 5.11 Panel B*) which was utilised to determine gene expression levels within the cDNA samples as described in *Section 2.2.26b.*

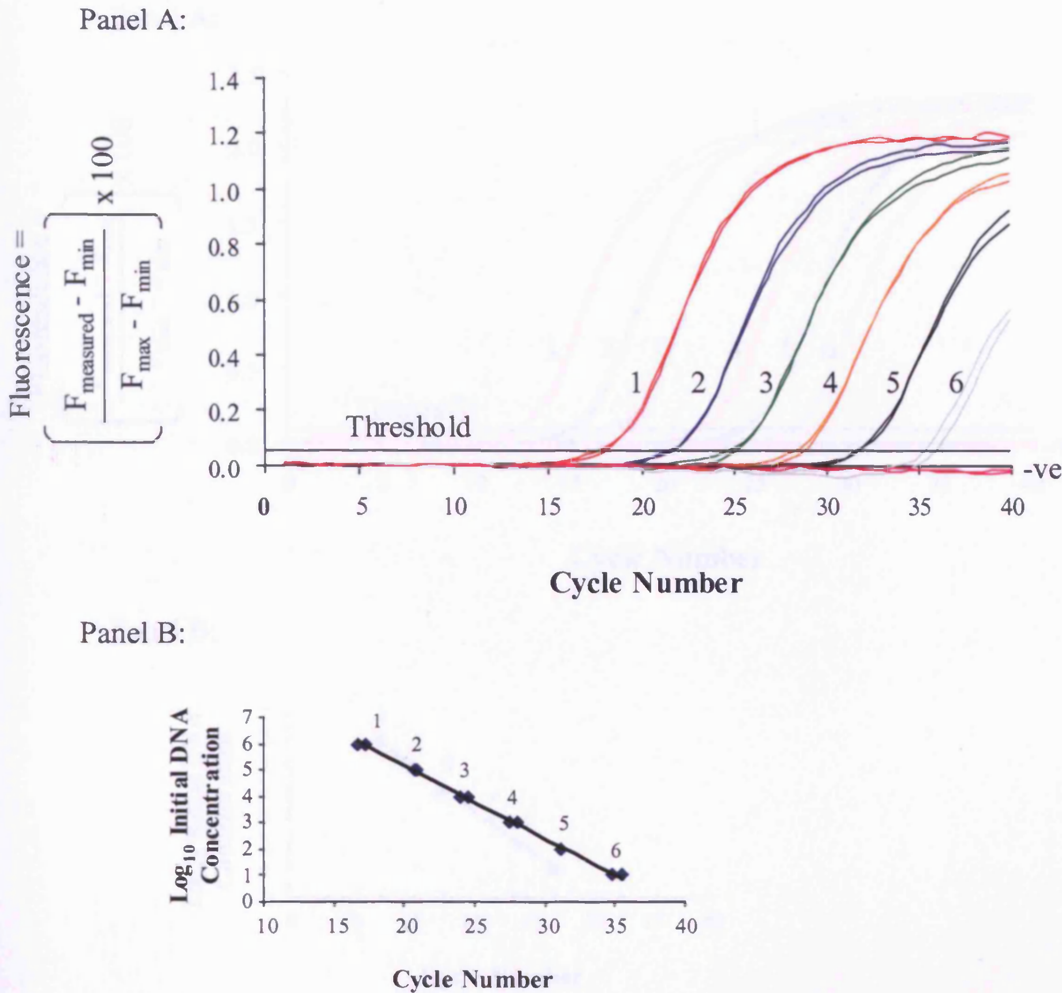


Figure 5.9: Quantitative PCR amplification of a serial dilution of β-actin standards isolated from *Lumbricus rubellus*. Panel A: shows the PCR amplification of a serial dilution of the β-actin standards (1 – 6). The template concentrations in each standard are represented by the numbers 1 – 6, which corresponded to a range of 100 ρg/µl to 0.001 ρg/µl. The amplification plot labelled "-ve" corresponds to the PCR reaction performed with no template DNA. **Panel B:** represents the computer generated regression analysis taken from the PCR amplification of the β-actin serial dilutions (1 – 6). The numbers 1 – 6 represent the same initial template concentrations as shown in Panel A.

Panel A:

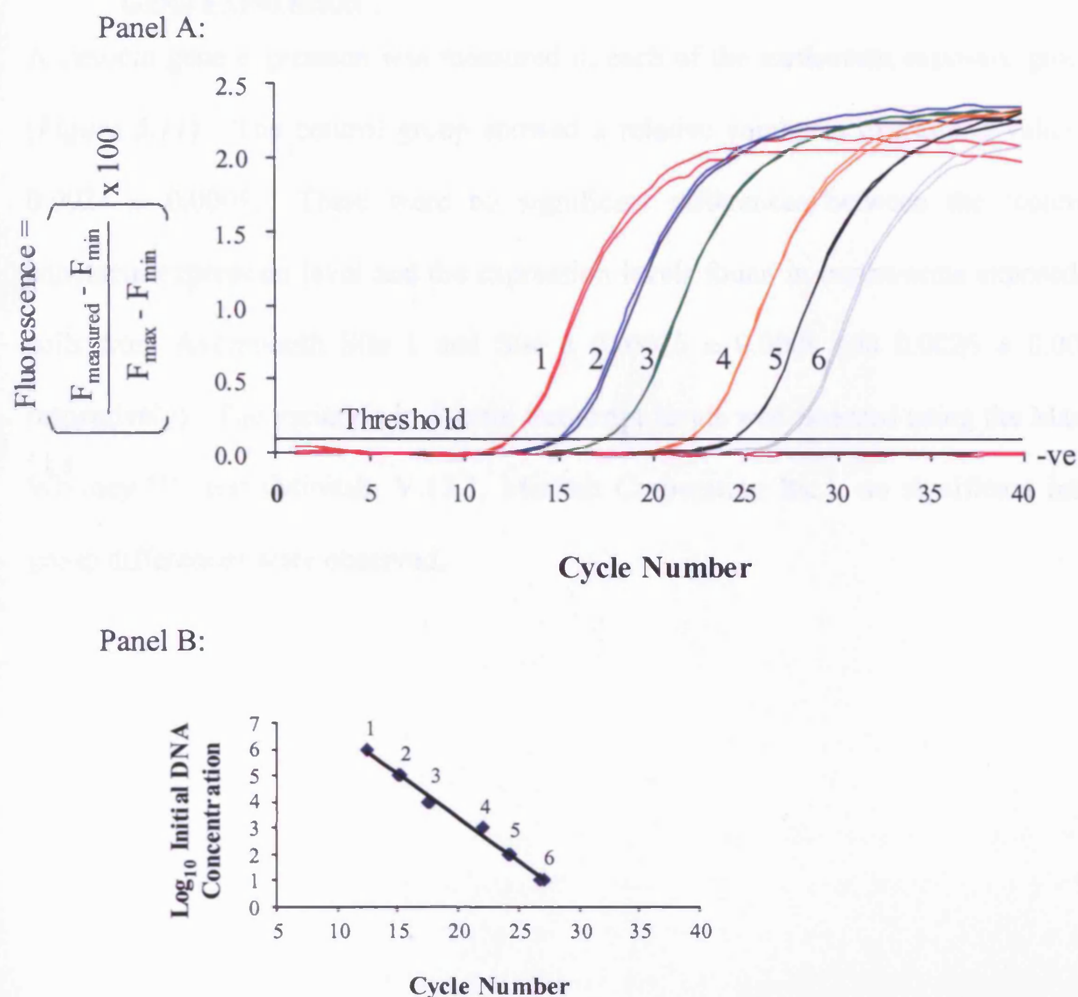


Figure 5.10: Quantitative PCR amplification of a serial dilution of annetocin standards isolated from *Lumbricus rubellus*. Panel A: shows the PCR amplification of a serial dilution of the annetocin standards (1 – 6). The template concentrations in each standard are represented by the numbers 1 – 6, which corresponded to a range of 100 ρg/μl to 0.001 ρg/μl. The amplification plot labelled "-ve" corresponds to the PCR reaction performed with no template DNA. **Panel B:** represents the computer generated regression analysis taken from the PCR amplification of the annetocin serial dilutions (1 – 6). The numbers 1 – 6 represent the same initial template concentrations as shown in Panel A.

5.2.9 MOLECULAR-GENETIC MEASUREMENT OF REPRODUCTION – ANNETOCIN GENE EXPRESSION

Annetocin gene expression was measured in each of the earthworm exposure groups (*Figure 5.11*). The control group showed a relative annetocin expression value of 0.0024 ± 0.0008. There were no significant differences between the 'control' annetocin expression level and the expression levels found in earthworms exposed to soils from Avonmouth Site 1 and Site 2 (0.0015 ± 0.0008 and 0.0026 ± 0.0006 respectively). The variation in β-actin transcript levels was assessed using the Mann-Whitney 'U' test (Minitab, V.13.1, Minitab Corporation Inc.); no significant inter-group differences were observed.

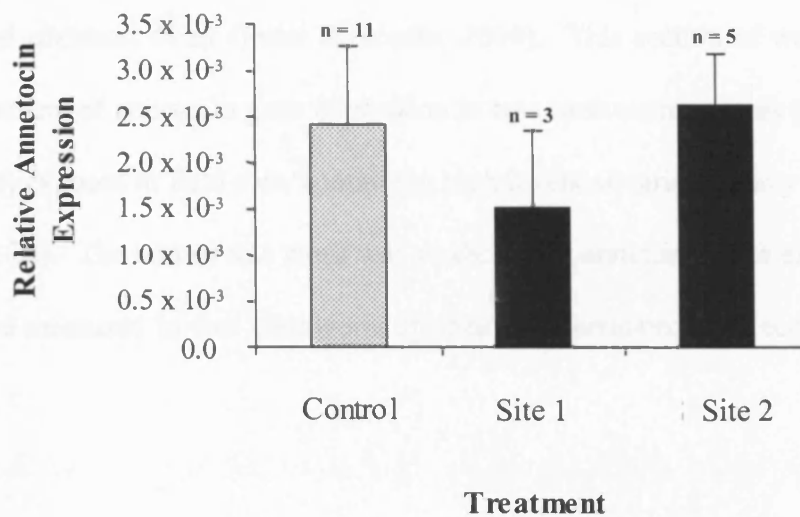


Figure 5.11: Relative annetocin gene expression in adult *Lumbricus rubellus* exposed to soils collected from the vicinity of the Avonmouth smelting works. Relative annetocin gene expression was determined by quantitative PCR using a dual-labelled 5'-exonuclease hybridisation probe. An internal, invariant control (β-actin) was used to measure variations between transcription efficiency. Therefore, relative annetocin gene expression is a ratio of annetocin transcript levels to β-actin transcript levels. No statistically significant differences were observed between groups (Mann-Whitney 'U' test). Error bars indicate the Standard Error of the Mean.

5.3 DISCUSSION

For a molecular genetic biomarker to be deemed valid in ecotoxicology it is critical that the marker can be measured in different species being exposed to different chemical toxicants (Van Gestel & Weeks, 2004). This section of work covered the measurement of annetocin gene expression in two earthworm species (*E. fetida* and *L. rubellus*) exposed to field soils containing high levels of various heavy metals (Pb, Zn, Cd and Cu). The aim of this work was to show that annetocin gene expression levels could be measured in two earthworm species and correlated with cocoon production rates.

5.3.1 ANNETOCIN GENE EXPRESSION LEVELS IN *EISENIA FETIDA*

A field soil containing lead and zinc was used in a laboratory exposure protocol. These metals caused a high level of mortality in *E. fetida*, indicating a toxic effect. Although mortality is a valid and ecologically relevant test parameter for toxicity of metals and other chemicals, in recent years sub-lethal parameters, such as growth and reproduction, have been used because they are deemed to be more sensitive (Moriarty, 1983; Spurgeon *et al.*, 2000b). Therefore, cocoon production was measured in unexposed and exposed animals (*Figure 5.5; Panel B*).

Cocoon production in those animals exposed to control soil was within the range normally seen in *E. fetida* (for example; Spurgeon & Hopkin, 1996a). However, upon exposure a significant decrease in cocoon production was observed (*Figure 5.5; Panel B*). These data showed that exposure to Pb/Zn had a negative effect on the reproductive rate of *E. fetida*, although there is no evidence to suggest that Pb and Zn are capable of interacting with the physiological reproductive processes. Therefore,

we can presume that any effects on reproduction from Pb and Zn are as a result of indirect action. For example, these metals are toxic to the earthworm. Therefore, at sub-lethal exposure concentrations the animal will be defending itself against the negative effects of the metals. That is, detoxification mechanisms will become active and energy will be diverted into this rather than growth and/or reproduction. Simplistically, this would result in the individual surviving the stress of toxic exposure, but the population as a whole would decrease as no reproduction would take place.

Annetocin gene expression levels were measured in both of the treatment groups (*Figure 5.5; Panel C*). The level of gene expression correlated with cocoon production measurements. That is, in unexposed animals annetocin levels were high and significantly reduced in the exposed animals suggesting that there may be a direct mechanistic link between annetocin and reproduction. This study was the first to examine the expression levels of an invertebrate neurohormone, known to orchestrate reproductive physiology, in organisms in which pollutant exposure has been shown to reduce reproductive rate.

5.3.2 ANNETOCIN GENE EXPRESSION LEVELS IN *LUMBRICUS RUBELLUS*

For this exposure field soil was collected from the Avonmouth smelting works in the U.K. (see *Section 2.2.3*). Survival of adult *L. rubellus* exposed to the soil from Site 2 (containing the highest concentration of heavy metals) was low in comparison with animals maintained on control (clean) soil (*Figures 5.7 & 5.6*).

The low survival of animals exposed to soil from Site 2 was reflected in the growth rates of these earthworms (*Figure 5.8; Panel A*). Growth was retarded in *L. rubellus* exposed to soil from Site 2, probably due to lower feeding rates and disruption of metabolism, thereby causing changes in the energy budget of the exposed animals. Cocoon production was also effected upon exposure to soil from Site 2 (*Figure 5.8; Panel B*). This was due to the toxicity of the metals found in this soil, as at this site *in situ* no worms/cocoons are found (Spurgeon *et al.*, 1994).

When annetocin gene expression levels were measured in each of the treatment groups (*Figure 5.11*) no differences were seen. It was also found that annetocin expression levels were much lower than those found in *E. fetida* (see previous section). *E. fetida* has a much greater reproductive output than *L. rubellus*; for example, Satchell (1958) observed a cocoon production rate of 0.92 cocoons/worm/week for *L. rubellus* in culture; while Van Gestel *et al.* (1989) recorded a cocoon production rate of 2.0 cocoons/worm/week for *E. fetida*. Therefore, if the annetocin gene product is involved in reproduction/cocoon production in earthworms it would be expected that the gene level would be lower in *L. rubellus* than *E. fetida*.

Another factor in the low annetocin levels in *L. rubellus*, is that the measurements were made during the month of May. According to Evans & Guild (1948) *L. rubellus* cocoon production is beginning to decrease at this time of the year. Cocoon production in this animal has two peaks; one during the spring and one during the autumn. Therefore, annetocin gene expression would also be expected to be lower during this time of the year. The fact that annetocin gene expression (and therefore reproduction) seemed to be seasonal would limit its value as a biomarker (although

this could be overcome to some extent by fully characterising its expression profile over a year/reproductive cycle). However, these findings do increase the power of annetocin as a tool to address the reproductive biology and population dynamics of earthworms.

The results from the first study in this Chapter indicate that annetocin gene expression levels are correlated with cocoon production rates. However, the study conducted with *L. rubellus* suggests that annetocin expression may vary between species. Before annetocin can be accepted as a predictive molecular biomarker, a full-scale investigation of annetocin expression patterns in different species at different times of the year must be conducted.

The two studies detailed above were conducted to assess the potential of annetocin as a molecular genetic biomarker. However, heavy metal toxicity was most probably the cause of reduced cocoon production and hence annetocin gene expression levels. Therefore, a hypothesis was formulated that linked endocrine disrupting chemicals with the expression of the annetocin gene.

CHAPTER 6

DISRUPTION TO EARTHWORM REPRODUCTION BY POTENTIALLY ENDOCRINE DISRUPTING CHEMICALS

6.1 INTRODUCTION

The field of chemical-based endocrine disruption research has been rapidly developing over the past twenty years (Sumpter, 1998). In fact, work carried out during the 1930s by Dodds & Lawson (1938) detailed the structural basis of oestrogenic activity by 'look-a-like' (that is, similar in structure to oestradiol) chemicals (such as, some biphenolic chemicals and alkylphenols). Such chemicals have since been shown to mimic or antagonise small hormones, particularly involved in reproductive control in a wide spectrum of organisms (Tyler *et al.*, 1998).

A number of critical studies have been made recently that have raised concerns over the possible impact to wildlife and human health of chemicals (both natural and man-made) with the potential to interfere with the endocrine system. Guillette *et al.* (1994) published a study detailing the abnormalities of the gonad and abnormal sex hormone concentrations observed in juvenile alligators from Lake Apopka, Florida, and postulated that these were caused by a pesticide spill into the lake (for more detail see *Section 1.6*). A second study by Gimeno *et al.* (1996) identified that feminisation of male carp was due to oestrogenic chemicals within the water. Purdom *et al.* (1994) showed the oestrogenic effects of effluents from sewage-treatment works on male fish (see *Section 1.6* for more detailed review).

Therefore, endocrine disruption research has centred on the effects of EDCs on aquatic organisms (particularly those freshwater species). This is due to the fact that aquatic sediments are generally the final sink for most chemicals. So, other environments have been neglected to some extent in endocrine disruption research. The terrestrial environment is one such neglected area, despite the fact that potentially endocrine disrupting chemicals are applied to the land in many countries (e.g. chemicals in human sewage sludge and pesticides).

The application of human sewage sludge is of great importance to the agricultural industry as it increases the organic matter content of poor soils and the levels of such nutrients as nitrogen, potassium and phosphorous (Marmo, 1999). However, the application of sludge to agricultural land does have draw backs. Sewage sludge is often contaminated with heavy metals, which originate from waste waters containing industrial discharges, detergents and medicines. For example, the surfactant nonylphenol (see below) has been found in sewage sludge at concentrations of up to 2,500 mg kg^{-1} dry mass (McGrath, 1999). Bisphenol A has been found to be at a concentration of 36.7 μg g^{-1} dry weight in some Canadian sewage sludge (Lee & Peart, 2000). 17β-oestradiol has been reported at a concentration of 37 ng g^{-1} (dry weight) in sewage sludge, whilst 17α-ethynylestradiol was found to be at a concentration of 17 ng g^{-1} dry weight. Animal manure also contains oestrogens; it has been estimated that chicken mature can contain 30 ng/g 17β – oestradiol (Hanselman *et al.*, 2003). Laying chickens may also secrete testosterone at the rate of 50 ng g^{-1} dry manure/day (Christiansen *et al.*, 2002).

Therefore, this section deals with the threat of 'classic' endocrine disrupting chemicals to the key terrestrial invertebrate, the earthworm. As the earthworm is a key organism in soil quality (see *Section 1.1*) it would be of major environment importance if it was affected by EDCs found in human sewage sludge.

6.2 APPROACH

Five 'classic' endocrine disrupting chemicals were chosen to determine their effects on the earthworm, *Eisenia andrei*. To begin with a 14-day 'range-finding' exposure experiment was utilised following the OECD earthworm acute toxicity test (1984) protocol (see *Section 2.2.4*). The data and experience gained from this initial experiment was then used to develop a test strategy for the reproduction test (OECD, 2000) (see *Section 2.2.5*).

The five chemicals chosen for the study were selected on the basis of their potential to disrupt endocrine function in animals, although they had never been shown to effect earthworm reproduction. These chemicals were; Bisphenol A, Nonylphenol, 17α – Ethynylestradiol, 17β – Oestradiol and Testosterone.

1. **Bisphenol A (BPA):** is a monomer of epoxy resins and polycarbonates. It is also used as a stabilising agent or antioxidant in food-can coatings, plastic products and dental sealants (Staples *et al.*, 1998). BPA has been shown to have oestrogenic activities by competing with 17β-oestradiol for binding to the human oestrogen receptor β (ER β) (Matthews *et al.*, 2001). Dodds & Lawson (1936) found that BPA stimulated the growth of the rodent uterus.

2. **Nonylphenol (NP):** 4-alkylphenol ethoxylates are a widely used class of nonionic surfactants with an annual worldwide production of approximately 650,000 tons (Guenther *et al.*, 2002). The most commonly used subgroup of these compounds are the nonylphenol ethoxylates, which are widely used in cleaning products and as industrial process aids (Thiele *et al.*, 1997). They have also been identified in many foods (they are used as antioxidants in plastic food packaging) (Guenther *et al.*, 2002). During sewage treatment alkylphenol ethoxylates undergo complex biodegration processes resulting in the formation of several metabolites. These compounds are persistent, strongly lipophilic and are more toxic than their parent compounds (Thiele *et al.*, 1997).

3. **17α – Ethynylestradiol (EE2):** is a synthetic oestrogen widely used in the pharmaceutical industry in the contraceptive pill and drugs used to treat cancer, hormonal imbalance and osteoporosis (Snyder *et al.*, 1999). On the basis of *in vivo* studies Johnson & Sumpter (2001) were prompted to designate EE2 as the oestrogenic compound causing most concern in the environment.

4. **17β – Oestradiol (E2):** is a natural steroid hormone synthesised and excreted by animals. It is synthesised from androgens (testosterone) via hydroxylation by NADPH/O_2 – dependent cytochrome P450 enzymes (Stryer, 1995). 17β – oestradiol works via a number of pathways, but perhaps the most well understood is the nuclear-receptor mediated activation of gene expression (Lewin, 1997). It has been shown to be excreted by a number of organisms, including cattle, poultry and humans (Casey *et al.*, 2003). It has been estimated that chicken mature can contain 30 ng g^{-1} 17β – oestradiol (Hanselman *et al.*, 2003).

5. **Testosterone (TES):** is an androgen, i.e, a natural steroid hormone synthesised from cholesterol by animals (Stryer, 1995). Testosterone can be converted to 17β-oestradiol via hydroxylation by the P450 enzyme, aromatase (Stryer, 1995).

6.3 RESULTS

(A) *EISENIA ANDREI* 14-DAY OECD ACUTE TOXICITY TEST

6.3.1 *EISENIA ANDREI* 14-DAY SURVIVAL AND GROWTH – EDC TOXICITY

A 'range-finding' experiment was conducted in order to derive fitted concentration-response relationships for toxicity of the five chemicals used (one replicate/dose). The effect concentration at which 50% of the treatment group died (LC_{50}) was determined by probit analysis (see *Appendix F*). The survival and growth patterns are shown in *Figures 6.1 – 6.5*.

Survival of 100% and 90% was observed in the 'Control' and 'Solvent Control' replicates respectively. Therefore, the test was deemed valid following the guidelines set out in the OECD protocol (1984). Also, an average (two replicates) weight change of 8.0% (increased weight) was seen in the 'Control' earthworms, whilst an average weight change of 12.0% (decreased weight) was noted in the 'Solvent Control' earthworms.

Bisphenol A: (*Figure 6.1*) No data was available for those earthworms exposed to 1.0 mg BPA kg^{-1} soil. A marked reduction in earthworm survival was observed in those earthworms exposed to 100 mg BPA kg^{-1} soil (only 10% of the earthworms survived). At $\geq$1,000 mg BPA kg^{-1} soil no earthworms survived, indicating that BPA

was toxic to earthworms above this concentration in soil. An LC_{50} value of 103 mg BPA kg^{-1} soil was found. This was reflected in the observation of a loss of weight in those earthworms exposed to higher BPA concentrations (*Figure 6.1, Panel B*).

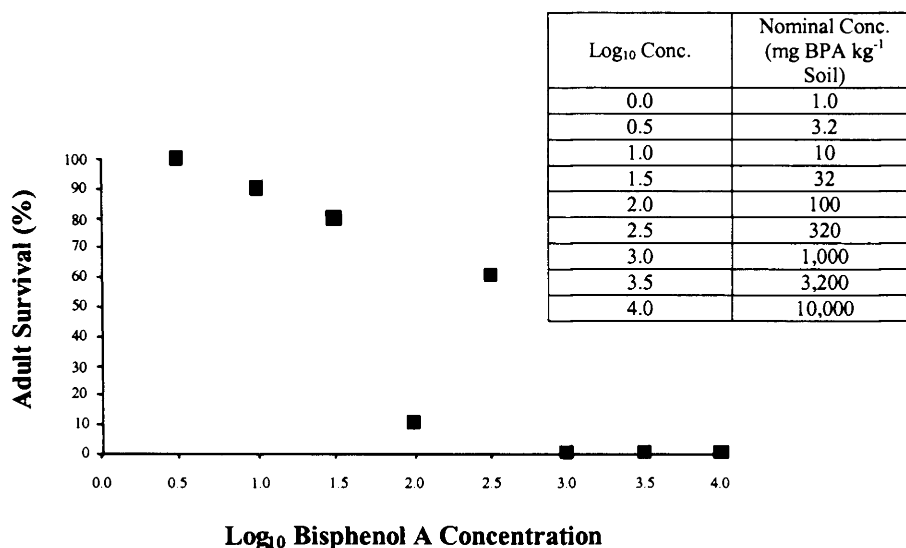
Nonylphenol: (*Figure 6.2*) Survival at NP concentrations < 100 mg NP kg^{-1} soil was high (> 90%). However, a reduction in survival was seen at 100 mg NP kg^{-1} soil with only 30% of the earthworms surviving the 14-day exposure period (*Figure 6.2, Panel A*). Full toxicity (i.e. no survival) was seen at concentrations ≥1,000 mg NP kg^{-1} soil. An LC_{50} value of 51 mg NP kg^{-1} soil was found. Growth of *Eisenia andrei* was not as markedly effected by exposure to nonylphenol as seen when earthworms were exposed to BPA (*Figure 6.2, Panel B*).

17α-Ethynylestradiol: (*Figure 6.3*) Survival of earthworms was not effected by exposure to 17α-ethynylestradiol, apart from at the top concentration used in this protocol, i.e. 1,000 mg EE2 kg^{-1} soil (*Figure 6.3, Panel A*), where 100 % mortality was observed. An LC_{50} value of > 320 mg EE2 kg^{-1} soil was found. Growth was similarly unaffected by exposure to EE2 (*Figure 6.3, Panel B*).

17β-Oestradiol: (*Figure 6.4*) No mortality was seen at the majority of concentrations of E2 (*Figure 6.4, Panel A*). However, 90% survival of *Eisenia andrei* was noted at 10 and 320 mg E2 kg^{-1} soil. An LC_{50} value of > 1,000 mg E2 kg^{-1} soil was found. Growth was determined as a percentage of initial wet body weight, and there seemed to be no pattern of weight change at the concentration range used (*Figure 6.4, Panel B*).

Testosterone: (*Figure 6.5*) No earthworm mortality was observed at the highest concentration used (1,000 mg TES kg^{-1} soil). However, a 10% reduction in survival was seen in those earthworms exposed to a concentration of 1.0 mg TES kg^{-1} soil (*Figure 6.5, Panel A*). An LC_{50} value of > 1,000 mg TES kg^{-1} soil was found. Some weight loss was observed at low concentrations of TES (*Figure 6.5, Panel B*). However, as mentioned previously it is impossible to verify whether this is statistically significant.

Panel A:



Log$_{10}$ Conc.	Nominal Conc. (mg BPA kg^{-1} Soil)
0.0	1.0
0.5	3.2
1.0	10
1.5	32
2.0	100
2.5	320
3.0	1,000
3.5	3,200
4.0	10,000

Panel B:

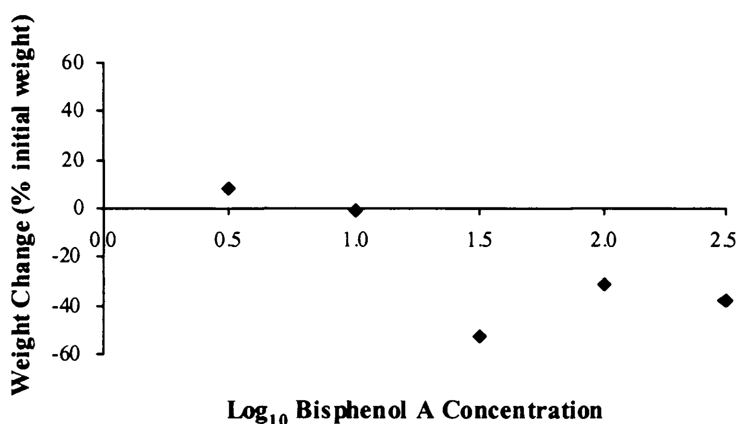
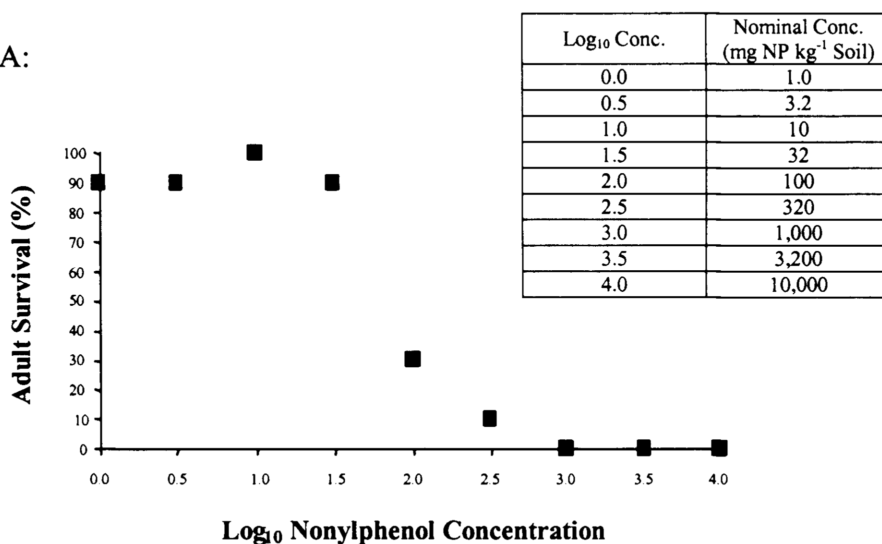


Figure 6.1: Growth and survival data obtained after the 14-day exposure of *E. andrei* to a range of Bisphenol A (BPA) concentrations. Panel A: Shows the survival of earthworms exposed to BPA. All BPA concentrations are shown as Log$_{10}$ and represent nominal concentrations of between 1.0 mg BPA kg^{-1} soil and 10,000 mg BPA kg^{-1} soil. Due to experimental error no data is available for the earthworms exposed to 1.0 mg BPA kg^{-1} soil. **Panel B:** The average change in wet weight of *E. andrei* over the exposure period (14-days) expressed as a percentage change in weight from the initial weight.

Panel A:



Log_{10} Conc.	Nominal Conc. (mg NP kg^{-1} Soil)
0.0	1.0
0.5	3.2
1.0	10
1.5	32
2.0	100
2.5	320
3.0	1,000
3.5	3,200
4.0	10,000

Panel B:

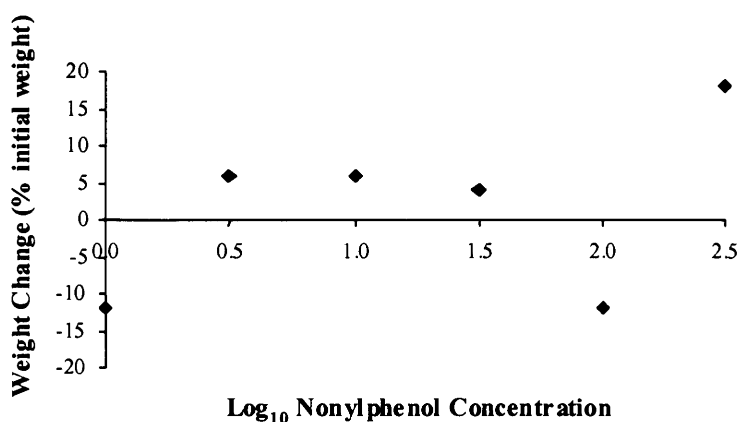
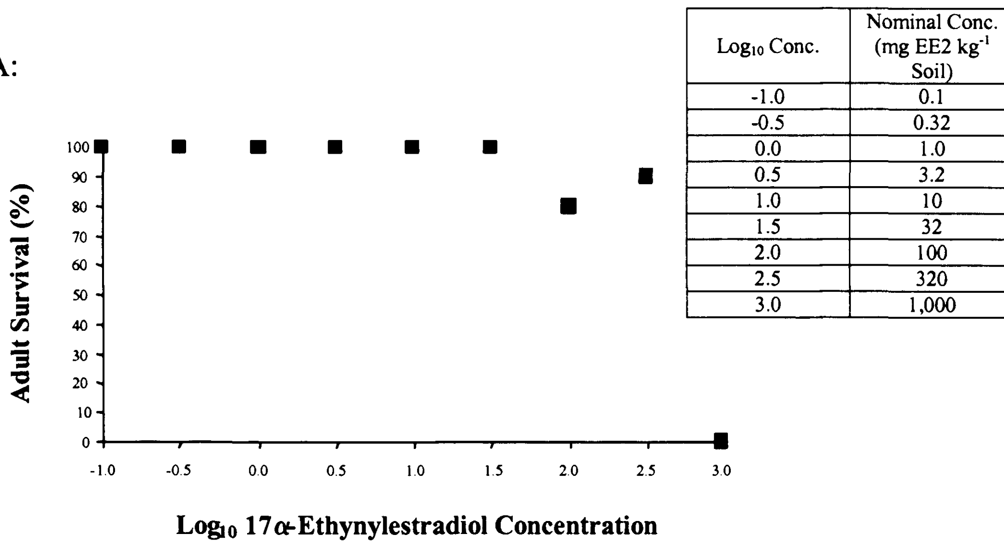


Figure 6.2: Growth and survival data obtained after the 14-day exposure of *E. andrei* to a range of Nonylphenol (NP) concentrations. Panel A: Toxicity of NP to *E. andrei* in soil over the range of 1.0 mg NP kg^{-1} soil to 1,000 mg NP kg^{-1} soil. **Panel B:** Weight change of those earthworms surviving a 14-day period of exposure to NP.

Panel A:



Log_{10} Conc.	Nominal Conc. (mg EE2 kg^{-1} Soil)
-1.0	0.1
-0.5	0.32
0.0	1.0
0.5	3.2
1.0	10
1.5	32
2.0	100
2.5	320
3.0	1,000

Panel B:

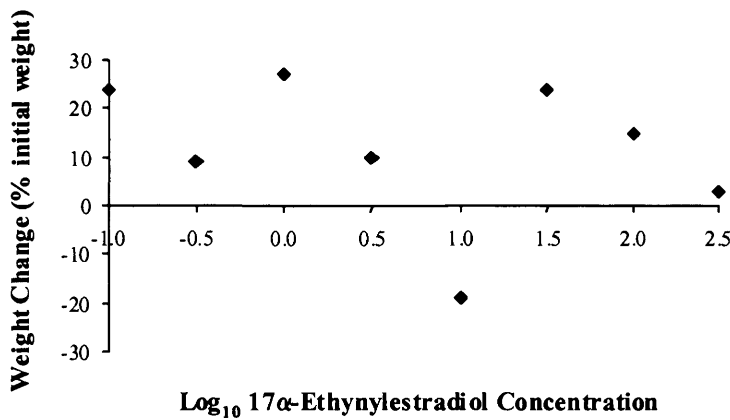
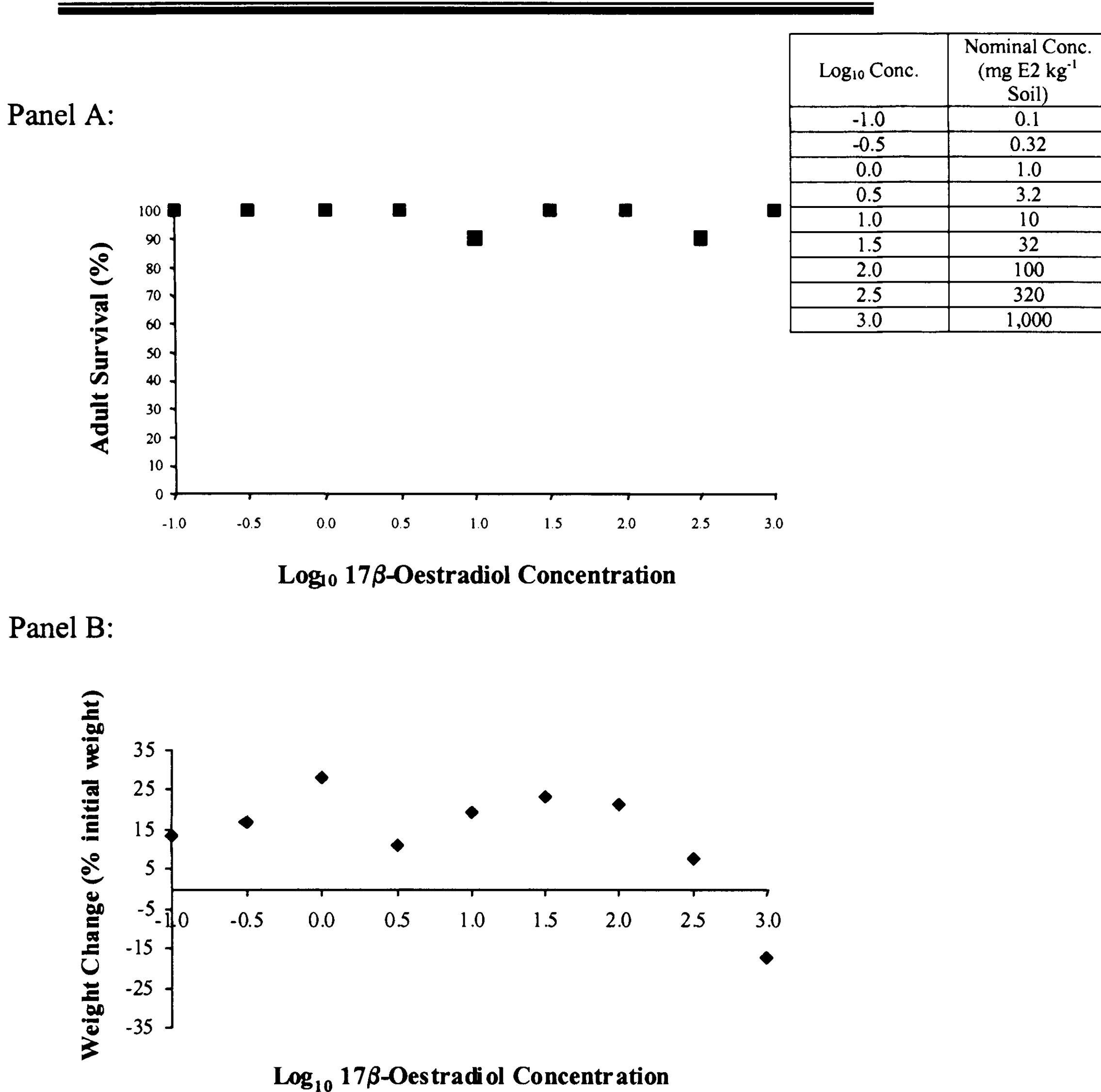


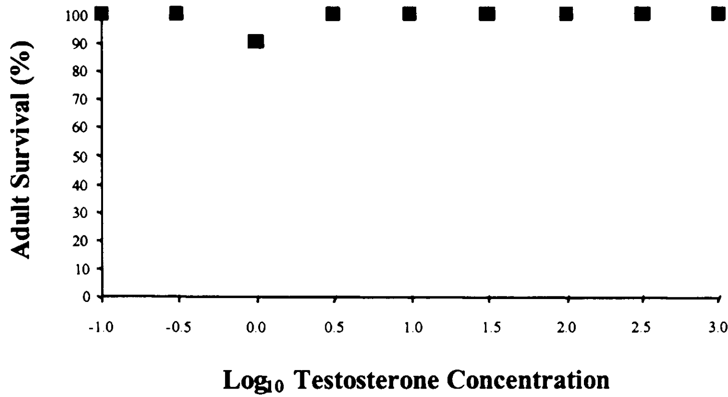
Figure 6.3: Growth and survival data obtained after the 14-day exposure of *E. andrei* to a range of 17α-ethynylestradiol (EE2) concentrations. Panel A: Toxicity of EE2 to the earthworm, *E. andrei*. These are nominal EE2 concentrations in OECD artificial soil. **Panel B:** Percentage weight change in earthworms exposed to the EE2 concentration range previously mentioned. Weight change was measured as a percentage of the mean initial wet weight of the earthworm exposure groups.



Log_{10} Conc.	Nominal Conc. (mg E2 kg^{-1} Soil)
-1.0	0.1
-0.5	0.32
0.0	1.0
0.5	3.2
1.0	10
1.5	32
2.0	100
2.5	320
3.0	1,000

Figure 6.4: Growth and survival data obtained after the 14-day exposure of *E. andrei* to a range of 17β-oestradiol (E2) concentrations. Panel A: Mortality of those earthworm groups exposed to a concentration range of the natural steroid hormone, E2. **Panel B:** Percentage wet weight change of the earthworm groups exposed to the above concentration range in OECD artificial soil.

Panel A:



Log$_{10}$ Conc.	Nominal Conc. (mg TES kg^{-1} Soil)
-1.0	0.1
-0.5	0.32
0.0	1.0
0.5	3.2
1.0	10
1.5	32
2.0	100
2.5	320
3.0	1,000

Panel B:

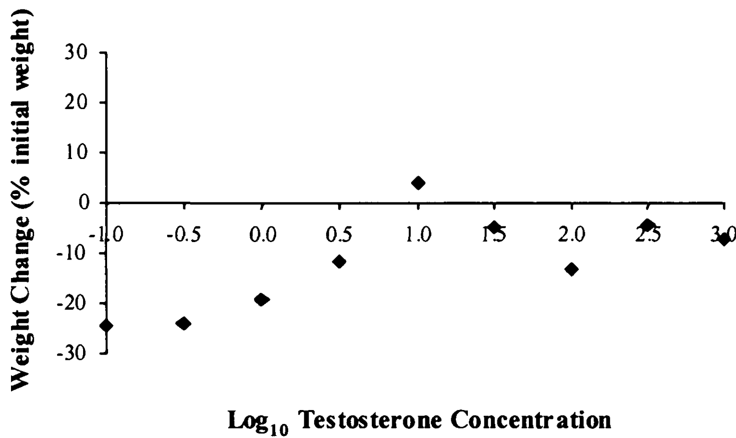


Figure 6.5: Growth and survival data obtained after the 14-day exposure of *E. andrei* to a range of Testosterone (TES) concentrations. Panel A: Adult *E. andrei* survival in artificial soil spiked with nominal concentrations of the natural steroid hormone, Testosterone. **Panel B:** The percentage change in weight of earthworms exposed to a concentration range of Testosterone.

6.3.2 TOTAL RNA ISOLATION AND CDNA SYNTHESIS

Total RNA was isolated from adult, fully clitellate *Eisenia andrei*, as described in *Section 2.2.8*. Quality control was performed on samples using agarose gel electrophoresis (*Section 2.2.11*) and visualisation under U.V. light. RNA was quantified as described in *Section 2.2.13*, and it was found that, on average, approximately 2.0 μg/μl of total RNA had been purified.

First-strand cDNA was synthesised in accordance with the protocol described in *Section 2.2.15*. An appropriate volume of total RNA was added to each reverse transcription reaction to give 2.0 μg of RNA. In order to establish whether the cDNA synthesis reaction was successful a selection of cDNA samples were chosen at random and a fragment of the *β*-actin gene was amplified using a gene-specific oligonucleotide primer-pair (data not shown; see *Chapter 4*, *Section 4.3.2*). Therefore, it was established that the cDNA was of high enough quality to proceed with downstream applications.

6.3.3 REPRODUCTIVE EFFECTS AT THE MOLECULAR LEVEL – ANNETOCIN GENE EXPRESSION

Gene expression measurements in *Eisenia andrei* (adult, fully clitellate) were made using dual-labelled, 5'-exonuclease oligonucleotide probe quantitative PCR technology (as described in *Section 2.2.26*). All quantitative PCR reactions were first optimised as detailed in *Section 2.2.26* using the appropriate cloned gene fragments (*Section 2.2.17*). The final concentrations of oligonucleotide primers and dual-labelled probes used in all real-time PCR reactions are given in *Table 2.5; Section 2.2.26*. Real-time quantitative PCR reactions were performed using a 1:20 dilution of

cDNA synthesised from total RNA (*Section 6.3.2*). Expression measurements were made using two genes; the 'housekeeping' *β*-actin gene was used as an invariant internal control, whilst the 'test' gene was the earthworm 'sex hormone', annetocin. *β*-actin was deemed a suitable invariant control as no variation was seen as being significant on statistical manipulation using the non-parametric Kruskal-Wallis test (Minitab v.13.1).

The ratio of 'test' gene expression to 'control' gene expression (i.e. annetocin:*β*-actin) gave a measure of the relative annetocin gene expression level in *Eisenia andrei*, which had either been exposed or not to EDCs. Relative annetocin gene expression values were measured in all earthworm populations available (*Figures 6.6 – 6.10*). These expression levels were averaged in each population (i.e. giving the mean relative annetocin expression level for a population of earthworms). Statistical significance between groups was measured using first, the Kruskal-Wallis non-parametric test (*Section 2.2.7*), then the Mann-Whitney U-test (*Section 2.2.7*) (both, Minitab, v.13.1).

1) Control/Solvent Control *Eisenia andrei*

Those earthworms deemed 'controls' were maintained on artificial soil for the 14-day period, but were not exposed to any chemical within the test regime (*Section 2.2.4*). Those earthworm populations labelled 'solvent controls' were maintained on artificial soil spiked with the highest amount of the solvent (*Section 2.2.4*) (acetone) used in other exposure populations, i.e. 3.5 mls acetone in 500 g of soil (see *Section 2.2.4*). In both cases two replicates, each containing ten earthworms were used. Gene expression measurements were made in each earthworm available after the 14-day

exposure period (see above for control/solvent control survival data) and replicates were pooled to give the mean expression values (see *Figures 6.6 – 6.10*).

The greatest annetocin gene expression (relative to β-actin gene expression) was seen in the control earthworm population (1.99 ± 0.33, n = 18). This expression level was in keeping with previously measured relative annetocin gene expression in control animals of this type (see *Section 5.2.5*). Two PCR reactions were deemed as failures, hence n = 18. The solvent control earthworm population showed an approximately 50% reduction in relative annetocin gene expression (0.83 ± 0.22, n = 17). A single PCR reaction was deemed as a failure, hence n = 17. This difference was shown to be significant ($p \leq 0.05$) using the Mann-Whitney non-parametric U-test.

2) Bisphenol A Exposed *Eisenia andrei*

The relative annetocin gene expression levels of earthworms exposed to BPA in artificial soil are shown in *Figure 6.6*. Two treatment groups were measured (those exposed to 3.2 and 10.0 mg BPA kg^{-1} soil) due to toxicity at higher concentrations (*Figure 6.1*).

A significant difference (compared with that in the control population) in relative annetocin gene expression at 3.2 mg BPA kg^{-1} soil and 10.0 mg BPA kg^{-1} soil (0.45 ± 0.08 and 0.62 ± 0.27, respectively) was found. These differences were not significantly different to the expression levels found in the solvent control earthworm population, suggesting an additional solvent effect on annetocin expression.

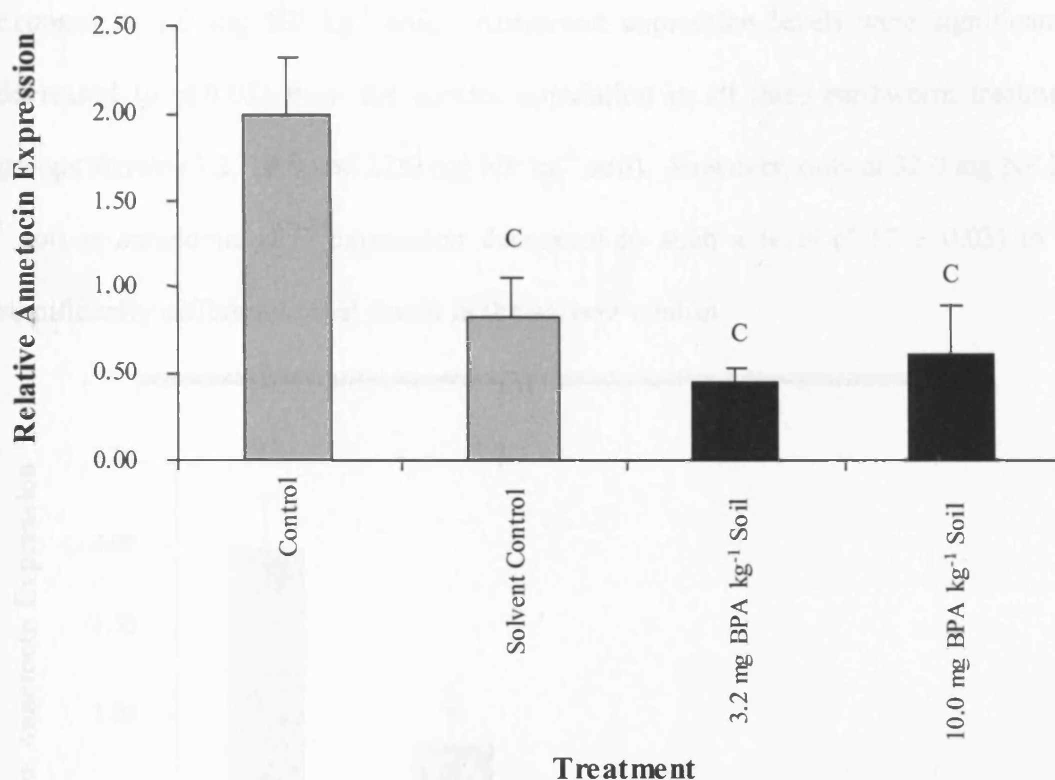


Figure 6.6: Relative annetocin gene expression in those earthworms exposed over a 14-day period to Bisphenol A (BPA). Annetocin gene expression was measured using dual-labelled fluorescent oligonucleotide quantitative PCR. Gene expression levels found in those earthworms exposed to BPA were compared with those values found in control and solvent control populations. Statistically significant differences (Mann-Whitney U-test, $p \leq 0.05$) are represented by the letter C. The letter C signifies a difference between the control population and the 'exposed' populations. ($n = 8$, 3.2 mg BPA kg^{-1} soil and $n = 8$, 10.0 mg BPA kg^{-1} soil). Standard Error of the Mean (SEM) is shown by error bars.

3) 4-Nonylphenol Exposed *Eisenia andrei*

Relative annetocin gene expression levels found in earthworms exposed to 4-nonylphenol are shown in *Figure 6.7*, expression levels were not measured in worms exposed to 1.0 mg NP kg^{-1} soil. Annetocin expression levels were significantly decreased ($p \leq 0.05$) from the control population in all three earthworm treatment groups shown (3.2, 10.0 and 32.0 mg NP kg^{-1} soil). However, only at 32.0 mg NP kg^{-1} soil is annetocin gene expression decreased to such a level (0.17 ± 0.03) to be significantly different to that found in the solvent control.

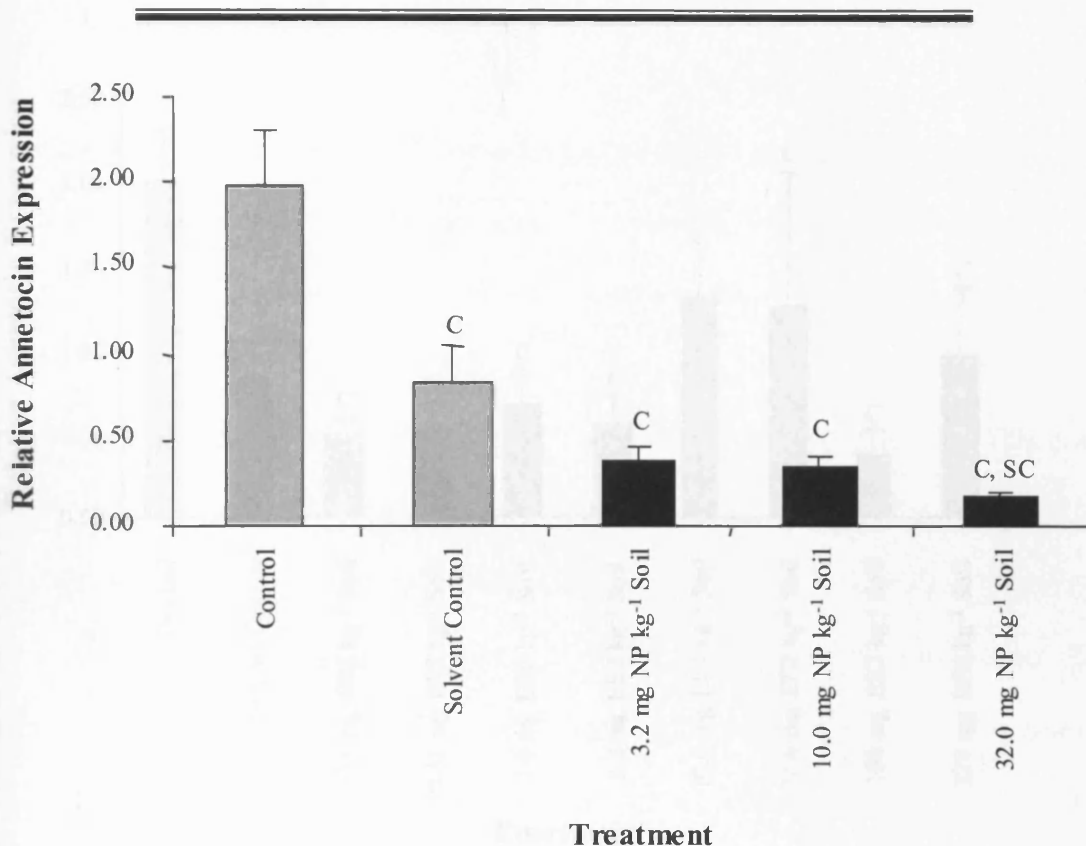


Figure 6.7: Relative annetocin gene expression in those earthworms exposed to sub-lethal 4-nonylphenol (NP) concentrations. Annetocin gene expression levels were measured in those earthworms exposed to NP in artificial soil and a mean expression level calculated for each population. C = statistically significant difference ($p \leq 0.05$) from control earthworms, SC = statistically significant difference ($p \leq 0.05$) from solvent control earthworms. (n = 8 for all three exposed populations). Standard Error of the Mean (SEM) is shown by error bars.

4) 17α-Ethynylestradiol (EE2) Exposed *Eisenia andrei*

Relative annetocin expression levels found in earthworms exposed to EE2 are shown in *Figure 6.8*. At all EE2 concentrations (apart from 10.0 mg EE2 kg^{-1} soil) relative annetocin gene expression was significantly lower than that found in the control population of earthworms. However, none of the test populations showed a difference in annetocin gene expression when compared to the solvent control earthworm population.

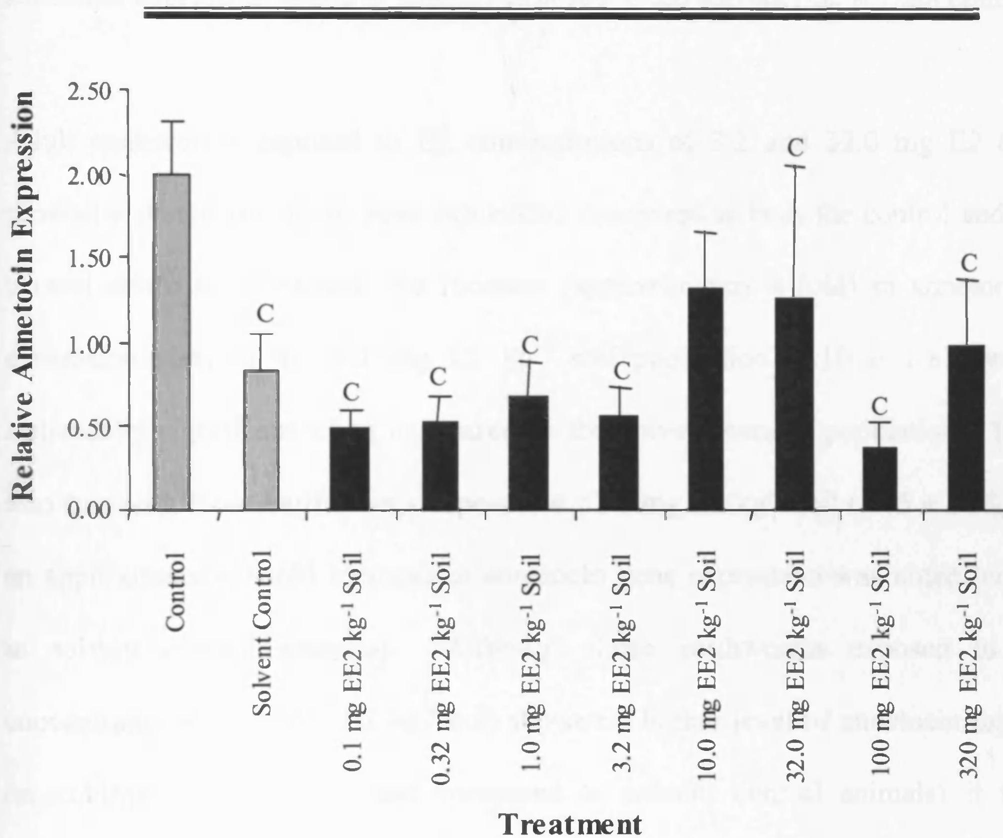


Figure 6.8: Relative annetocin gene expression in those earthworms exposed to sub-lethal 17α-Ethynylestradiol (EE2) concentrations. Relative annetocin gene expression levels were measured in those animals exposed to EE2 in artificial soil and a mean expression level was calculated for each population. C = statistically different ($p \leq 0.05$) from control earthworm population. Standard Error of the Mean (SEM) is shown by error bars.

5) 17β-Oestradiol (E2) Exposed *Eisenia andrei*

The mean relative annetocin gene expression levels found in those earthworms exposed to sub-lethal concentrations of the natural steroid hormone, 17β-Oestradiol (E2) are shown in *Figure 6.9*. These levels were statistically compared with the annetocin gene expression levels found in those unexposed animals. Those animals exposed to E2 concentrations of 0.1, 0.32, 1.0, 100 and 1,000 mg E2 kg^{-1} soil all showed a statistically lowered ($p \leq 0.05$) annetocin gene expression in comparison to the control animals. However, these were not statistically lower than the relative annetocin expression found in animals exposed to the solvent (i.e. solvent controls).

Adult earthworms exposed to E2 concentrations of 3.2 and 32.0 mg E2 kg^{-1} soil showed elevated annetocin gene expression compared to both the control and solvent control animals. However, the increase (approximately 4-fold) in annetocin gene expression seen in the 3.2 mg E2 kg^{-1} soil population (4.10 ± 1.81) was only statistically significant when compared to the solvent control population. This was also true with those earthworms exposed to 32.0 mg E2 kg^{-1} soil (3.05 ± 0.91), where an approximately 3-fold increase in annetocin gene expression was noted (compared to solvent control animals). Although those earthworms exposed to an E2 concentration of 10.0 mg E2 kg^{-1} soil showed a higher level of annetocin expression (approximately 3-fold increase compared to solvent control animals) it was not significantly different from that found in control or solvent control animals.

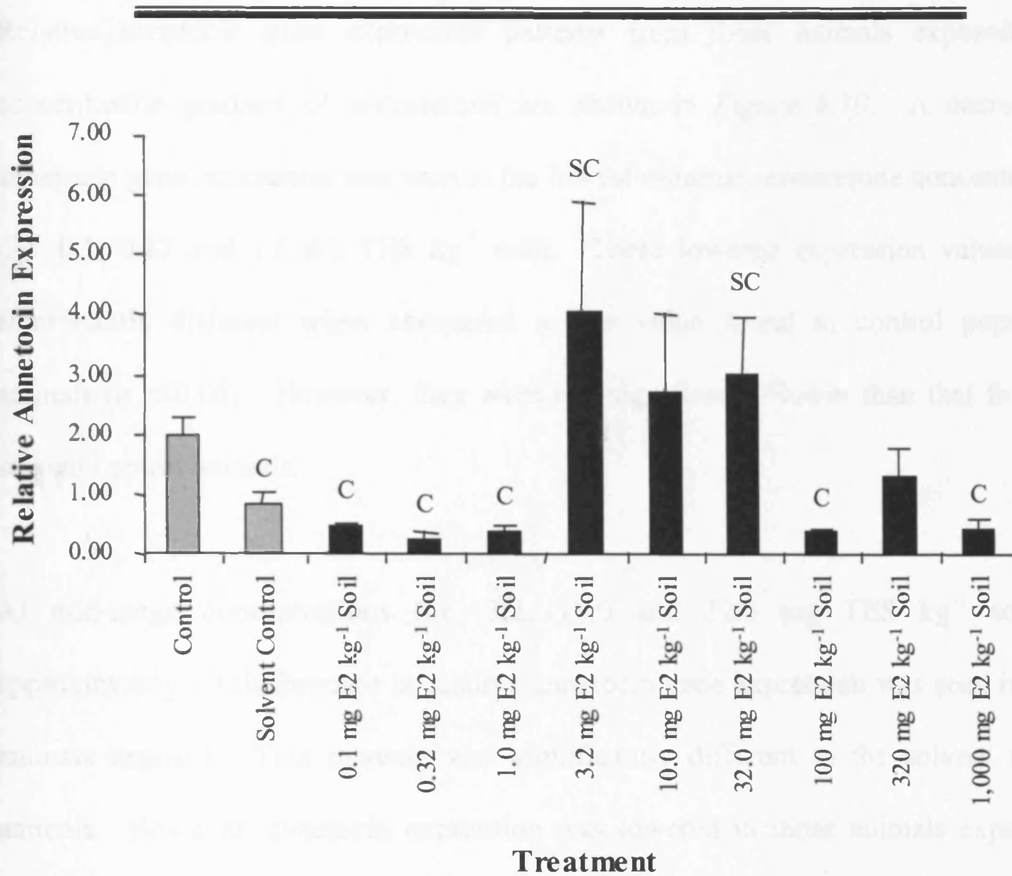


Figure 6.9: Relative annetocin gene expression in those earthworms exposed to sub-lethal 17β-oestradiol (E2) concentrations. Annetocin gene expression levels were measured in those earthworms exposed to E2 in artificial soil and a mean expression level calculated for each population. C = statistically significant difference ($p \leq 0.05$) from control earthworms, SC = statistically significant difference ($p \leq 0.05$) from solvent control earthworms. (0.1 n = 7, 0.32 n = 7, 1.0 n = 10, 3.2 n = 10, 10.0 n = 9, 32.0 n = 10, 100 n = 9, 320 n = 7 and 1,000 n = 9). Standard Error of the Mean (SEM) is shown by error bars.

6) Testosterone Exposed *Eisenia andrei*

Relative annetocin gene expression patterns from those animals exposed to a concentration gradient of testosterone are shown in *Figure 6.10*. A decrease in annetocin gene expression was seen at the lowest nominal testosterone concentrations (i.e. 0.1, 0.32 and 1.0 mg TES kg^{-1} soil). These lowered expression values were significantly different when compared to the value found in control population animals ($p \leq 0.05$). However, they were not significantly lower than that found in solvent control animals.

At mid-range concentrations (i.e. 3.2, 10.0 and 32.0 mg TES kg^{-1} soil) an approximately 5-fold increase in relative annetocin gene expression was seen in those animals exposed. This increase was significantly different to the solvent control animals. However, annetocin expression was lowered in those animals exposed to nominal testosterone concentrations of 100, 320 and 1,000 mg TES kg^{-1} soil. This was not different from control or solvent control annetocin expression.

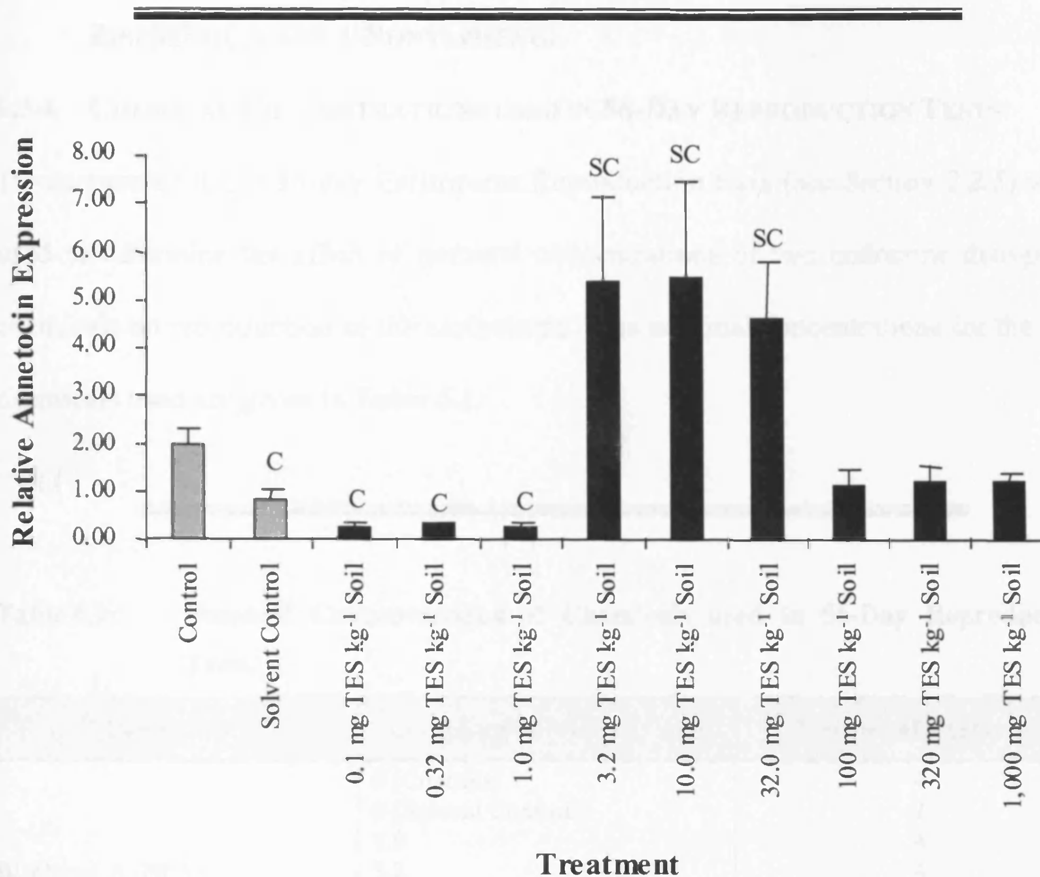


Figure 6.10: Relative annetocin gene expression in those earthworms exposed to sub-lethal testosterone (TES) concentrations. Annetocin gene expression levels were measured in those earthworms exposed to TES in artificial soil and a mean expression level calculated for each population. C = statistically significant difference ($p \leq 0.05$) from control earthworms, SC = statistically significant difference ($p \leq 0.05$) from solvent control earthworms. (0.1 n = 9, 0.32 n = 9, 1.0 n = 9, 3.2 n = 7, 10.0 n = 10, 32.0 n = 8, 100 n = 8, 320 n = 10 and 1,000 n = 9). Standard Error of the Mean (SEM) is shown by error bars.

(B) *EISENIA ANDREI* 56-DAY OECD EARTHWORM REPRODUCTION TEST BISPHENOL A AND 4-NONYLPHENOL

6.3.4 CHEMICAL CONCENTRATIONS USED IN 56-DAY REPRODUCTION TESTS

Two separate OECD 56-day Earthworm Reproduction tests (see *Section 2.2.5*) were used to determine the effect of nominal concentrations of two endocrine disrupting chemicals on reproduction in the earthworm. The nominal concentrations for the two chemicals used are given in *Table 6.1*.

Table 6.1: Nominal Concentrations of Chemicals used in 56-Day Reproduction Tests.

Compound	Concentration (mg kg^{-1} soil)	Number of Replicates
Bisphenol A (BPA)	0 (Controls)	4
	0 (Solvent Controls)	2
	1.0	4
	3.2	4
	10.0	4
	32.0	4
	100	4
4-Nonylphenol (NP)	0 (Controls)	4
	0 (Solvent Controls)	2
	1.0	4
	3.2	4
	10.0	4
	32.0	4
	100	4

6.3.5 28-DAY GROWTH AND SURVIVAL OF EARTHWORMS EXPOSED TO BISPHENOL A

After 28-days of exposure, earthworms were removed from the soil media, counted and weighed (*Section 2.2.5*). Those animals maintained on 'control' artificial soil showed an average 28-day survival of 100 % (data not shown). There was no significant mortality in those animals exposed to the 'solvent control' (survival was an

average of 95 ± 5 % SEM; data not shown). No significant mortality (survival was greater than 90% in all replicates) was observed in any of the 'test' replicates during the 28-day exposure period (data not shown).

Growth of adult *Eisenia andrei* was not effected over the 28-day exposure period (data not shown). Growth was assessed by determining average weight change of the earthworms. Weight changes in all test and control earthworm populations were deemed not significant using the Kruskal-Wallis non-parametric statistical test (Minitab, v.13.1).

6.3.6 REPRODUCTIVE EFFECTS IN *EISENIA ANDREI* UPON EXPOSURE TO BISPHENOL A – COCOON AND JUVENILE PRODUCTION

After 56-days of exposure to BPA, the number of cocoons in each replicate were counted (*Figure 6.11*) (see *Section 2.2.5*) along with the number of juveniles produced over the exposure period (*Figure 6.12*).

Cocoon production in the control group of animals (0.281 ± 0.094 cocoons/worm/week; n = 4) was not significantly different from that found in any of the test groups or the solvent control groups. The BPA exposure and the NP exposure (see *Section 6.3.11*) were run in parallel. Therefore, when adult *Eisenia andrei* were removed from the soil (only the control groups) after 28-days they were pooled from both exposures (i.e. four replicates from each exposure giving a total of 80 earthworms). This did not include those animals exposed for 28-days in the solvent controls; in fact these earthworms were discarded. The pooling of these animals allowed for more cost effective use of downstream applications (see *Section 6.3.7*).

In *Figure 6.11* the cocoon production in the control groups from both exposures have been averaged to give an 'All Controls' value (0.331 ± 0.050 cocoons/worm/week; n = 8). This value was not significantly different from any of the test or solvent control groups. However, a decrease (approximately 50 % reduction) in cocoon production was observed in those earthworms exposed to the solvent control, indicating that the solvent used affected reproduction rates in earthworms. In all the test groups of animals cocoon production was greater (not significantly) than that found in the solvent controls. This could indicate that exposure to BPA may increase the reproductive output of *Eisenia andrei*.

Cocoon production was greatest in those animals exposed to a 100 mg BPA kg^{-1} soil (0.444 ± 0.059 cocoons/worm/week; n = 4). This was despite the observation that only 10 % of the animals exposed to this concentration during the 14-day exposure survived (see *Section 6.3.1* and *Figure 6.2*).

The number of juveniles produced per replicate is shown in *Figure 6.12*. According to OECD testing guidelines (OECD, 2000) the earthworm reproductive test is only valid if ≥30 juveniles are produced per replicate in the controls. In this exposure an average of 14.75 ± 3.64 juveniles/replicate (n = 4) were produced. Therefore, this exposure cannot be deemed valid following OECD guidelines. However, molecular analysis was performed on adult earthworms removed from the exposure after 28-days (see *Section 6.3.7*).

Cocoon viability was not affected by exposure to BPA. The number of juvenile earthworms emerging from each cocoon laid remained constant (approximately 2 juveniles/cocoon) (data not shown).

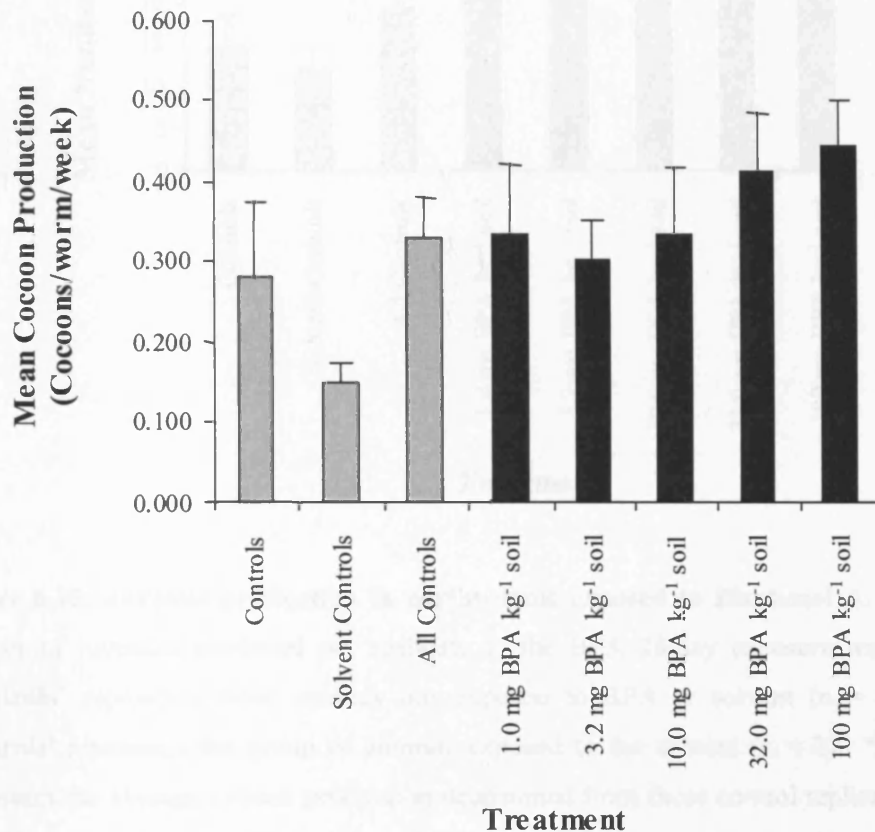


Figure 6.11: Cocoon production rates in earthworms exposed to nominal concentrations of Bisphenol A (BPA). Cocoon production rates were determined by counting the number of cocoons in each replicate and using the number of adult earthworms at the beginning of the exposure test. **'Controls'** represents those animals not exposed to BPA or solvent (n = 4). **'Solvent Controls'** represent the group of animals exposed to the solvent (n = 2). **'All Controls'** represents the average cocoon production determined from those control replicates from both the 28-day exposure to BPA and the 28-day exposure to NP (n = 8). No significant differences were found between the cocoon production rates in the control animals and the test animals (Mann-Whitney, $p \leq 0.05$). Standard Error of the Mean (SEM) is shown by error bars.

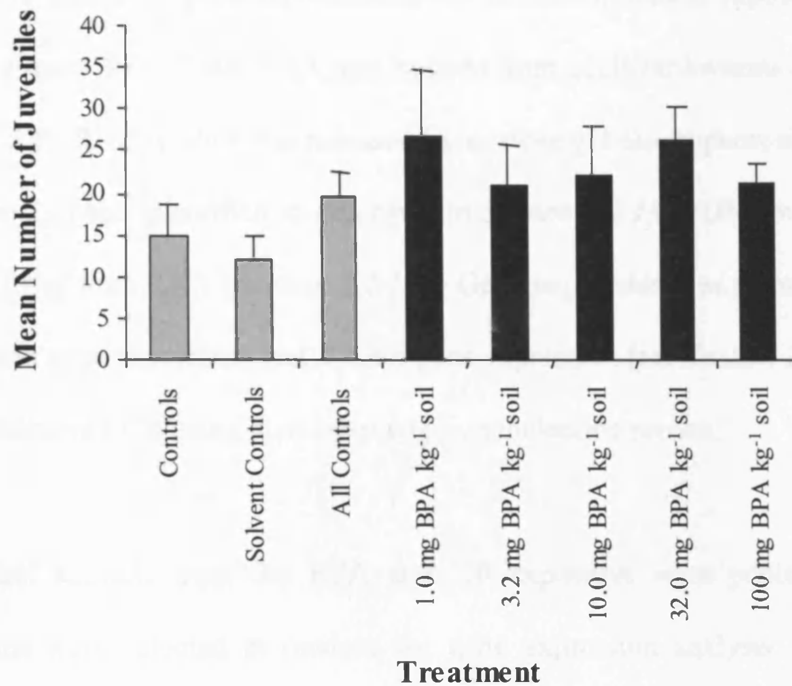


Figure 6.12: Juvenile production in earthworms exposed to Bisphenol A. The average number of juveniles produced per replicate in the BPA 28-day exposure was determined. **'Controls'** represents those animals not exposed to BPA or solvent (n = 4). **'Solvent Controls'** represents the group of animals exposed to the solvent (n = 2). **'All Controls'** represents the average cocoon production determined from those control replicates from both the 28-day exposure to BPA and the 28-day exposure to NP (n = 8). No significant differences were found between the average number of juveniles produced in the control animals and the test animals (Mann-Whitney, $p \leq 0.05$). Standard Error of the Mean (SEM) is shown by error bars.

6.3.7 REPRODUCTIVE EFFECTS IN *EISENIA ANDREI* UPON EXPOSURE TO BISPHENOL A – ANNETOCIN GENE EXPRESSION

The relative annetocin gene expression levels in *Eisenia andrei* exposed to BPA are shown in *Figure 6.13*. Total RNA was isolated from adult earthworms as described in *Section 2.2.8*. RNA quality was assessed by agarose gel electrophoresis (for example see *Figure 2.2*) and quantified as described in *Section 2.2.13*. cDNA was synthesised from 2.0 μg of total RNA (*Section 2.2.15*). Gene expression was measured as a ratio of annetocin gene expression and *β*-actin gene expression (see *Section 2.2.26)* by real-time quantitative PCR using dual-labelled oligonucleotide probes.

The control animals from the BPA and NP exposures were pooled and sixteen earthworms were selected at random for gene expression analysis. Therefore, in *Figure 6.13* the 'All Controls' group is representative of the relative annetocin gene expression in earthworms removed from eight replicates. Upon analysis of the qPCR data obtained from these earthworms, two PCR reactions were found to be failures. Therefore, the number of animals used in the 'All Controls' group was $n = 14$.

Relative annetocin gene expression was found to be greatest in the control animals (1.91 ± 0.48 SEM). Only one of the five test populations analysed showed a significant difference in annetocin gene expression when compared to the "All Controls" group. Those earthworms exposed to 3.2 mg BPA kg^{-1} soil showed an approximately four-fold (0.45 ± 0.13 SEM) reduction in relative annetocin gene expression compared to the "All Controls" group. Although those earthworms exposed to BPA at other concentrations (*Table 6.1*; *Figure 6.13*) showed no significantly different annetocin gene expression profiles, there was a general trend of

reduced gene expression upon BPA exposure. All the earthworm groups exposed to BPA showed an approximately 50 % reduction in annetocin gene expression.

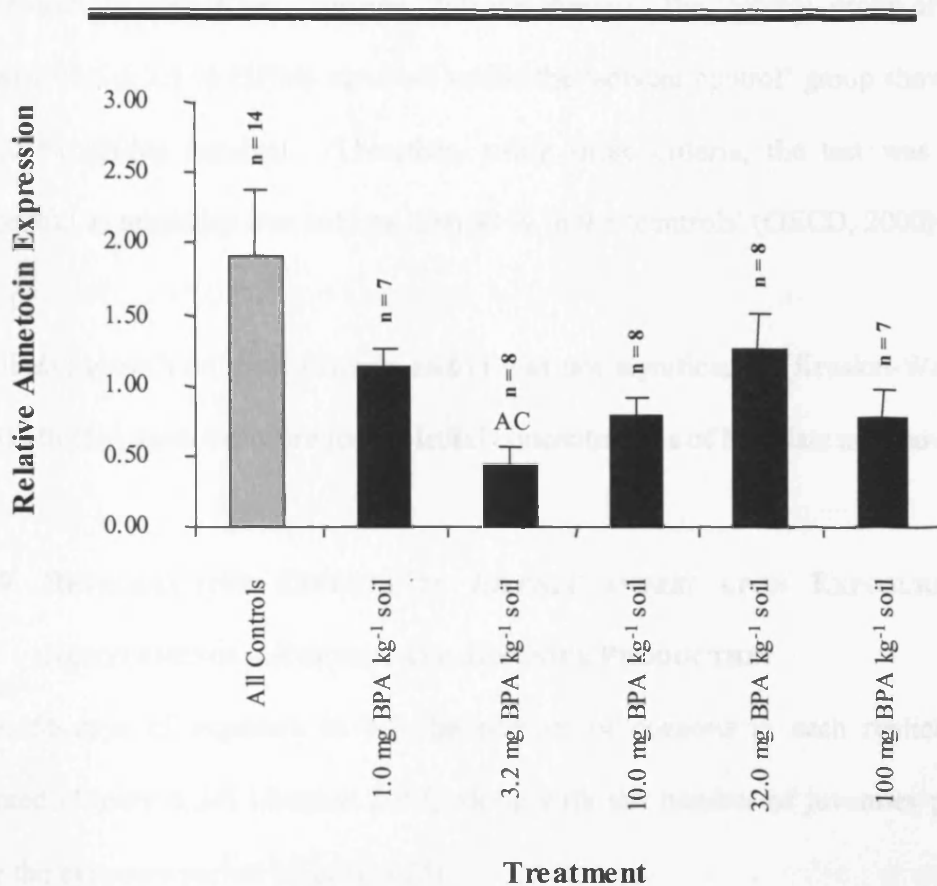


Figure 6.13: Mean relative annetocin gene expression in *Eisenia andrei* exposed to BPA over a 28-day period. Relative annetocin gene expression is the ratio of β-actin gene expression to annetocin gene expression. Transcript levels were determined using dual-labelled fluorescent oligonucleotide real-time PCR. Individual animals were used to determine relative annetocin gene expression (relative to β-actin) and a mean relative annetocin gene expression value was determined (Standard Error of the Mean was also calculated). "All Controls" represents the mean annetocin gene expression found in those earthworms from the control and solvent control populations (n = 14). AC = significantly different from the "All Controls" group. Standard Error of the Mean (SEM) is shown by error bars.

6.3.8 28-DAY GROWTH AND SURVIVAL OF EARTHWORMS EXPOSED TO 4 NONYLPHENOL

No significant mortality ($p \leq 0.05$) was seen in any replicates, at any of the nominal concentrations used in this exposure (data not shown). The 'control' group of animals showed 97.5 ± 2.5 % (SEM) survival, whilst the 'solvent control' group showed 95.5 ± 5.0 % (SEM) survival. Therefore, using these criteria, the test was deemed successful as mortality was not less then 90 % in the 'controls' (OECD, 2000).

Similarly, growth of adult *Eisenia andrei* was not significantly (Kruskal-Wallis, $p \leq 0.05$) effected upon exposure to sub-lethal concentrations of NP (data not shown).

6.3.9 REPRODUCTIVE EFFECTS IN *EISENIA ANDREI* UPON EXPOSURE TO 4 NONYLPHENOL – COCOON AND JUVENILE PRODUCTION

After 56-days of exposure to NP the number of cocoons in each replicate were counted (*Figure 6.14*) (*Section 2.2.5*) along with the number of juveniles produced over the exposure period (*Figure 6.15*).

Figure 6.15 shows the mean cocoon production rate (number of cocoons/worm/week) in the control and exposed earthworm populations. As with the cocoon production rates of those earthworms exposed to BPA (*Figure 6.11*), an "All Controls" group was produced. This consisted of the cocoon production rates from each of the BPA and NP control replicates (n = 8). The control group of earthworms (n = 4) gave a mean cocoon production rate over the 28-day exposure period of 0.381 ± 0.033 cocoons/worm/week. Cocoon production was reduced by over half in the solvent control group to, 0.150 ± 0.025 cocoons/worm/week (n = 2). However, this was not

significantly different ($p \leq 0.05$) from any of the control groups (controls or "All Controls").

The greatest cocoon production rate was seen in the earthworm population exposed to 1.0 mg NP kg^{-1} soil (0.413 ± 0.062 cocoons/worm/week, n = 4). However, this was not a significant increase when compared to any of the control groups. All the earthworm populations exposed to NP gave an approximately two-fold increase in cocoon production rate when compared to that found in the solvent control group. These values were not significantly different ($p \leq 0.05$) from the value found in the control or solvent control earthworm populations after statistical analysis was performed using the Kruskal-Wallis and Mann-Whitney non-parametric tests (Minitab V. 13.1, *Section 2.2.7*).

A similar pattern was observed when the mean number of juveniles produced per replicate was calculated (*Figure 6.15*). Juvenile production was lowest in the solvent control group of earthworms (12.00 ± 3.00 juveniles/replicate, n = 2) compared with a value of 24.25 ± 4.03 juveniles/replicate (n = 4) in the control population. No significant differences ($p \leq 0.05$) were observed between the test populations and the control groups (using Kruskal-Wallis and Mann-Whitney statistical tests). However, juvenile production was higher in all test groups than that observed in the solvent control populations (in most cases it was approximately double that of the solvent control group). The number of juveniles produced per cocoon remained constant in all replicates (data not shown) at approximately 2 juveniles/cocoon, indicating that cocoon viability was not affected upon exposure to NP.

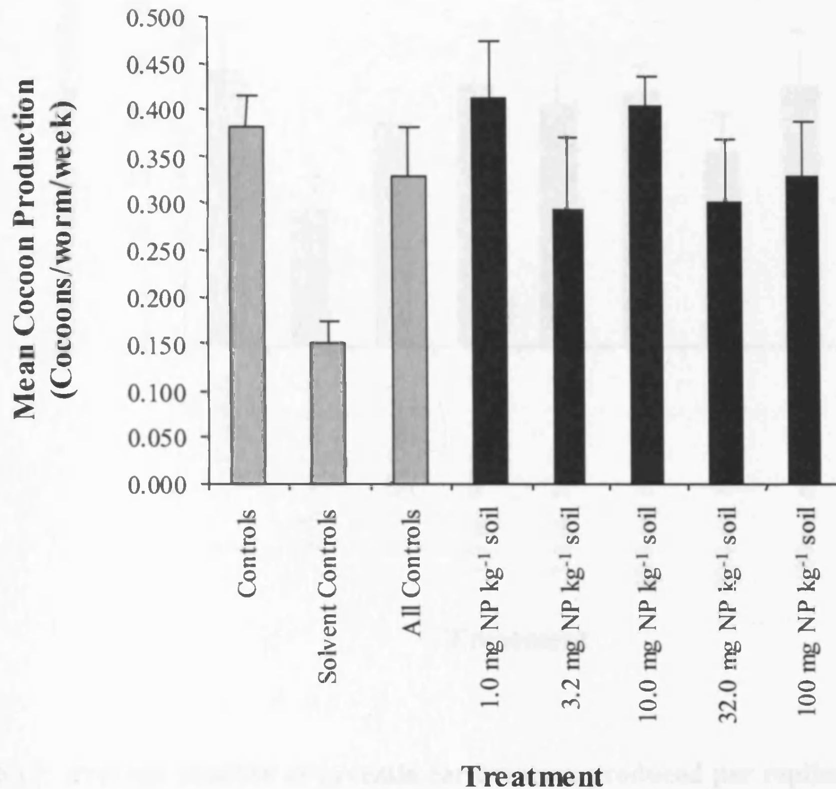


Figure 6.14: Cocoon production rate of earthworms exposed to 4-nonylphenol (NP) over a 28-day period. Adult earthworms (*Eisenia andrei*) were exposed to NP for 28-days in OCED (2000) artificial soil. After 28-days all adults were removed from the soils, but any cocoons were left for a further 28-day period. After a total of 56-days exposure cocoons were removed from the soil and counted. A cocoon production rate (number of cocoons/worm/week) was calculated for each replicate, and a mean for each treatment. At each treatment there were four replicates (i.e. n = 4), but the "All Controls" group contains eight replicates (n = 8). There were no significant differences ($p \leq 0.05$) between cocoon production rates in any of the test or control groups after statistical analysis using the non-parametric tests, Kruskal-Wallis and Mann-Whitney (Minitab V. 13.1). Standard Error of the Mean (SEM) is shown by error bars.

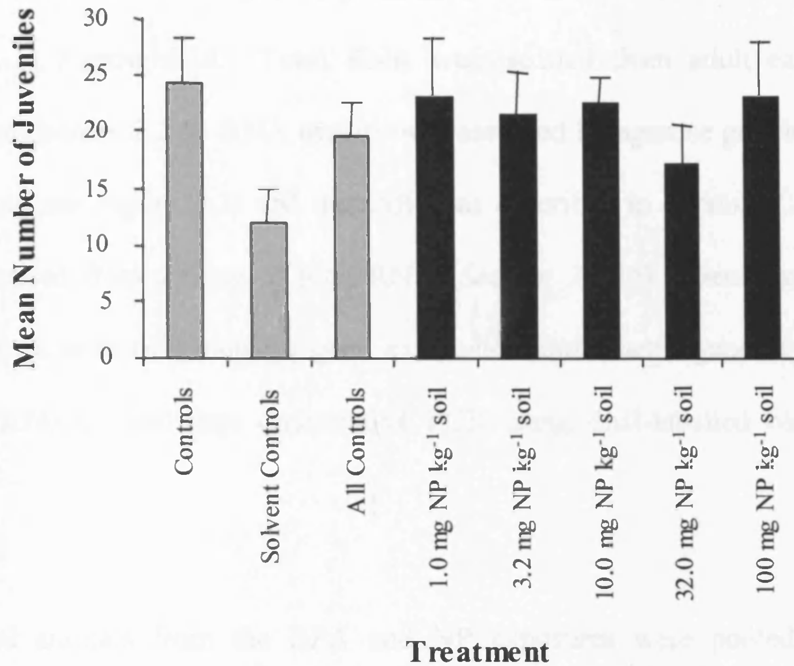


Figure 6.15: Average number of juvenile earthworms produced per replicate over a 28-day expose to 4-nonylphenol (NP). The number of juveniles present in each replicate at the end of the 56-day exposure period was calculated along with the standard error of the mean. Each treatment contained four replicates, excluding the "All Controls" group which contained data from eight replicates. No significant differences were observed between any of the control and test treatments (after Kruskal-Wallis and Mann-Whitney statistical analysis, Minitab V. 13.1). Standard Error of the Mean (SEM) is shown by error bars.

6.3.10 REPRODUCTIVE EFFECTS IN *EISENIA ANDREI* UPON EXPOSURE TO NONYLPHENOL – ANNETOCIN GENE EXPRESSION

The mean relative annetocin gene expression levels in *Eisenia andrei* exposed to NP are shown in *Figure 6.16*. Total RNA was isolated from adult earthworms as described in *Section 2.2.8*. RNA quality was assessed by agarose gel electrophoresis (for example see *Figure 2.2*) and quantified as described in *Section 2.2.13*. cDNA was synthesised from 2.0 μg of total RNA (*Section 2.2.15*). Gene expression was measured as a ratio of annetocin gene expression and *β*-actin gene expression (see *Section 2.2.26)* by real-time quantitative PCR using dual-labelled oligonucleotide probes.

The control animals from the BPA and NP exposures were pooled and sixteen earthworms were selected at random for gene expression analysis. Therefore, in *Figure 6.16* the 'All Controls' group is representative of the relative annetocin gene expression in earthworms removed from eight replicates. Upon analysis of the qPCR data obtained from these earthworms, two PCR reactions were found to be failures. Therefore, the number of animals used in the 'All Controls' group was n = 14.

The highest relative annetocin gene expression was seen in those earthworms that were not exposed to NP, i.e. the "All Controls" group (1.91 ± 0.48) (*Figure 6.16*). Relative annetocin gene expression was reduced in all the test treatments, but not significantly ($p \leq 0.05$).

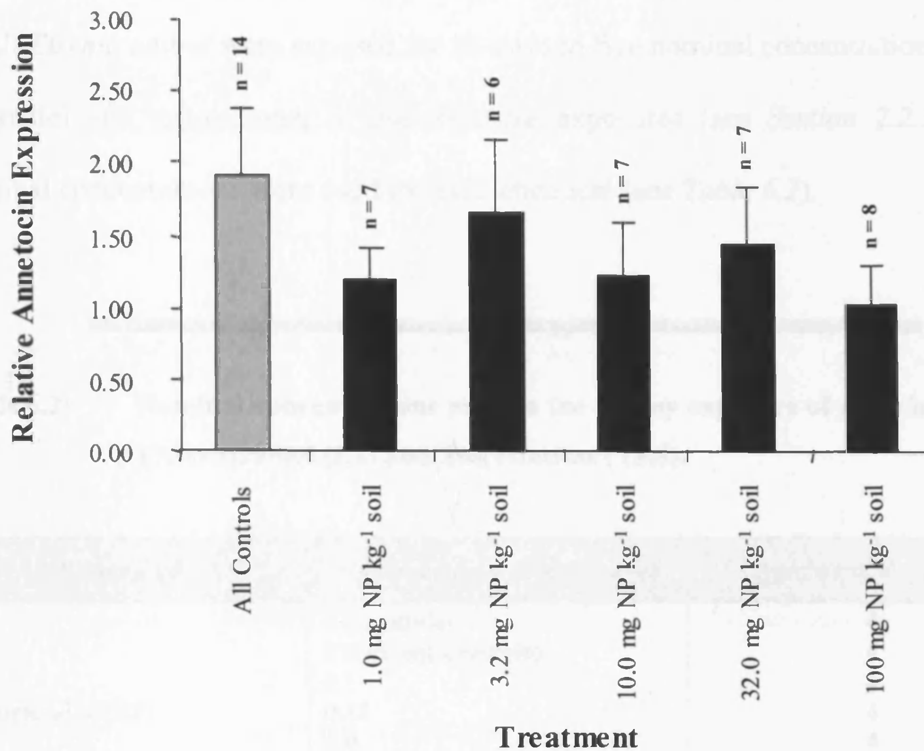


Figure 6.16: Relative annetocin gene expression in earthworms exposed for 28-days to 4-nonylphenol. Relative annetocin gene expression is the ratio of β-actin gene expression to annetocin gene expression. Transcript levels were determined using dual-labelled fluorescent oligonucleotide real-time PCR. Individual animals were used to determine relative annetocin gene expression (relative to β-actin) and a mean relative annetocin gene expression value was determined (Standard Error of the Mean was also calculated). "All Controls" represents the mean annetocin gene expression found in those earthworms from the control and solvent control populations (n = 14). AC = significantly different from the "All Controls" group. Standard Error of the Mean (SEM) is shown by error bars.

(C) *EISENIA ANDREI* 56-DAY OECD EARTHWORM REPRODUCTION TEST – 17β OESTRADIOL AND TESTOSTERONE

6.3.11 CHEMICAL CONCENTRATIONS USED IN 56-DAY REPRODUCTION TESTS

Adult *Eisenia andrei* were exposed for 28-days to five nominal concentrations of 17β-oestradiol and testosterone, in two separate exposures (see *Section 2.2.5*). Five nominal concentrations were used for each chemical (see *Table 6.2*).

Table 6.2: Nominal concentrations used in the 28-day exposure of *Eisenia andrei* to 17β-oestradiol (E2) and Testosterone (TES).

Compound	Concentration (mg kg^{-1} soil)	Number of Replicates
17β-oestradiol (E2)	0 (Controls)	4
	0 (Solvent Controls)	4
	0.1	4
	0.32	4
	1.0	4
	3.2	4
	10.0	4
Testosterone (TES)	0 (Controls)	4
	0 (Solvent Controls)	4
	0.1	4
	0.32	4
	1.0	4
	3.2	4
	10.0	4

6.3.12 28-DAY GROWTH AND SURVIVAL OF EARTHWORMS EXPOSED TO 17β OESTRADIOL AND TESTOSTERONE

After a 28-day period of exposure in OECD artificial soil (see *Section 2.2.5*) adult earthworms were removed from the soil and counted. Survival was determined as a percentage of the number of earthworms placed on the soil at the start of the exposure period and the number of earthworms remaining on the soil after 28 days. Average survival in each of the replicates is shown in *Table 6.3*.

Table 6.3: Survival of Adult *Eisenia andrei* Exposed to Nominal Concentrations of two Endocrine-related Compounds

Compound	Concentration (mg kg^{-1} soil)	Average Survival (% ± SEM)
17β-oestradiol (E2)	0 (Controls)	97.5 ± 2.5
	0 (Solvent Controls)	90.0 ± 4.1
	0.1	95.0 ± 2.9
	0.32	97.5 ± 2.5
	1.0	100 ± 0.0
	3.2	100 ± 0.0
	10.0	75.0 ± 21.8
Testosterone (TES)	0 (Controls)	90.0 ± 0.0
	0 (Solvent Controls)	92.5 ± 2.5
	0.1	85.0 ± 2.9
	0.32	90.0 ± 0.0
	1.0	90.0 ± 0.0
	3.2	90.0 ± 0.0
	10.0	87.5 ± 2.5

Earthworm survival in both the control population and solvent control population (in both exposures) was well within the OECD guidelines (2000). Therefore, the tests were valid. Earthworm survival, in general, was not effected upon exposure to either of the two compounds used in the separate experiments. Although a reduced survival rate was observed in those earthworms exposed to 10.0 mg E2 kg^{-1} soil, this mortality was not deemed significantly different from the control or solvent control populations. These finds were in keeping with those observed during the 14-day 'range-finding' experiment conducted using these chemicals (*Section 6.3.1; Figures 6.4 & 6.5*).

Growth of *Eisenia andrei* was unaffected upon exposure to both chemicals (data not shown). Growth was measured by comparing the average weight of the earthworms at the start of the test with that at the end of the test. At the end of the 28-day exposure period those animals from the E2 control group had an average wet weight of, 0.647 ±

0.031 g; whilst the E2 'solvent control' group had an average wet weight of, 0.569 g ± 0.016 g. A similar pattern was seen in the TES exposed control and 'solvent control' groups, 0.477 g ± 0.009 g and 0.438 g ± 0.027 g respectively. These data suggest that the animals used in these exposures were healthy.

6.3.13 REPRODUCTIVE SUCCESS OF *EISENIA FETIDA* EXPOSED TO 17β–OESTRADIOL AND TESTOSTERONE OVER A 28-DAY PERIOD

In accordance with the protocol set out in *Section 2.2.5* cocoons were removed from the soils and counted after 56 days (from the start of the exposure period). Each soil was wet sieved (see *Section 2.2.5*) to remove all cocoons laid during the exposure period. This enabled a cocoon production rate (number of cocoons/worm/week) to be calculated for each replicate (each replicate containing ten worms at the start of the exposure).

The number of juvenile earthworms produced at the end of the test was also determined. OECD guidelines (2000) recommend that a successful earthworm reproductive test should yield ≥ 30 juveniles/replicate in the control replicates. However, this was not the case in either of the 28-day exposures using the two compounds listed above. In fact, the average number of juveniles produced per replicate in the control group from the 17β-oestradiol experiment was 4.50 ± 2.25 juveniles/replicate. In the solvent control group from this experiment a figure of 2.25 ± 0.48 juveniles/replicate was observed. In the testosterone exposed treatment groups a similar pattern was observed. Control replicates produced 0.00 ± 0.00 juveniles/replicate and the solvent controls produced 0.30 ± 0.25 juveniles/replicate. The juvenile production rates in each of the exposed replicates were similar in both

experiments and no significant changes were seen (Mann-Whitney non-parametric test). Therefore, the test was deemed to be unsuccessful following the OECD guidelines (2000).

6.3.14 ANNETOCIN GENE EXPRESSION IN CONTROL POPULATIONS OF *EISENIA FETIDA*

As cocoon production rates, and hence juvenile numbers, were low in both the 17*β*-oestradiol and testosterone exposures it was decided that downstream processing of earthworms for annetocin gene expression analysis was not necessary. However, those earthworms from the control and solvent control populations were processed for annetocin gene expression profiling. This was done to compare annetocin gene expression at different times of the year.

The BPA and NP exposure experiments (earthworm reproduction tests, "All Controls" group) were carried out during September/October. During autumn earthworms (of all species) in 'natural' conditions would be at the peak of their reproductive output (Sims & Gerard, 1985). The 17*β*-oestradiol exposure was carried out during May/June, a period when reproductive output is classically low (Sims & Gerrard, 1985). The testosterone exposure was run during July/August, again when reproductive output is low in natural populations. Although the exposures were carried out under laboratory conditions (20 °C; 12 hours light, 12 hours dark), the earthworms had been collected from populations living under natural conditions immediately prior to exposure.

As previously mentioned the "All Controls" group was used as a comparison for the two other tests. The "All Controls" group showed the mean relative annetocin expression in those earthworms maintained on control artificial soil during the BPA and NP 56-day exposures (*Sections 6.3.7* and *6.3.10*). This group showed a mean relative annetocin gene expression value of 1.91 ± 0.48 (n = 14) (*Section 6.3.7*), which correlated with a cocoon production rate of, 0.331 ± 0.050 cocoons/worm/week (n = 8). The control group from the 17*β*-oestradiol experiment (run during May/June) showed a relative annetocin gene expression of 0.06 ± 0.02 (n = 6), whilst the control group from the testosterone exposure experiment (run during July/August) showed an annetocin expression of 0.38 ± 0.14 (n = 8). The two relative annetocin gene expression values from May/June and July/August were not significantly different from each other ($p \leq 0.05$), whilst they both were significantly different from the value obtained in September/October. These values correlated with low cocoon production rates from both experiments (Control E2 = 0.09 ± 0.02 cocoons/worm/week, n = 4; Control TES = 0.00 ± 0.00 cocoons/worm/week, n = 4).

Figure 6.17 shows the relative annetocin gene expression as a function of cocoon production rates of control earthworms from the three 28-day exposures detailed in this chapter (i.e. the 'All Controls' group from the BPA/NP exposure [run during September/October], the control group from the 17*β*-oestradiol exposure [run during May/June] and the control group from the testosterone exposure [run during July/August]).

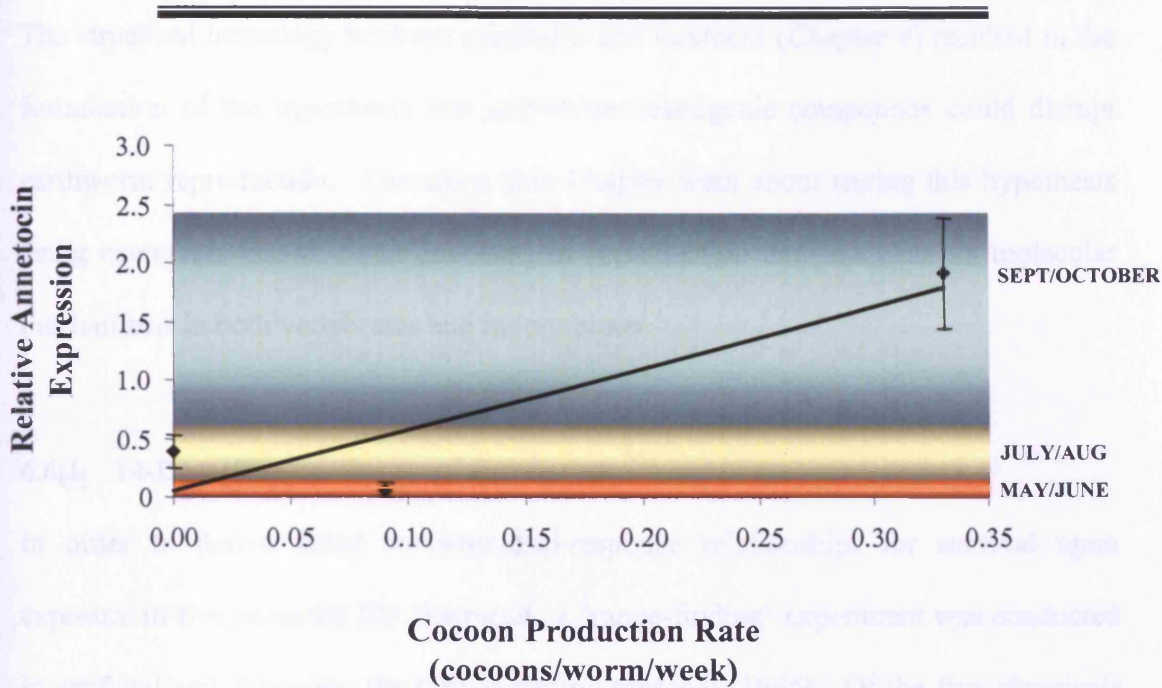


Figure 6.17: Relative Annetocin Gene Expression Compared to the Cocoon Production Rates from Earthworms at Different times of the Year. *Eisenia andrei* earthworms were maintained on OECD artificial soil for a 28-day period at standard conditions. After 28-days adult earthworms were removed from the soil, but any cocoons or juveniles present in the soil were left for a further 28-day period. After a total of 56-days the soils were sieved and the number of cocoons and juveniles were counted. A cocoon production rate was calculated (number of cocoons/worm/week). At different times of the year *Eisenia andrei* produced cocoons at varying rates.

6.4 DISCUSSION

The structural homology between annetocin and oxytocin (*Chapter 4*) resulted in the formulation of the hypothesis that soil-borne oestrogenic compounds could disrupt earthworm reproduction. Therefore, this Chapter went about testing this hypothesis using chemicals known to be involved in reproduction and modulate its molecular mechanisms in both vertebrates and invertebrates.

6.4.1 14-DAY 'RANGE-FINDING' EXPOSURE

In order to derive fitted concentration-response relationships for survival upon exposure to five potential ED chemicals, a 'range-finding' experiment was conducted in artificial soil following the OECD testing protocol (1984). Of the five chemicals used in this experiment only three showed toxicity to the earthworm. BPA and NP were the most toxic with LC_{50} values of 103 and 51 mg kg^{-1} soil respectively (see *Figures 6.1 & 6.2*). Whilst EE2 only produced mortality at the highest dose (*Figure 6.3*) and E2/TES exposure resulted in no mortality (*Figures 6.4 & 6.5*).

Each of the five treatment groups were analysed for annetocin gene expression to verify whether these chemicals were effecting earthworm reproduction over the 14-day exposure period. It was observed that gene expression in the 'solvent control' treatment group had decreased by approximately half when compared to the control group (see *Figures 6.7 – 6.10*) and meant that control treatment group data was deemed void. There is no evidence that acetone (the solvent used in this case; see *Section 2.2.4 & 2.2.5*) is capable of directly interfering with the reproductive mechanisms in earthworms, but it may have been toxic to the organisms, particularly if it had not fully evaporated before the earthworms were placed on the soil.

Annetocin gene expression in those animals exposed to BPA and NP was measured in those groups with greater than 90 % survival (*Figures 6.7 & 6.8*). BPA exposure reduced the relative annetocin gene expression levels by approximately 75 % of the control group expression level. However, these levels were statistically different from the expression levels found in the 'solvent control' treatment group. Therefore, although BPA was toxic to earthworms at concentrations > 10 mg kg^{-1} soil, it did not seem to effect annetocin gene expression. Any differences in expression levels can be put down to the use of a solvent in the test regime. Exposure to NP resulted in lower annetocin gene expression levels (*Figure 6.7*). At 32.0 mg NP kg^{-1} soil annetocin gene expression was approximately 25 % of that found in the 'solvent control' treatment group. This was despite the observation that survival at this NP concentration was high (90 %) (*Figure 6.2; Panel A*). This finding suggested that annetocin gene expression could potentially be a sensitive biomarker of harmful effects of chemicals to earthworms. A biomarker must serve as an 'early-warning' system, and this is shown in this example.

The annetocin expression pattern from earthworms exposed to the synthetic hormone 17α-ethynylestradiol (EE2) showed that the chemical could potentially increase the expression of this reproductive gene. At 'mid-range' EE2 concentrations (i.e. 10.0 and 32.0 mg EE2 kg^{-1} soil) an increase in gene expression was observed (*Figure 6.8*). This increase was not greater than that found in the control treatment group, but it was greater than that found in the 'solvent control' group. This is valid if we take into account the solvent effect on annetocin gene expression levels observed in the 'solvent control' group. At the highest EE2 concentrations gene expression levels were

reduced, probably due to the increasing toxic effect of this chemical (LC_{50} > 320 mg EE2 kg^{-1} soil).

On exposure to 17β-oestradiol (E2) and testosterone (TES) a similar annetocin gene expression pattern was observed as with exposure to EE2. Both of these chemicals had LC_{50} values > 1,000 mg kg^{-1} soil, indicating that they were not toxic to earthworms in soil. However, at 'mid-range' chemical concentrations (between 3.2 and 32.0 mg kg^{-1} soil) annetocin gene expression was significantly increased in comparison to the levels seen in the 'solvent control' treatment group (*Figures 6.9 & 6.10*). This observation was particularly marked in exposure to TES at these concentrations (*Figure 6.10*), indicating that TES may be involved in the regulation of the annetocin gene. However, there is a possibility that TES may be converted to oestrogen (specifically E2) by the earthworm's natural synthesis pathways (i.e. via the aromatisation of TES catalysed by the P450 enzyme, aromatase).

These exposure regimes allowed the identification of concentration ranges at which annetocin expression could be measured without toxic effect to the animals. *E. andrei* have a reproductive cycle of approximately 21 days (Sims & Gerard, 1985), so an exposure regime was used that would allow cocoons measurements to be made, i.e. a 28-day OECD earthworm reproduction test (2001).

6.4.2 56-DAY REPRODUCTION EXPOSURE – BISPHENOL A

From the 14-day exposure an LC_{50} value of 100 mg BPA kg^{-1} soil was determined (*Figure 6.1; Panel A*). Therefore, the highest concentration for the 56-day exposure was chosen as 100 mg BPA kg^{-1} soil (*Table 6.1*). During a study of sewage sludge in

Canada a maximum BPA concentration of 36.7 mg kg^{-1} dry sludge was observed (Lee & Peart, 2000). Therefore, the concentrations used in this study reflect these observations. No significant mortality was observed at any of the test concentrations (*Section 6.3.6*). This was not expected, particularly at the highest BPA concentration used and pointed to the fact that the exposure protocol may have been sub-optimal. Cocoon numbers were high in all treatment groups (*Figure 6.11*), expect in the 'solvent control' group, where cocoon production rates were approximately half of those found in the control treatment. This reflected the results observed in the 14-day exposure and reaffirmed the fact that the solvent, acetone, may effect earthworm reproductive rates. If this is the case we would expect that cocoon production rates would be low in the test treatment groups as well. However, in all treatment groups cocoon production rates were greater than that found in the 'solvent control' group (*Figure 6.11*). This suggested that BPA increased the reproductive output of those earthworms within the test regime. If this was the case annetocin gene expression levels should have been greater than the 'solvent control' group. Unfortunately, those animals exposed in the 'solvent control' group were not genetically analysed. Rather the controls were the only group tested for annetocin gene expression. Therefore, it was not possible to compare annetocin gene expression levels in the 'solvent control' animals and the test animals.

Only those animals exposed to 3.2 mg BPA kg^{-1} soil showed a statistically lower annetocin gene expression level compared with the control animals (*Figure 6.13*). The other test treatment groups showed lower (compared to control animals) annetocin expression levels, but this was not significant. These results indicate that annetocin gene expression is not effected by exposure to BPA, but the decreased

levels of the gene may be due to the solvent effect. However, the annetocin gene levels did not correlate with the cocoon production rates as these were greater in the test treatments compared with both the control treatments (*Figure 6.11*). Lowered annetocin gene expression was observed in animals exposed to BPA during the 14-day exposure, but during this test cocoon production rates were not recorded. It may be that cocoon production rates are maintained above a threshold annetocin gene expression level and they only drop when the expression level falls below this point.

6.4.3 56-Day Reproduction Exposure – 4-Nonylphenol

As with the 56-day BPA exposure a maximum concentration of 100 mg kg^{-1} soil of nonylphenol was used (*Table 6.1*). Concentrations in the order of µg/L are required in water to cause effects in fish (Jobling *et al.,* 1996). Therefore, it was expected that greater concentrations would be required in soil if similar effects were to be observed.

A similar pattern of cocoon production rates to that found in the 56-day BPA exposure was observed when animals were exposed to NP. Cocoon production rates were low in the 'solvent control' treatment group, indicating that acetone certainly effected earthworm reproductive rates. However, cocoon production rates in the test groups were higher than that found in the 'solvent control' group. Once again, on the face of it this would indicate that NP had the effect of increasing earthworm reproductive output. However, no toxicity was observed in the highest concentration treatment group (100 mg NP kg^{-1} soil), despite this concentration causing 70 % mortality during the 14-day exposure (*Figure 6.2; Panel A*). This would tend to suggest that the animals were not exposed to the NP during the exposure period. Thiele *et al.* (1997) reviewed the presence of NP in a number of different sites. In one study the level of

NP found within a field soil had decreased to 20 % of its original level during a three week period. If this was the case in the 28-day exposure period it would be valid to suggest that the levels of NP within the test soils had diminished to such an extent that no toxic effect was left after two to three weeks. Therefore, the animals exposed to the test concentrations would have had time to begin reproduction once again before the end of the test and this hypothesis could also be applied to the situation with BPA.

6.4.4 56-DAY REPRODUCTION EXPOSURE – 17β-OESTRADIOL AND TESTOSTERONE

These two naturally occurring hormones are found at low levels within sewage effluent (concentrations in the low ng/L range cause effects in fish; Purdom *et al.*, 1994). Therefore, a lower concentration gradient than in previous studies was used (see *Table 6.2*).

Earthworm survival was generally high in these exposures (*Table 6.3*). Although, reduced survival was observed in the highest concentrations used (10 mg kg^{-1} soil), but these were not statistically different from the survival observed in the control groups. This data along with that from the 14-day exposure confirmed that E2 and TES do not appear to be toxic to earthworms at the concentrations used.

As detailed in *Section 6.3.13* cocoon production rates and hence juvenile production (in the control treatment groups) in both of these exposure protocols were low. Hence, the exposure was deemed invalid according to OECD requirements (2000). In the test treatment groups of these exposures no cocoons were produced, which was not the finding that was expected after seeing increased annetocin gene expression in some earthworm treatment groups in the 14-day exposure. A possible explanation for

this is that the earthworms used in this testing regime were not in the full reproductive cycle at the start of the exposure period (although wet weights at the end of the test suggested that the earthworms were in good health). Hence, reproductive output was low – this would also suggest that E2 and TES did not increase annetocin/reproductive levels upon soil exposure. This could either be due to poor exposure in the soil or that E2 and TES do not play a role in earthworm reproductive mechanisms.

With the question of seasonal fluctuations of reproductive rates in mind, control group annetocin gene expression levels were measured in animals from three different studies. *Figure 6.17* shows the cocoon production rates versus the relative annetocin gene expression of thee control group animals from the three exposure periods (taking place at different times of the year). Although this data set is limited it suggests that cocoon production is greatest during the autumn period (September/October) and much lower during the summer (May through to August). Annetocin gene expression values correlate with this observation. This would suggest that annetocin is an integral factor in earthworm reproduction, but that other stimuli and mechanisms are involved in the complex process of reproduction.

Published work prior to this study had focussed on the toxicity of heavy metals found in sewage sludge to the earthworm. This was despite the finding of additional chemicals capable of disrupting vertebrate and invertebrate reproduction (Johnson, 2003). The toxic effect of NP in sewage sludge on the springtail, *Folsomia fimetaria* (Collembola: Isotomidae) has been investigated (Scott-Fordsmand & Krogh, 2003) and reproductive effects were observed at concentrations above approximately 5 mg NP kg^{-1} dry soil.

This study was conducted to increase the knowledge of reproductive effects on terrestrial organisms resulting from EDCs found in sewage sludge and from animal manure. This broad aim was achieved here, but these tests uncovered a number of interesting observations. Firstly, seasonal variation in annetocin gene expression and hence reproductive output is of major importance when chemical toxicity is to be considered. Therefore, before annetocin can become a strong biomarker of reproduction in earthworm ecotoxicology further investigation of this is required. Secondarily, annetocin gene expression was increased (in some cases) upon exposure to EE2, E2 and TES. This suggested that the annetocin gene was modulated by these chemicals, either indirectly or directly and that this hypothesis warranted further investigation. The following sections detail the investigation of the annetocin gene promoter in *E. fetida*.

CHAPTER 7

THE ELUCIDATION OF THE ANNETOCIN GENOMIC STRUCTURE

7.1 INTRODUCTION

Eukaryotic gene structure, in general, follows a well defined pattern. The gene is usually divided into a number of introns and exons and upstream of the first exon is the promoter region. The promoter region is not transcribed during gene expression to yield RNA, but it is this section of the 'gene' which controls gene expression. The promoter of eukaryotic genes contains regions of DNA that interact with the cells transcription machinery (i.e. RNA polymerase, transcription factors, etc.) and hence drive (or repress) gene transcription (production of an RNA copy of the gene).

The promoter region of a gene can therefore allow the identification of the function (or part of the function) of a particular gene, e.g. the metallothionein gene promoter, a metal binding protein found in a wide range of organisms including earthworms, contains transcription factor binding sites which bind metal responsive transcription factors that enhance gene expression. Therefore, this section of work details the analysis of the promoter region of annetocin from the earthworm, *Eisenia fetida*.

The Universal GenomeWalker™ kit (Clontech) allows the analysis of promoter regions of any gene when DNA sequence information of the gene is known (i.e. if the DNA sequence of the cDNA is known). The method involved in the use of this kit is described in *Section 7.2*. However, a schematic flow diagram detailing the processes involved in genome walking is shown in *Figure 7.1*.

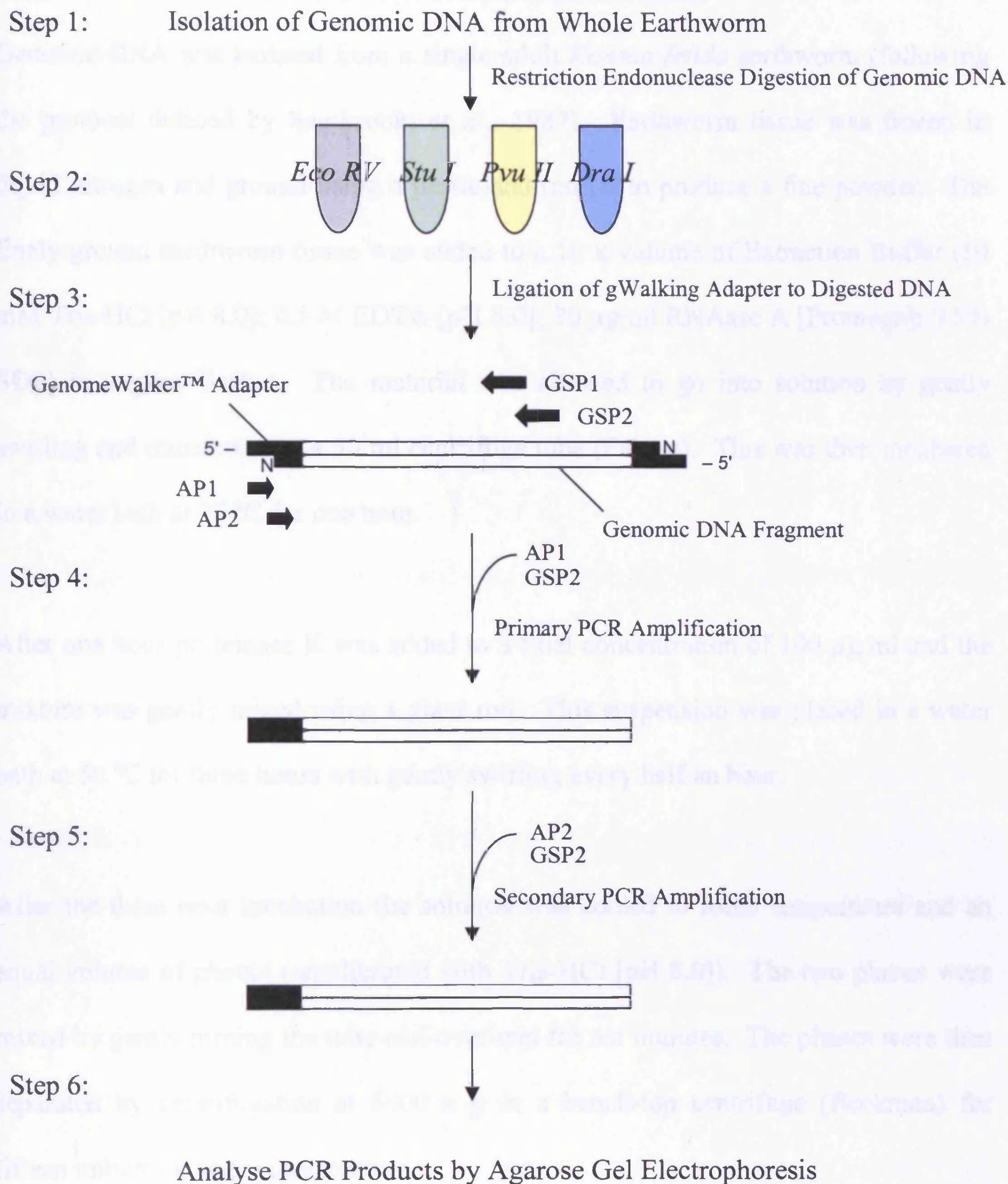


Figure 7.1: Flow chart representing the Genome Walking protocol. Genome 'walking' allows the amplification and analysis of genomic DNA up- or downstream of known DNA sequences. AP1 and AP2 represent adapter primer 1 and 2 respectively, whilst GSP1 and GSP2 represent gene-specific primer 1 and 2 respectively.

7.2 METHOD

7.2.1 ISOLATION OF GENOMIC DNA FROM *EISENIA FETIDA*

Genomic DNA was isolated from a single adult *Eisenia fetida* earthworm (following the protocol defined by Sambrook, *et al.*, 1987). Earthworm tissue was frozen in liquid nitrogen and ground using a pestle and mortar to produce a fine powder. The finely ground earthworm tissue was added to a 10 x volume of Extraction Buffer (10 mM Tris-HCl [pH 8.0]; 0.1 M EDTA [pH 8.0]; 20 μg/ml RNAase A [Promega]; 0.5% SDS) in a glass beaker. The material was allowed to go into solution by gently swirling and transferred to a 50 ml centrifuge tube (Falcon). This was then incubated in a water bath at 37 °C for one hour.

After one hour proteinase K was added to a final concentration of 100 μg/ml and the mixture was gently mixed using a glass rod. This suspension was placed in a water bath at 50 °C for three hours with gently swirling every half an hour.

After the three hour incubation the solution was cooled to room temperature and an equal volume of phenol (equilibrated with Tris-HCl [pH 8.0]). The two phases were mixed by gently turning the tube end-over-end for ten minutes. The phases were then separated by centrifugation at 5000 x *g* in a bench-top centrifuge (Beckman) for fifteen minutes at room temperature.

The viscous aqueous phase was then removed using a wide-bore pipette as not to shear the genomic DNA. This was transferred to a clean 50 ml centrifuge tube (Falcon) and the extraction process with phenol was repeated twice.

After the third extraction with phenol the aqueous phase was removed, transferred to a clean 50 ml centrifuge tube (Falcon) and a 0.2 volume of 10 M ammonium acetate was added. To this solution two volumes of room temperature ethanol was added. The solution was mixed by swirling gently and the genomic DNA formed a precipitate. This precipitated gDNA was removed from the solution using a plastic pipette bent into a U-shape, and placed in a clean 15 ml centrifuge tube (Falcon). This was allowed to dry at room temperature for approximately ten minutes before resuspending in 1.0 ml of TE buffer (pH 8.0). Resuspension was performed overnight at room temperature with gentle rocking.

7.2.2 ENZYMATIC DIGESTION AND PURIFICATION OF *EISENIA FETIDA* GENOMIC DNA

The four blunt-end restriction digestion enzymes used to fragment the earthworm gDNA were; *Dra* I, *EcoR* V, *Pvu* II and *Stu* I (all Promega). Each digestion reaction was performed in a 200 µl volume containing 7.5 µg of gDNA. Each reaction was incubated at 37 °C for two hours. They were removed from the water bath and gently vortexed, then returned to the water bath at 37 °C and incubated overnight.

The digested gDNA was purified using a standard phenol/chloroform extraction method (see *Section 2.2.9*), followed by ethanol precipitation (*Section 2*) using 20 µg of glycogen (Sigma) as a carrier. After precipitation the gDNA pellets were resuspended in a 20 µl volume of TE (10 mM Tris, 0.1 mM EDTA; pH 7.5).

7.2.3 LIGATION OF THE GENOME WALKING ADAPTERS

The genome walking (gWalking) adapter was designed according to the criteria defined in the Universal GenomeWalker Kit (Clontech, 2000). For sequence information refer to *Appendix C*.

The two single stranded gWalking oligonucleotides were mixed in equal amounts (25 µl of each oligonucleotide at 100 µM). This was made up to 100 µl with Buffer E (Promega). The mixture was incubated in a water bath at 100 °C for ten minutes, then allowed to cool to room temperature. Duplex formation was assessed by agarose gel electrophoresis on a 4.0 % gel (v/w) and visualisation under U.V. light.

Each sample of gDNA digested with the four restriction endonuclease enzymes was subjected to the ligation reaction separately. The reaction took place in an 8.0 µl volume containing 4.0 µl of each digested gDNA sample (in TE buffer). T4 DNA ligase (6 units/µl; Promega) was used. The reactions were incubated at 16 °C on a PCR thermal cycler (Perkin Elmer 480) overnight. The reactions were halted by heating to 70 °C for five minutes. A volume of 72 µl of TE (10 mM Tris; 1 mM EDTA; pH 7.5) was added to each reaction tube and the samples were mixed by vortexing gently.

7.3 RESULTS

7.3.1 GENOMIC DNA ISOLATION AND ENDONUCLEASE RESTRICTION DIGESTION

Genomic DNA was isolated from three adult *Eisenia fetida* earthworms, each with a wet weight of approximately 250 mg. These earthworms were ground under liquid nitrogen as described in *Section 7.2.1*, and the ground material was combined to give a

total approximate weight of tissue of 750 mg. After extraction gDNA (resuspended in 2.0 ml of TE Buffer, see above) concentration was estimated following the procedure described in *Section 2.2.13*. Before quantification a 1:10 dilution of the pure gDNA was made. This dilution was heated to 65 °C for ten minutes to remove gDNA secondary structure. This was then used to make the gDNA concentration measurement.

Quantification showed that approximately 276.2 µg of gDNA (138.1 µg/ml) had been isolated from the starting 750 mg of earthworm tissue. This DNA was of high purity as the A_{260}:A_{280} ratio as greater than 1.75 (*Section 2.2.13*). Further quality assessment of isolated gDNA was carried out by agarose gel electrophoresis on a 0.5 % (w/v) agarose gel (*Figure 7.2*). This showed that the gDNA isolated was of the correct size and that it migrated in the agarose gel much slower than linear DNA.

An appropriate volume of the resuspended gDNA was taken to give 7.5 µg and added to the four separate restriction endonuclease reactions. These reactions were performed as described in *Section 7.2.2*. The success of the restriction digest was assessed by agarose gel electrophoresis on a 0.5 % (w/v) agarose gel (*Figure 7.2*). A proportion of each digestion reaction was taken to give 0.375 µg of gDNA on the agarose gel. The digestion reactions were deemed successful as a smear of gDNA was seen on the agarose gel (*Figure 7.2*).

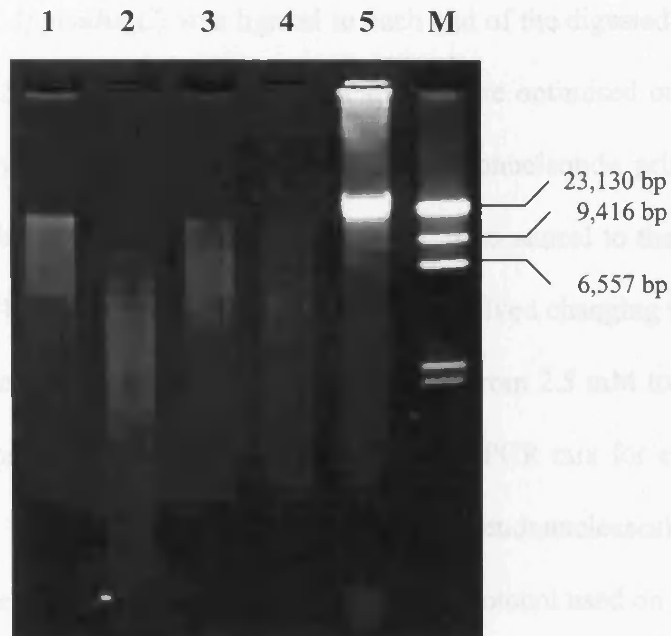


Figure 7.2: Genomic DNA isolated from *Eisenia fetida*. Genomic DNA (7.5 µg undigested) (lane 5) isolated from three, adult *Eisenia fetida* earthworms. Nucleic acids were run on a 0.5 % (w/v) agarose gel containing EtBr (0.4 µg/ml) and visualised under U.V. light. Lane 1 represents genomic DNA (0.375 µg) digested with the restriction endonuclease enzyme, *Eco RV*. Lane 2 shows the genomic DNA (0.375 µg) digested with *Stu I*. Lane 3 shows the genomic DNA (0.375 µg) digested with *Dra I*. Lane 4 shows the genomic DNA (0.375 µg) digested with *Pvu II*. Lane M represents the molecular weight DNA marker used to estimate DNA size (λ DNA digested with *Hind III*) (Promega).

7.3.2 PRIMARY AND SECONDARY PCR AMPLIFICATION OF THE ANNETOCIN PROMOTER REGION

The gWalking adapter (*Appendix C*) was ligated to each end of the digested gDNA as described in *Section 7.2.3*. Primary PCR amplifications were optimised on a Perkin Elmer 480 Thermal Cycler. Primary PCR used two oligonucleotide primers; one designed to anneal to the gWalking adapter (AP1) and one to anneal to the 5'-end of the annetocin gene (GSP1) (*Appendix C*). Optimisation involved changing the $MgCl_2$ concentration in each reaction (final concentrations ranged from 2.5 mM to 1.0 mM), and the annealing temperature used (60 °C or 62 °C). The PCR mix for each of the four reactions (gDNA digested with different restriction endonucleases) used the protocol described in *Section 2.2.16*. The thermal cycling protocol used on the Perkin Elmer 480 is described below;

95°C	for	10 minutes	
95°C	for	1 minute	x 35
60/62°C	for	1 minute	
72°C	for	2 minute	
72°C	for	5 minutes	

After primary PCR amplification it was found that the optimal final $MgCl_2$ concentration was 2.5 mM and an annealing temperature of 60 °C gave best results (data not shown).

Secondary PCR amplification utilised the 'nested' oligonucleotide primers designed to anneal to the gWalking adapter sequence (AP2) and the 5'-end of the annetocin gene (GSP2) (*Appendix C*). Both of these oligonucleotide primers were designed to anneal

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