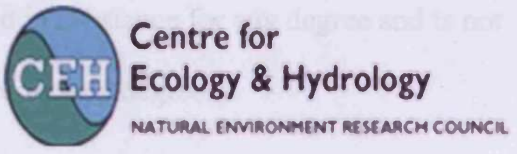

LIFE-CYCLE TOXICITY TESTING IN *CAENORHABDITIS ELEGANS* -- COMPARATIVE EFFECTS ON TRAITS AND THEIR MECHANISTIC BASIS

SUBMITTED FOR THE DEGREE OF Ph.D.

BY

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The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not 'Eureka!' but 'That's funny...'

(Isaac Asimov)

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Summary

A major aim of ecotoxicology is to discover how toxicants impact populations. Often lethality is used as an endpoint in toxicity tests; however this in isolation is inadequate to predict consequences for populations. The combination of life cycle toxicity testing and demographic modelling offers an opportunity to provide a solution to this problem, whilst the use of transcriptomic profiling offers the chance to understand how changes in life history are mediated at the molecular level.

The nematode *Caenorhabditis elegans* was utilised to elucidate the effects of environmental toxicants on the life history of individuals, population growth rate, energy utilisation, and gene expression. Toxicity of four common pollutants (cadmium, fluoranthene, atrazine and aldicarb) was assessed in full life-cycle toxicity tests. The impact of each chemical on life history parameters including; reproductive output/ period, time to maturity, growth and lifespan, was determined. These experiments revealed complex dose dependent responses indicating the most sensitive trait to be reproductive output. The influence of temperature and strain was investigated on cadmium toxicity indicated an increase in overall sensitivity at higher temperature and strain specific response profiles.

Integration of life history data into a demographic model provided a solution to translating effects on individuals into meaningful population responses. Decreases in juvenile survival and reproduction were identified as the traits which

caused the largest impacts on population growth rate. Life-cycle toxicity data was also integrated into a process-based model (DEBtox) to assess the effects of the toxicants on energy utilisation by the organisms. Finally, the mechanistic basis of observed life-history responses for the toxicant aldicarb was examined using transcriptomic profiling to identify single genes and biological and energetic pathways responsive to toxicant stress. Analysis of the molecular responses revealed novel mechanisms of toxicity.

Contents

Chapter 3 - The effects of a model toxicant on a nematode: A comparison of temperature and strain influences

Abbreviations and Conventions

aadUTP	Amino allyl dUTP
AChE	Acetylcholinesterase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
C. elegans	*Caenorhabditis elegans*
Cd	Cadmium
cDNA	Complementary DNA
CEH	Centre for Ecology and Hydrology
Cy	Cyanine
CYP P450	Cytochrome P450
D. magna	*Daphnia magna*
D. melanogaster	*Drosophila melanogaster*
dATP	Deoxy-Adenosine-Tri-Phosphate
DAVID	Database for Annotation, Visualisation Integrates Discovery
DCA	3, 4-dichloroaniline
dCTP	Deoxy-Cytosine-Tri-Phosphate
dGTP	Deoxy-Guanidine-Tri-Phosphate
dH20	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double stranded

dTTP	Deoxy-Thymine-Tri-Phosphate
dUTP	Deoxy-Uracil-Tri-Phosphate
EASE	Expression Analysis Systematic Explorer
ECx	Effect concentration
g	Gravity
GABA	Gamma animobutyric acid
GFP	Green fluorescent protein
GO	Gene Ontology
GST	Glutathione-s-transferase
HEPES	(N-[2-HydroxyEthyl]-Piperazine-N'-[2-EthaneSulphonic acid]
HPLC	High performance liquid chromatography
HSP	Heat shock protein
kb	Nucleotide kilobase pairs
LB	Luria Broth (Luria Bertani Media)
LCx	Lethal concentration
LTx	Lethal time
M	Molar concentration
M9	Buffer
MMLV-RT	Moloney-Murine Leukaemia Virus-Reverse Transcriptase
mRNA	Messenger RNA
MTL	Metallothionein
N	Number of samples analysed
n	Nucleotide

NCBI	National Centre for Biotechnology Information
NERC	Natural Environment Research Council
NGM	Nematode growth medium
NOEC	No effect concentration
nt	Nucleotide
OD	Optical Density
OP	Organophosphate
OP50	*E. coli* strain
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PNEC	Predicted no effect concentration
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
Sa	Adult survival
Sa	Survival of adults
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
Sj	Juvenile survival
Sj	Survival of juveniles
SNP	Single nucleotide polymorphism
Ss	Single stranded
SSC	Saline Sodium Citrate buffer

Ta	Time as a reproductive adult
TAE	Tris Acetate-EDTA buffer
TIFF	Tagged image file format
Tj	Time as a juvenile
Tm	Melting temperature
tRNA	Transfer RNA
U	Enzyme units
UTR	Untranscribed region
UV	Ultraviolet
X. laevis	*Xenopus laevis*
Zn	Zinc
λ	Lambda, population growth rate

The one and three letter abbreviations for nucleotides and amino acids are those recommended for use by the Biochemical Journal. Policy of the Journal and Instructions to Authors (1983). *Biochemical journal.* **209**. 1-27.

Genes are discriminated from proteins by the use of italicisation.

Chapter 1

Introduction

1.1 Environmental pollution

During the 20th century the Western world saw a vast expansion in industrialisation, and the consequential widespread pollution of the atmosphere as well as aquatic and terrestrial ecosystems. The need to monitor pollution in the environment and to have a greater understanding of the underlying ecology of organisms being challenged by pollution spawned a new field of research, ecotoxicology.

1.1.1 Ecotoxicology

Ecotoxicology grew out of the two research areas of ecology and toxicology as the need for understanding how toxicants impact the fidelity and health of natural ecosystems. Ecotoxicology forms the basis of regulatory testing of new and existing chemicals, and assists in the characterisation of the effects of potentially toxic chemicals, singularly or as mixtures, on individual organisms, populations, and communities.

Ecotoxicology not only covers the regulatory testing of chemicals, it also endeavours to understand the physiological and molecular effects of environmental chemicals. Effective pollution monitoring can be achieved by the

cumulative understanding of effects at molecular, individual, population and ecosystem levels.

1.1.2 Ecotoxicology Testing

Ecotoxicological testing often aims at measuring lethality or sublethal effects on organisms. Toxicity testing yields expressions of toxicity in terms of LC_x, LT_x, EC_x, NOEC and NEC values, amongst others. LC_x is the concentration at which x % of the test individuals die (lethal concentration). LT_x is the time at which x % of the test individuals die (lethal time). EC_x is the concentration at which there is an x % effect in an endpoint, commonly expressed as EC_{50} and EC_{10}. NEC is the no-effect-concentration, and NOEC is the no-observable-effect concentration.

The aim of regulatory ecotoxicology is to test chemicals in standardised tests. There are a number of such tests developed and optimised for terrestrial ecotoxicology, and they are based on the need to test new compounds on a few endpoints in a few species, including microbes, earthworms, and springtails. There are also standardized aquatic toxicity tests using the water flea *Daphnia magna* for assessing effluents and plant protection products.

Earthworms have long been recognised to have major contributions to soil fertility and structure (Edwards and Bohlen, 1996; Syers and Springett, 1984) and thus are deemed of high priority in regulatory testing. The OECD (Organisation for Economic Co-operation and Development) earthworm test is a 14 day LC_{50} test using the earthworm *Eisenia fetida*. A longer term earthworm reproduction test is also commonly used which can assess weight change, feeding activity, and

reproduction. A third standardized test used in regulatory testing is that with the soil dwelling collembolan *Folsomia candida.* This test which focuses on reproduction as an endpoint was produced by the International Standards Organisation (ISO, 1999). Microbes are also of importance in the maintenance of soil fertility and the decomposition of organic matter (Nannipien et al., 2003), and as such it is important to assess impacts on the soil microfauna. One standardised acute toxicity test, Microtox™, assesses the activity of luminescent bacteria to estimate the toxicity of environmental samples (Chang et al., 1981). Cellular activity in the bacteria emits light (Uitzar and Dunlap, 1995); when exposed to toxic compounds respiration and thus cellular activity is reduced which in turn reduces the amount of light emitted .

The aim of ecotoxicology research is to understand the toxicological effects of compounds in a range of organisms and their effects on individuals, populations, and ecosystems. There are a large number of sublethal toxicity tests used in terrestrial ecotoxicology which have been thoroughly reviewed by Spurgeon et al. (2002). One goal of ecotoxicological research is to develop biomarkers which can be used as environmental diagnostic tools for identifying stressed organisms, and thus provide early warning of toxicant-induced perturbations. An excellent review of the principles and practice of the application of biomarkers was published by Kammenga et al. (2000b). Organisms respond to chemical stress in many specific and non-specific ways, which can and have been assessed using cellular assays, enzyme assays, protein and molecular responses and alterations to behaviour, life history and demographics.

One cellular assay which has been developed as a potential biomarker in earthworms is the neutral red retention assay (Svendsen et al., 2004), which assesses lysosomal membrane stability of coelomocytes (the immuno-active cells in earthworms) which can be disrupted by exposure to inorganic and organic residues. Enzyme assays can be useful in detecting exposure to specific chemicals; a good example is provided by the reduction in acetylcholinesterase activity caused by organophosphate pesticides (Engenheiro et al., 2005).

The identification and measurement of the expression of specific genes involved in stress response, or linked to certain other functional parameters, are potentially useful biomarkers. The expression levels of a small number of genes have been quantified in terrestrial invertebrates exposed to sub-lethal concentrations of environmental toxicants; these genes include metal-inducible metallothioneins (Spurgeon et al., 2004; Swain et al., 2004), heat shock proteins (Kammenga et al., 1998; Stringham and Candido, 1994), and cytochrome P450s (CYP450s) (Menzel et al., 2005). Genes which have been shown to be mechanistically linked to life history parameters, such a reproduction, have also been assessed as potential biomarkers. One such example is annetocin, a gene implicated in the induction of egg-laying behaviour in the earthworm *Eisenia fetida*; its level of expression was down-regulated after exposure to lead and zinc, and corresponded with a reduction in reproductive output (Ricketts et al., 2004).

The most direct way of assessing the impacts of pollutants on organisms is to measure life cycle parameters such as reproduction, growth, or lifespan. This has high ecological relevance; however, a practical drawback with such performance

indicators is that in many organisms obtaining the necessary data can be time and resource consuming. In ecotoxicology, single life-cycle endpoints (e.g. mortality, growth, reproductive output) are often used in acute or chronic exposures, with the primary aim of these toxicity tests being to estimate critical effect levels (EC_x, LC_x) from concentration-response relationships (Kammenga et al., 1997a). The weakness of focussing on single end-points in toxicity testing is that there is no single parameter that is consistently the most sensitive trait across a range of chemicals, a range of species, or different life stages. Forbes and Calow (1999) analysed data from a large number of life-cycle experiments, and found no general pattern with respect to the sensitivity of life history traits of individuals. This illustrates the difficulty of making generalised rules concerning the effects of toxicants on life history.

The use of full life cycle toxicity tests can give invaluable information on the sublethal effects of toxicants on an organism's life cycle. Life cycle toxicity tests have added value in that the data collected can be used to predict toxicant consequences on population fitness, which is a useful ecological risk assessment metric for making predictions about long-term impacts on populations (Calow et al., 1997). Changes in population growth rates after exposure to environmental contaminants have been assessed in nematodes (Kammenga et al., 2000a; Kammenga et al., 1997b), polychaetes (Forbes et al., 2003; Linke-Gamenick et al., 1999), earthworms (Baveco and DeRoos, 1996; Klok et al., 1997; Spurgeon et al., 2003), collembola (Herbert et al., 2004; Noel et al., 2006), and isopods (Kammenga et al., 2001). Full life-cycle toxicity tests are an excellent way of

assessing toxicant impact on individuals with short life spans but are not practical for long-lived species.

Using molecular techniques stress responses to toxicants can be measured in order to gain greater understanding of the underlying biology associated with chemical exposure and can potentially also be exploited for use as biomarkers in regulatory ecotoxicology. New molecular biology methods, alongside more traditional life cycle assessments in ecotoxicology, will provide a fuller picture of an animal's responses to toxicosis.

1.1.3 Pollutants

Toxicants found in the terrestrial environment may be natural, or more commonly, anthropogenic. This project investigates the effects of compounds from three different major pollutant classes, and two compounds that have different biological targets from within one class (the specifically acting compounds). Table 1.1 details the compounds used.

Pollutants can have very diverse impacts on communities of organisms and can influence the ecological functioning of soil systems at virtually all trophic levels including affecting microbes (Brookes, 1995), invertebrates that live in the soil (Spurgeon and Hopkin, 1996) and invertebrates that live on the soil (Fountain and Hopkin, 2004). They can each potentially affect biodiversity in soil, which in turn affects the functional role of organisms in soil ecosystems leading to poor quality soil. Impacts on soil biodiversity can also have impacts on food webs affecting predator/prey relationships. The cumulative effects of chemicals may also

Table 1.1: Pollutant classes and compounds examined.

Major Class	Group	Compound	Pollution Source
Metals		Cadmium	Phosphate based fertilizers, industrial waste
Non-specifically acting compounds	Polycyclic Aromatic Hydrocarbons (PAHs)	Fluoranthene	Natural deposits of petroleum and gas. Formation during incomplete combustion of organic materials, e.g. from petrol, coal and tree fires.
Specifically acting compounds - Pesticides	Metabolic uncoupler	Atrazine (a triazine herbicide)	Run-off from agricultural practices. Atrazine is the highest usage herbicide in the USA.
	Acetylcholinesterase inhibitor	Aldicarb (a carbamate pesticide)	Aldicarb is used as a pesticide to control nematodes, mites and aphids.

impact on organisms higher up the food chain, a classic example being the organochlorine pesticide, DDT, which accumulated and caused major reproductive disruptions in birds of prey (Peakall, 1970).

1.2 Introduction to *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living bacterivorous nematode that is found in almost every type of terrestrial environment (Wood, 1988) (Figure 1.1). It is normally found as a self-fertilising hermaphrodite, although males are also found in low numbers. Males occur in about 0.2% in the population, arising spontaneously in hermaphroditic populations by X-chromosome non-disjunction at meiosis.

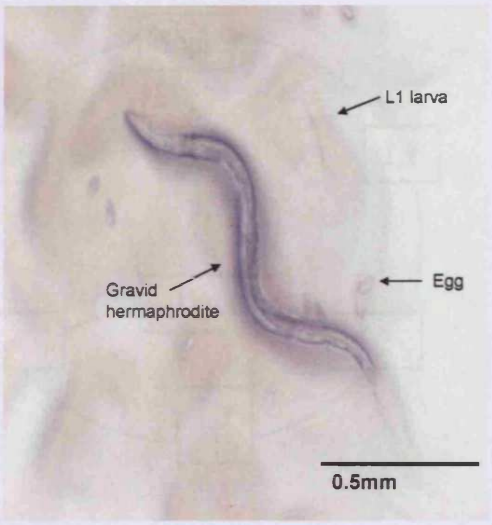


Figure 1.1: *Caenorhabditis elegans*. This photograph illustrates the hermaphrodite adult nematode, alongside a number of eggs that it has laid and an L1 stage larva, approximately 12 hours after it was laid as an egg.

C. elegans goes through 4 larval moults before becoming adult, taking approximately 3.5 days at 20°C. It has a reproductively active period of about 3-4 days, and then spends up to 12 days in senescence. Maximal adult length is usually about 1mm (Byerly et al., 1976). If food is abundant, and population density is not too great, the nematode proceeds through larval stages, L1, L2, L3 and L4 (it is during the L4 stage that sperm and egg cells are produced) to the reproductive adult phase (Figure 1.2). If food is limited or some other stressor, such as temperature, is present the larvae may take an alternative route, becoming dauer larvae at the second moult (Wood, 1988). Dauer larvae do not feed, they disperse and when they come into contact with food again development continues straight away. They can survive for up to 3 months until food is found, living off lipid reserves in their bodies. A single hermaphrodite worm produces about 280

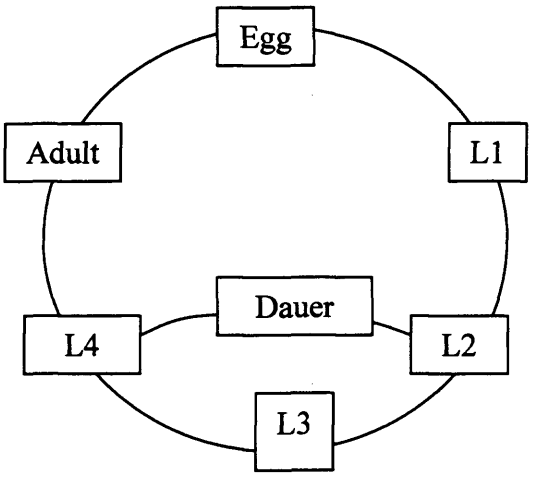


Figure 1.2: The life-cycle of *Caenorhabditis elegans*

eggs, which can increase to up to 1000 eggs by mating with a male, as sperm is the limiting factor for hermaphrodites (Riddle et al., 1997).

C. elegans was the first multi-cellular organism to have its complete genome sequenced (Consortium, 1998). It is the most well characterised metazoan to date, being a model organism for genetic, development, and neurobiology studies. Sydney Brenner pioneered it as a tool for research in 1965 and its value as a model organism can be attributed to a number of features: its small size, each hermaphrodite adult is composed of only 959 cells; the developmental cell lineage is known and invariant; its brief life span; low maintenance cost, and ease of laboratory culture. It is also amenable to genetic manipulation, and it possesses the advantage of the ability to both self-fertilise and cross-fertilise. There is also a central *C. elegans* strain bank (*Caenorhabditis* Genetics Centre in USA) which contains a vast number of wild-type strains isolated from the field, and laboratory-generated mutant strains that can be freely distributed to the research community.

Strain N2 is the laboratory standard wild-type strain which was isolated from mushroom compost near Bristol, England, by L.N. Staniland, pre-1956. It was subsequently cultured by W.L. Nicholas, C.E. Dougherty, and Sydney Brenner, and sub-cultured by Don Riddle in 1973 (www.wormbase.org).

C. elegans possesses a number of other traits that make it an ideal experimental organism for the specific purposes of the present study. Notably, its short life-span facilitates full life-cycle studies, and its transparency aids in the identification of specific life stages. *C. elegans* has an important function in this project due to the depth and breadth of knowledge about its genome, and the advanced molecular resources, and particularly proprietary DNA microarray chips, available to probe genomic changes induced by exposures to potential toxic chemicals.

In its natural habitat this nematode is found living in the interstitial spaces of soil. As most pollutants are transported by water movement throughout the terrestrial environment it follows that these nematodes would be directly exposed to xenobiotics, giving an ecotoxicological perspective to the use of *C. elegans*. As a result, *C. elegans* has also been used by several research groups for studies in terrestrial ecotoxicology. Although *C. elegans* is not the most sensitive nematode species to chemical perturbation (Kammenga et al., 1994) it has a number of advantages as an ecotoxicology testing species. The tests are rapid and can be performed in soil (Peredney and Williams, 2000), sediment (Hoss et al., 1999), agar (Popham and Webster, 1979), or liquid (Williams and Dusenbery, 1990) substrates. Moreover, the nematode can tolerate wide variations in salinity and pH without disturbing its capacity to respond to chemical stressors (Khanna et al.,

1997). *C. elegans* has been used to examine the toxicity of chemicals from a range of classes: metals (Jonker et al., 2004a; Peredney and Williams, 2000; Tatara et al., 1997); non-specific acting compounds, including phenols (Tominaga et al., 2003), tributylin (Hoshi et al., 2003), and 4-nonylphenol (Hoss et al., 2002); and specifically acting compounds, including a fungicide (Easton et al., 2001), and the estrogen mimic 17-β estradiol (Hoshi et al., 2003).

Whilst many of the toxicity tests performed with *C. elegans* are acute toxicity tests examining lethality, a range of sublethal endpoints have also been investigated (Anderson et al., 2001; Dhawan et al., 1999), such as growth and reproduction (Hoss et al., 1999). Behavioural endpoints have been examined in response to environmental chemicals including movement (Dhawan et al., 2000) and feeding rate (Jones and Candido, 1999). Further endpoints reported for toxicity studies with *C. elegans* are ultrastructural changes (Popham and Webster, 1979), molecular endpoints such as RNA/DNA ratios (Ibiam and Grant, 2005) and stress responsive gene expression (Custodia et al., 2001; Kwon et al., 2004; Menzel et al., 2005; Reichert and Menzel, 2005; Swain et al., 2004), and stress protein induction via the use of transgenic nematodes. Transgenic *C. elegans* have been assessed as potential biosensors for environmental pollution (Candido and Jones, 1996), these nematodes also provide the opportunity to identify the cellular sites of the stress response. Nematodes which carry the fusion gene *lacZ-hsp16* express β-galactosidase when stressed, which can then be measured to quantify the response of *C. elegans* to a wide range of stressors including the fungicide captan (Jones et al., 1996) and heavy metals (Stringham and Candido, 1994). A different transgenic strain that contains an *mtl-2-lacZ* transgene responds to heavy

metal perturbation (Cioci et al., 2000). However the majority of these studies are short-term taking from 24 - 96 hours to complete and examine only one life stage of the nematodes, normally adults, and thus are not representative of what is likely to be occurring in the field, as it would be expected that organisms are exposed to environmental chemicals throughout their lifespan.

The effect of toxicants on life-cycle events has become an increasingly popular study area in recent years. A number of life-cycle toxicity studies have been completed on nematodes, looking specifically at fitness and population dynamics (Kammenga et al., 1996; Kammenga et al., 1994; Kammenga et al., 1997b) and alterations to energy allocation (Alvarez et al., 2005; Jager et al., 2005). The effects of multiple toxicants on life history traits and fitness in *C. elegans* has also been studied (Jonker et al., 2004a; Jonker et al., 2004b).

1.3 Life-history

1.3.1 Life history Traits and Theory

Life histories refer to the age specific schedule of fecundity and mortality of organisms (Stearns, 1989). Life history theory aims to explain patterns in the evolution of reproduction, growth and survival traits. It does this by explaining the features of a life cycle and relating the phenotype to fitness of an individual and analysing how variation in life history traits lead to variation in fitness among individuals (Stearns, 1992). The key life cycle parameters that are normally measured are size at birth, growth, size and age at maturity, reproduction rate and reproductive period length and life span. The theoretical measure of fitness most

widely used, r, is called the intrinsic rate of natural increase, or Malthusian parameter (Stearns, 1992).

1.3.2 Demography

Demography is central to life history theory; it connects age- and stage- specific variation in mortality and fecundity to variation in fitness (Stearns, 1992). The timing of life-cycle events can be combined to gain an understanding of how changes within individuals lead to a change in population growth rate. The intrinsic rate of population increase, r_m, (also known as the Malthusian parameter) can be calculated from survival and reproduction schedules using the Euler-Lotka equation, as described in Kammenga and Laskowski (2000):

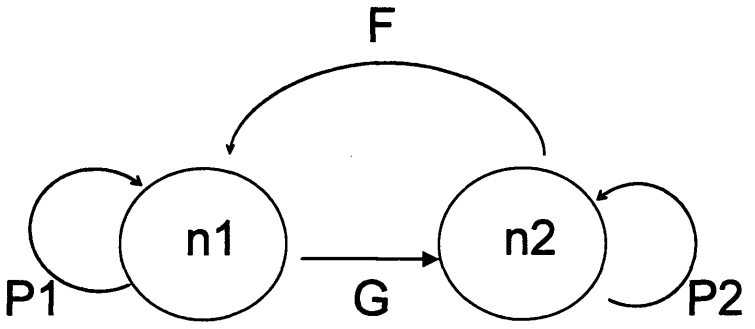
$$\int_{\alpha}^{\omega} e^{-r \cdot t} l_t \cdot m_t dt = 1$$

Where α is age at maturity, ω is age at last reproduction, t is age in general, l_t age-specific survival, and m_t is age specific offspring number.

More often the measure of population growth is documented as the population multiplication rate, λ, which gives a specific growth value over a set time. It is calculated from the *Euler-Lotka* equation, $\lambda = e^r$ (Calow et al., 1997). Disadvantages of population growth rate modelling are that it can only predict the potential performance of a population in an unlimited environment, as it does not take into account density dependence. In terms of the potential use of demography in ecotoxicology, population growth models do not take into account that chemical concentration may change over time or that subsequent generations may not respond identically (Stark and Banks, 2000).

There are two types of population growth models used to estimate *r*: age-specific and stage-specific population growth models. Each type has its own advantages and disadvantages. The stage-specific model includes time to maturity as a parameter, which has been shown to be of particular importance in changes in population growth rate as a result of toxicant exposure (Kammenga et al., 1996). Staged models do not require as much information as age-structured models; however the disadvantage of this is that stage-structured models do not take into account changes in reproduction over time. With age-specific models it is possible to determine the most important age classes affecting population growth rate; however, its main disadvantage is the requirement for data taken at regular time points.

The approach taken in this thesis to assess population growth rates is using a simple two stage population growth model (Caswell, 2001; Neubert and Caswell, 2000). The two stages are juveniles (pre-reproductive animals) and adults (reproducing animals). The model illustrated in Figure 1.3 shows the two stages, and the parameters required for the model: time to maturity, time to reproduce, number of offspring and the survival of juveniles and adults.



$$n_1(t+1) = P_1 n_1(t) + F n_2(t)$$

$$n_2(t+1) = G n_1(t) + P_2 n_2(t)$$

Figure 1.3: Diagrammatic representation of a two stage matrix model for population growth rate and equations. Where n_1 is the number of juveniles, n_2 is the number of adults, P_1 and P_2 are the probabilities of juvenile and adult survival respectively, G is the probability of a juvenile becoming adult and begin to reproduce, F is the reproductive output of the adults.

1.4 Dynamic Energy Budgets

1.4.1 DEB

The theory of Dynamic Energy Budgets (DEB) (Kooijman, 1993) describes how individuals acquire and utilize energy, based on a set of simple rules for metabolic organisation. According to the principle of allocation (Levins, 1968) and the dynamic energy budget theory (Kooijmann, 1993) energy resources in an organism are partitioned between reproduction, somatic maintenance and growth. These traits are inextricably linked to each other in the competition between one trait and another for finite energy resources.

Animals obtain their energy from oxidation and reduction reactions and get carbon from organic compounds. The food ingested by an animal is digested and transformed into faeces and egested. The energy derived from the food is taken up via the blood, which exchanges it with storage, and delivers energy to somatic and reproductive tissues, as illustrated in Figure 1.4. A central assumption in the DEB model is that growth and maintenance compete more directly with each other for available energy than with reproduction, known as the *k*-rule for allocation. A fixed part of the energy, *k*, is used firstly to maintain the animal (this includes all the processes that are necessary to keep the organism alive) and secondly for growth. The rest, 1-*k*, is used for development or reproduction. Energy requirements for maintenance always has priority over growth, so growth ceases if all energy available for maintenance plus growth is used for maintenance (Kooijman and Bedaux, 2000).

It is assumed that in a favourable environment the energy in an individual is partitioned so that the combination of these life cycle traits produces the optimal fitness for the individual. When an organism is stressed in some way then the combination of these traits may alter, caused by alterations in energetics through increased maintenance costs or decreased assimilation of energy. When an organism encounters a polluted environment, it needs to detoxify and repair damage, which it does by producing a range of stress responsive proteins which deal with this, examples of which are discussed in Section 1.5.5. The production of these proteins to combat and reduce stress is energetically costly. It follows then that the energy used in the production of these proteins would be diverted from energy that could have been used for some other function such as growth, or

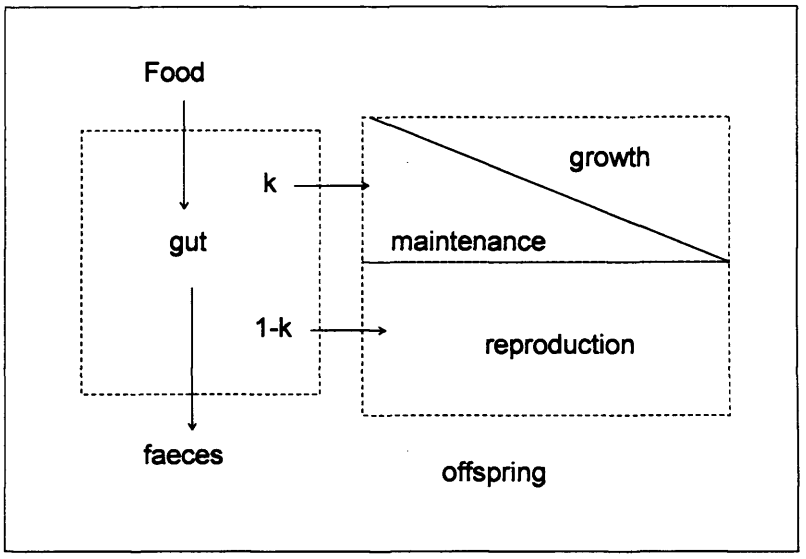


Figure 1.4: Energy channelling in the dynamic energy budget theory, taken from Kooijman and Metz (1984).

reproduction. Evidence for the reduction of energy reserves in toxicant exposed organisms comes from De Coen and Jansen (2003) who exposed *Daphnia magna* to cadmium chloride and found that lipid reserves were significantly reduced compared to controls. Similar effects have been described by Ribeiro et al. (2001) who found that parathion exposed *Porcellio dilatatus* (woodlice) had significantly decreased protein, glycogen and lipid contents, all of which are energy reserves or used in energy generation.

DEB has been used to describe how toxicants impact energy usage in an attempt to bridge the gap between individual and ecosystem-based approaches to environmental toxicology (Kooijman and Metz, 1984). They examined responses of individuals under chemical stress, in terms of individual energy balance. Their model framework allowed the distinction of how a chemical may affect the rate of

reproduction. The general model states that any chemical that hampers growth will necessarily affect reproductive rate. This model framework has been developed further to produce the DEBtox modelling software, both in a theoretical and practical way, to explain the allocation of energy under toxic stress (Kooijman and Bedaux, 1996).

1.4.2 DEBtox

DEBtox, a modelling program, developed by Kooijmann and Bedaux (1996) is used to evaluate how toxic substances alter the allocation of resources between the competing requirements of maintenance, growth and reproduction. Chemicals with different modes of action may change the allocation patterns and the software is designed to simulate such changes and their ultimate effects. It models multiple endpoints to give a single no effect concentration value. It also gives an indication as to the toxic mode of action of the chemical. There are 5 toxic modes of action covered by DEBtox (illustrated in Figure 1.5):

1 – Assimilation. The organism is unable to acquire as much energy, either from inefficient energy assimilation from the food, or the toxicant may directly affect the feeding rate.

2 – Maintenance costs. The energy costs required to keep the individual functioning properly is increased.

3 – Growth costs. The toxicant may exert its toxic effects on growth, in that it can either increase or reduce growth. As growth is inextricably linked to reproduction effects will be seen in this parameter also.

4 – Reproduction costs. There may be direct effect of the toxicant on reproduction, for example in the increase of energy costs of each egg.

5 – Hazard to embryo. The toxicant may directly impact the survival of embryos.

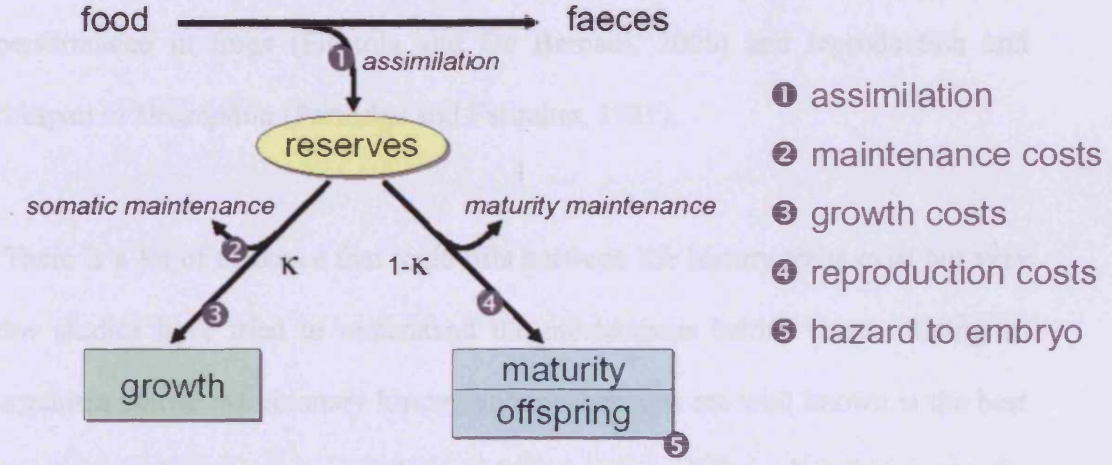


Figure 1.5: The possible places where toxicants can impact on energy that are described by the DEBtox program.

When reproduction is affected directly, as in 4 and 5 on Figure 1.5, growth assimilation and maintenance are not affected. However effects on assimilation, maintenance and growth have indirect effects on reproduction by delaying the onset of reproduction or by reducing energy available for the production of eggs. Allocation decisions between two or more processes that compete directly with one another for limited resources within a single individual can cause physiological trade-offs (Stearns, 1989).

1.4.3 Trade-offs

Trade-offs are the linkages between traits that constrain the simultaneous evolution of two or more traits (Stearns, 1989), they are measured as negative correlations between traits. Trade-offs between growth and survival have been measured in plants (Metcalfe et al., 2006), developmental rate and future performance in frogs (Ficetola and De Bernadi, 2006) and reproduction and lifespan in *Drosophila* (Partridge and Farquhar, 1981).

There is a lot of evidence that trade-offs between life history traits exist but very few studies have tried to understand the mechanisms behind them. Using an organism whose evolutionary history and mechanisms are well known is the best way to reveal mechanisms involved in life history evolution. For this reason the fruit fly *Drosophila melanogaster,* also a model organism, has been experimented with extensively. It would therefore make sense to use *C. elegans* for this purpose also and indeed in the last few years this has been increasing.

Gems *et al.* (1998) found that *daf-2* mutants had increased longevity without any significant reduction in fecundity, suggesting that the use of one energy resource, reproduction, is not obligately related to withdrawal of resources from the other, somatic maintenance. Another study using *C. elegans* suggests that DAF-16 mediates energy allocation between reproduction and maintenance in response to environmental change. Henderson and Johnson (2001) discovered that DAF-16 was found in the cytoplasm of cells when conditions were favourable, but was localized in the nucleus when conditions were stressing which suggests that this could be modulating resource allocation.

Recent challenges to the idea of resource allocation as the basis of life history trade-offs have come from work on the mechanistic basis of trade-offs. Leroi (2001) reviewed the evidence of antagonistic pleiotropy between longevity and fecundity and proposed that trade-offs may not be due to resource allocation at all, but rather to the effects of molecular signals. In *C. elegans* there are four gonad precursor cells present in the worm at hatching, Z1 and Z4 are precursors of the somatic gonad, Z2 and Z3 are germ line precursor cells. When the entire gonad was ablated there was no change in lifespan, suggesting that production of progeny does not affect the ageing process (Kenyon et al., 1993). When just the germ line precursor cells (Z2 ad Z3) were ablated, a 60% increase in longevity occurred suggesting that in normal worms a signal from the germ line inhibits DAF-16 activity. They then ablated the germ line cells of *daf-2* mutants and found that lifespan was four times greater than wild type. They have proposed that a signal from the germ line cells decreases lifespan by down regulating activity of *daf-16* and *daf-12* and an opposite acting signal from the somatic gonad increases lifespan by down regulating *daf-2* activity (Hsin and Kenyon, 1999). Barnes and Partridge (2003) have also reviewed the evidence surrounding this alternative hypothesis to trade-offs. They propose that both resource allocation and signalling pathways are responsible for trade-offs.

1.5 *Caenorhabditis elegans* Genetics and Life History

Life history traits are expected to be influenced by many genes (quantitative trait loci) as well as the environment. Quantitative trait loci, QTL, have been identified for many life history traits in *C. elegans,* including life span, fertility and age at

maturity in (Ayyadevara et al., 2001; Ebert et al., 1993; Gutteling et al., 2006; Shook et al., 1996; Shook and Johnson, 1999).

1.5.1 Regulation of fertility

The regulation of fertility in *C. elegans* is thought to be controlled at least in part by the insulin-signalling pathway. Mutations in the *age-1* and *daf-2* genes, which are part of the insulin-signalling pathway, give rise to worms with lower fertility, and also longer lifespan. Several experiments have been performed to locate QTLs for fertility. These studies locate important QTLs by composite interval mapping of recombinant inbred lines (RILs) of *C. elegans*, usually from initial crosses between the strains N2 (Bristol) and RW7000 (Bergerac) (Shook et al., 1996; Shook and Johnson, 1999), and more recently using N2 and CB4856 (Hawaii) RILs (Gutteling et al., 2006). QTLs for fertility have been identified on chromosomes II and IV by Shook and Johnson (1999) and on chromosomes IV and V by Gutteling at al. (2006).

1.5.2 Regulation of body size

Body size and growth is controlled via the interactions of many genes and environmental cues. The genetics behind body size has not been studied to any extent. Most small bodied mutant nematodes are defective in components of a TGF-β (transforming growth factor beta) – related signalling pathway, the Sma/Mab pathway (Flemming et al., 2000; Wang et al., 2002). There is strong evidence to suggest that the influence on environmental cues has a large impact on growth. Mutants that have defects in their sensory cilia exhibit abnormalities in

body size, which have been shown not to be caused by inability to locate food. Fujiwara et al. (2002) suggest that sensory perception can regulate neuro-endocrine functions that determine the growth and ultimate body size of nematodes.

1.5.3 Regulation of Life Span

The development of *C. elegans* has been studied for many years, from the first embryonic cell division to the mechanisms surrounding aging. Mutant nematodes have been isolated that can live up to twice as long in some cases as the wild type N2 and have been studied to identify genes involved with longevity which include *age-1* and *daf-2*. More than 50 genes have been identified so far that extend life span in *C. elegans*, including *daf-2* an insulin like receptor and *age-1*, a phosphatidyl inositol 3-OH kinase. These mutants become dauer larvae which extends their lifespan. A second type of long lived mutant are called the clock (clk) mutants. *Clk-1* mutants differ from the other mutants in that all aspects of their development are slowed down.

Apfeld and Kenyon (1999) have shown that mutations that cause defects in sensory cilia or their support cells, or in signal transduction extend life span (mutants in 5 genes were examined, *che-2, daf-6, daf-10, osm-3* and *osm-5)*. Hsin and Kenyon (1999) proposed that signals from the normal somatic gonad promote longevity by inhibiting DAF-2 receptor activity. Many mutants with extended life spans also exhibit reduced fecundity, e.g. *spe-10* (Cypser and Johnson, 1999), but this is not always the case (Gems et al., 1998; Lithgow et al., 1994).

1.6 Molecular Responses to Stress

Stress responses in organisms challenged by exposure to chemicals invariably involve various proteins that detoxify pollutants and repair damage to cells (Korsloot et al., 2004), many of which are induced, such as heat shock proteins, glutathione-S-transferases, cytochrome P450's and metallothioneins. Many of these response pathways have been investigated in *C. elegans* (Lindblom and Dodd, 2006).

Metallothioneins (MTLs): Two metallothionein genes have been identified in *C. elegans, mtl-1* and *mtl-2*. *mtl-1* is constitutively expressed in the bulb of the pharynx and is upregulated upon exposure to metals and can then be found in the intestine. *mtl-2* however, is not expressed under basal conditions but is rapidly expressed in the intestine when exposed to metals (Swain et al., 2004). Under normal conditions metallothioneins contribute to a cellular homeostasis of essential metals. Under metal exposure they act by binding to the metal ions.

Oxidative Stress Response: The components of the oxidative stress response decompose and scavenge reactive oxygen species (ROS) which are a by-product of respiration and metabolism. They function under toxicant exposure to scavenge oxidants, maintain cellular redox states and initiate repair of oxidative damage. Excess ROS lead to oxidative stress and damage to cellular structures such as membranes and can lead to apoptosis and necrosis (Buttke and Sandstrom, 1994). Superoxide dismutases (SOD) are a family of metalloenzymes responsible for quenching of superoxide radicals. MnSOD and Cu/ZnSOD mRNA levels

were found to increase in response to paraquat, a herbicide that produces oxidative stress (Tawe et al., 1998). Increased MnSOD gene expression has also been linked to extended life span in *C. elegans* (Honda and Honda, 2002).

Mixed Function Oxidases (MFOs): Under normal conditions MFOs are involved in the biotransformation and conjugation of xenobiotics, hormones and metabolites. MFOs are induced by chemicals that penetrate the cell and cause damage. One example of MFOs are the cytochrome P450s (CYP450s), which act in the phase I detoxification process. *C. elegans* has 80 CYP450 genes, which have been shown to be induced by a range of chemicals, including fluoranthene, atrazine and Toluene (Menzel et al., 2001; Menzel et al., 2005; Reichert and Menzel, 2005).

Glutathione-S-transferases (GSTs): GSTs are a family of detoxifying enzymes which are involved in the maintenance of cellular integrity. GSTs contribute to phase II biotransformation of xenobiotics by conjugating these compounds with reduced glutathione to facilitate dissolution in the aqueous cellular and extracelluar media, and from there, out of the body. GSTs have been shown to confer resistance to oxidative stress in *C. elegans* (Leiers et al., 2003).

Heat shock proteins (HSPs): Heat shock proteins are expressed under normal conditions and are involved in the secondary and tertiary folding and assembly of protein complexes and the intracellular transport of new proteins. They are general stress response proteins and are induced not only by heat as their name suggests but also by metals and other xenobiotics. They function to protect

essential proteins and to maintain cellular integrity. HSPs have been shown to be induced by heat, metal exposure and endocrine disrupting compounds in *C. elegans* (Custodia et al., 2001; Guven and Depomerai, 1995; Stringham and Candido, 1994)

1.6.1 Longevity and Stress Resistance

A universal trend in *C. elegans* is that increased longevity is associated with increased stress resistance (Johnson et al., 2001; Lithgow and Walker, 2002). This is also the case in fruit flies (Lin et al., 1998). Cypser and Johnson (1999) suggest that resistance to multiple forms of stress (UV, heat, and Reactive Oxygen Species (ROS) is essential for extended longevity. Lithgow et al. (1995) exposed N2 and *age-1*, *spe-26* and *daf-2* mutants to a temperature of 30°C for varying time periods. They found that the long-lived mutants were able to tolerate thermal stress better than wild type worms and that mild heat shocks lead to a small increase in life extension. *age-1, daf-2* and *daf-16* mutant alleles both extend life span and increase resistance to UV, heat and oxidative stress. Murakami and Johnson (1998) showed that OLD-1 is a positive regulator of longevity and stress resistance and is under DAF-16 regulation.

Longevity and heavy metal resistance has also been demonstrated in *daf-2* and *age-1* mutants of *C. elegans* (Barsyte et al., 2001). Resistance to cadmium and copper ions was significantly higher in these mutants than in wild type N2.

1.6.2 Cross-tolerance to stressors

It has been shown that exposure to a low level of stress can confer increased tolerance to further stressors. This has been demonstrated with *C. elegans* exposed to heat shock and then metal stress, the cross-tolerance is suggested to be mediated by the induction of heat shock proteins within the nematode. Exposure to heat shock at 35°C caused an induction of two gene products, HSP72 and HIF-1 (hypoxia inducible factor), which conferred cross tolerance to both cadmium and hypoxia stress (Treinin et al., 2003). *hif-1* was shown to be required for heat acclimation and the mediator of the cross-tolerance.

1.7 Ecotoxicogenomics

Genomics is the study of an organism's genetic makeup and the use of the genes, by transcriptomics, proteomics and metabolomics. It offers the opportunity to examine how an organism uses the genes it has under different conditions. Ecotoxicogenomics offers the possibility to identify the mechanism of action of a single chemical or a mixture of chemicals which is more environmentally realistic and may be able to identify conserved responses to toxicants between species. Ecotoxicogenomics, a term proposed by Snape et al. (2004) to describe the integration of genomic based science into ecotoxicology, is a recent and fast growing discipline. The combination of toxicology, ecology and molecular biology will lead to a fuller understanding of the molecular mechanisms of toxic response to chemicals, the impact this has on the life history of an individual and what this could potentially mean at the population level. The use of molecular

techniques may provide a powerful tool to detect changes within an individual due to toxicants.

1.7.1 Gene Expression

Advances in molecular biology and genomic technology in recent years is now enabling the study of the causes and mechanisms of complex biological pathways, not only the quantification as before. It is now possible to examine the expression of the entire genome of an organism, provided it has been fully sequenced, which for *C. elegans* is the case. This is known as transcriptomics. The amount of genomic information available on an organism limits the usefulness of transcriptomics; however, the amount of information is increasing rapidly with many environmental sentinel species being partially sequenced in EST (expression sequence tag) studies including the earthworm *Lumbricus rubellus* (www.earthworms.org), the springtail *Folsomia candida* and the water flea *Daphnia magna.* These are all organisms currently used in ecotoxicology testing.

1.7.2 Microarrays

One of the major recent advances in molecular biology has been the advent of microarray technology. DNA microarrays were first described by Schena et al. (1995). DNA microarrays allow the expression of thousands of genes to be monitored simultaneously, which potentially allows all of the genes in an organism to be studied simultaneously in one experiment (Primrose and Twyman, 2003). Currently there are only a few organisms whose genome has been fully sequenced, for example the model organism *C. elegans.*

The aim of transcription profiling is to develop a complete overview of all genes in a genome that are up- or down-regulated in response to some factor of interest in comparison with a designated reference expression (Van Straalen and Roelofs, 2006). In ecotoxicogenomics the factor of interest is toxicant exposure, with the aim of identifying toxicant responsive genes and pathways affected by the toxicant. There is also the possibility to look for patterns of expression that match patterns in the changes in life history traits. Chemicals interact directly with cellular components, which in turn affect the life history traits.

The starting point for microarrays is mRNA, which is the transcript of the genes. This mRNA is isolated from organisms taken from control and treatment conditions. The mRNA is reverse transcribed and labelled with fluorescent dyes, to produce labelled cDNA which is then hybridised onto the printed microarray slide. The microarray slide has immobilized on it 'target' nucleic acid sequences, either oligonucleotides or cDNA's. The labelled samples hybridized to the microarray are known as the 'probe'. Competitive binding of the cDNA's to the immobilized nucleotides then gives an indication of how much of the gene is being expressed between two fluorescent dyes in a semi-quantitative way.

The use of transcriptomics in ecotoxicology is in its infancy. However, there are examples of where gene expression studies have been used to examine responses to chemicals of environmental concern (Bartosiewicz et al., 2001). Williams et al. (2003) produced a 160 gene custom microarray which contained 110 stress-related and other genes to analyse environmentally sampled European flounder. The

fathead minnow is also being used to discern toxicant induced gene expression alterations (Miracle et al., 2003).

Examining the whole genome expression patterns of *C. elegans* exposed to xenobiotics to test if particular genomic regions contribute differently to changes in transcript profiles when living in a polluted environment is a relatively new technique. Custodia et al. (2001) have used DNA microarrays to examine genes in *C. elegans* which respond to exposure to estrogens, including 17-β-estradiol and progesterone, looking particularly at vitellogenin as a potential endpoint of xenobiotics responsivity. They also found other up-regulated stress responsive genes including those encoding metallothioneins, GSTs, CYP450s and hsp70. 17-β-estradiol exposure down-regulated about 1500 genes, including vitellogenin at the lowest level tested, 10^{-9}M. Genome response to ethanol has also been examined in *C. elegans* (Kwon et al., 2004), and it was found that heat shock proteins were induced as well as genes involved in lipid metabolism, alcohol dehydrogenases, and energy generation-related genes.

Using gene expression profiling, it would be possible to identify genes involved in life history traits when under stress. For example, it is possible to compare the deviations of the gene expression pattern when a reproducing organism is under stress to the gene expression pattern shown when reproduction occurs in a favourable environment. DNA microarrays can disclose global characteristics in the organism that cannot be revealed by single gene studies. Gene expression profiling will provide important insights into life history trait regulation.

1.8 Aims of the project

This project aimed to reveal changes in life-history traits in *C. elegans* when exposed to environmental toxicants, to reveal the molecular mechanisms behind these changes and to identify toxicant-induced responses at the molecular level. This was achieved through the following individual aims:

- To develop a robust repeatable life cycle toxicity testing system with the nematode *Caenorhabditis elegans* (Chapter 3).
- To examine the life history of wild type *C. elegans* strains from geographically distinct regions under control and cadmium exposed conditions (Chapter 3).
- To examine how temperature affects the life history of the nematode (Chapter 3).
- To examine the life history changes caused by toxicants from different chemical classes, cadmium, fluoranthene, atrazine and aldicarb (Chapter 4).
- To examine how changes in life history caused by environmental toxicants could affect population growth rate (Chapter 5).
- To examine how pollutants alter the energy allocations within exposed individuals and to predict the toxic mode of action in term of dynamic energy budgets (Chapter 5).
- To mechanistically investigate how a toxic compound affects gene expression in *C. elegans* using whole genomic oligo-arrays (Chapter 6).
- To link life history changes to changes at the gene expression level (Chapter 6).

Chapter 2

Materials and Methods

2.1 Materials and reagents

A summary of suppliers of reagents, consumables and buffers is given in Appendix 1.

2.1.1 Decontamination of equipment

All glassware used with toxicants were acid washed along with the filter equipment used in the extraction of the liquid fraction from the agar.

2.1.2 Sterilisation conditions

All media and equipment used in culturing and experiments with *C. elegans* and all consumables and solutions used in molecular work were sterilised by autoclaving for 20 minutes at 121°C and 15psi. Any heat-sensitive components were sterilised by filtration through a 0.22µm Nucleopore™ filter into a fresh sterile container. Worm picks were dipped in 70% ethanol prior to use. All solutions and consumables used for RNA work were double autoclaved or were taken from pre-sterilised RNA free sources.

2.1.3 Water

For culture media and stock solutions distilled water (dH_20) was used. For all applications involving nucleic acid manipulations filter sterilised HPLC water or RNAse free water was used.

2.1.4 Media

LB nutrient broth, LB agar and S-Gal/LB agar blend were prepared as described by the manufacturer (Sigma, Poole, UK) and sterilised before use. Media was cooled to 50°C and, where required, antibiotics were added at an appropriate concentration.

2.1.5 Antibiotics

Stock solutions of ampicillin and carbenicillin were dissolved in sterile dH_2O to the required concentration, passed through a 0.22μM Nucleopore™ filter and stored at -20°C. The stock concentrations were 100mg/ml ampicillin and 50mg/ml carbenicillin.

2.1.6 DNA markers

The DNA marker used was Φ174/*HaeIII* (Promega) the fragment sizes are 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp.

2.2 *Caenorhabditis elegans* culturing

2.2.1 Strains used

C. elegans strains as listed in Table 2.1 were obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, USA.

Table 2.1: *C. elegans* strains used in the study

Strain Name	Common Name	Origin	Genotype
N2	Bristol, Wild Type	Bristol, UK	WT
CB4856	Hawaii	Hawaii, USA	WT
RW7000	BO, Bergerac	Bergerac, France	WT
GE31	GE31	CGC, USA	cib-1(e2300)I.

N2 is the laboratory standard wild type strain which was isolated from mushroom compost near Bristol, England, by L.N. Staniland, pre 1956. It was subsequently cultured by W.L. Nicholas, C.E. Dougherty, Sydney Brenner and sub-cultured by Don Riddle in 1973 (www.wormbase.org). It has a low copy number of the transposable element Tc1. This strain was used as the standard wild type test strain in Chapters 3 and 6.

CB4856 is a wild type strain collected from pineapple fields in Hawaii in 1972 by L. Hollen. It has a low copy number of the transposable element Tc1. This strain was used in the experiments in Chapter 3 to compare wild type strains from geographically distinct regions.

RW7000 was collected from garden soil near Bergerac, France pre 1949. Bergerac strain is a commonly used strain for mapping studies, as it has a high copy number of Tc1 transposable elements. This strain was used in the experiments in Chapter 3 to compare wild type strains from geographically distinct regions.

GE-31 was produced by ethylmethane sulfonate mutagenesis of the *him-3* strain. GE-31 is wild type at 15°C, but maternal effect embryonic lethal at 25°C. The temperature sensitive period is during oocyte and early embryogenesis (Wormbase, 2005). This strain was used so that single stage adult populations of nematodes could be collected. Any eggs that were laid by the adults would not hatch so there would not be larvae contaminating the adult population.

2.2.2 Nematode Growth Medium (NGM) preparation

Nematodes were grown on agar, on a medium known as nematode growth medium (NGM). NGM was prepared by dissolving 1.7% bacto agar, 0.025% bacto peptone and 50mM $NaCl_2$ in dH_20 which was then sterilised by autoclaving. After sterilisation 0.005% cholesterol, 1mM $CaCl_2$. 1mM $MgSO_4$ and 25mM KH_2PO_4 (each of which had been previously sterilised by autoclaving) was added to the agar and mixed thoroughly prior to the agar being poured into Petri dishes or multiwell plates in a laminar flow cabinet to ensure sterility.

2.2.3 Food source

E. coli strain OP50 was used as a food source. OP50 is a uracil deficient strain which limits its growth so it will not overcrowd the culture plates (Brenner, 1974). OP50 was cultured overnight in Luria Bertani (LB) broth at 37°C. OP50 was spread onto NGM Petri plates or dropped onto NGM multiwell plates and were either incubated overnight at 37°C or for 2 days at room temperature before use.

2.2.4 Frozen stocks

(i) Worms

Strains were frozen at -80°C for long term storage.

Worms were washed from a 60mm Petri dish in 1ml M9 buffer when the majority were at L1-2 stage. Approximately 50 nematodes were put on the Petri dish to lay eggs, it is these worms that were frozen for storage 2-3 days after the eggs were laid. Harvested worms (0.75ml) were added to freezing solution (0.75ml; See Appendix 3) and vortexed. The worms were then frozen slowly, inside a Styrofoam block to -80°C for 24 hours.

(ii) Bacteria

A single *E. coli* OP50 colony was picked and grown up overnight at 37°C in LB. An aliquot of the overnight culture (1ml) was mixed with 190µl sterile glycerol (80% w/v) by inversion and stored at -80°C.

2.2.5 Stock cultures on agar

Nematodes were cultured on nematode growth medium (NGM) agar plates (as described in Appendix 1, Table 2.2) in either constant dark or constant light at

15°C. *E. coli* OP50 was inoculated onto each 60 mm / 90 mm diameter Petri plate of NGM and allowed to grow to a lawn (so that there was an even coverage of the inoculated area), 60mm Petri plates were inoculated with 25μl *E. coli* and 90mm Petri plates were inoculated with 60µl *E. coli*. Worms were transferred to fresh NGM plates weekly.

2.2.6 Isolation of eggs from nematodes

To obtain pure samples of nematode eggs adult worms were placed in a bleaching solution which kills and dissolves the worms but leaves the eggs unharmed due to their impermeable membrane. This procedure also kills bacteria and other contaminants such as fungi.

(i) Small scale egg prep

Nematodes were washed off a 60mm NGM plate with 1ml M9 buffer and transferred to a 1.5ml micro centrifuge tube and centrifuged at room temperature for 10 seconds at 13,000 x g. The supernatant was discarded without disturbing the worm pellet and 1ml bleaching solution (0.5M NaOH, 1% sodium hypochlorite) was added to the tube and vigorously shaken for 5 minutes to break up the worm bodies and release the eggs. The tube was centrifuged for 10 seconds at 13,000 x g and the supernatant removed. M9 buffer (1ml) was added to wash the pellet and then spun for 10s at 13,000 x g. The supernatant was removed and a further 2 washes in M9 buffer were performed; to remove remaining bleach from the egg pellet. The egg pellet was then transferred to a clean NGM plate previously inoculated with *E. coli* OP50 and incubated at 15°C to hatch.

(ii) Large scale egg prep

Nematodes were washed off large (90mm) NGM agar plates with M9 buffer, transferred to a 15ml falcon tube and centrifuged at 1500 x g at 4°C for 1 minute. The supernatant was removed without disturbing the worm pellet and 8ml of bleaching solution was added (0.5M KOH, 2.5% sodium hypochlorite). The tube was shaken vigorously for 4 minutes and then centrifuged at 1500 x g at 4°C for 1 minute and the supernatant removed. The egg pellet was resuspended in 12ml M9 buffer and centrifuged at 1500 x g at 4°C for one minute. This wash step was repeated 4 times. After the final wash the supernatant was removed and the pellet resuspended in 8ml M9 buffer and incubated overnight at 15°C, continuously rotating. The hatched L1 larvae were then centrifuged for 1 minute at 1500 x g, the supernatant removed and the larvae were transferred to a clean NGM plate previously inoculated with *E. coli* OP50.

2.3 *C. elegans* life cycle toxicity testing

2.3.1 Dosing the media

All chemicals were of the highest analytical grade available. Stock solutions of cadmium chloride and aldicarb were prepared in sterile bidistilled water and then filter sterilised through a 0.22μM Nucleopore™ filter. Cadmium chloride stock solution was kept at 4°C until use. Aldicarb stock solutions were used within 3 days of preparation. Fluoranthene was dissolved in ethanol and used immediately. Atrazine solutions were prepared in dimethyl sulfoxide (DMSO) and used immediately. For experiments where a solvent was used the corresponding

amount of solvent was also added to the control agar. Toxicants were added at the appropriate concentrations when the autoclaved NGM agar had cooled to 50°C, but before solidification.

2.3.2 Life cycle toxicity test setup

Nematodes used in life cycle toxicity tests were exposed from embryo by pre-exposing their mothers. Parental worms were firstly age synchronised by either the egg-prep procedure as described in Section 2.2.6 or by transferring 15 gravid nematodes onto dosed / control NGM plates to lay eggs for 4 hours. The adult worms were then removed and the eggs allowed to mature into adults. The second synchronisation step was carried out in the same way onto dosed / control NGM plates and adults allowed to lay eggs for 1 – 4 hours. This ensured that there would be no more than 4 hours difference in the age of all worms used in the experiment. The test worms were incubated at either 15°C or 25°C and individual nematodes were transferred from the NGM plates to 24 well multi-compartment plates before their reproductive period began. Throughout the reproductive period worms were transferred to a fresh dosed agar well each day. This allowed the time dependent patterns of reproduction and survival to be assessed.

Figure 2.1 shows a diagrammatic representation of the experimental procedure. Each well in the multi-compartment plate contained 2ml dosed agar. *E. coli* OP50 (15µl) was dropped onto the centre of the agar and the plates were incubated overnight at 37°C. Test plates were kept at room temperature until needed.

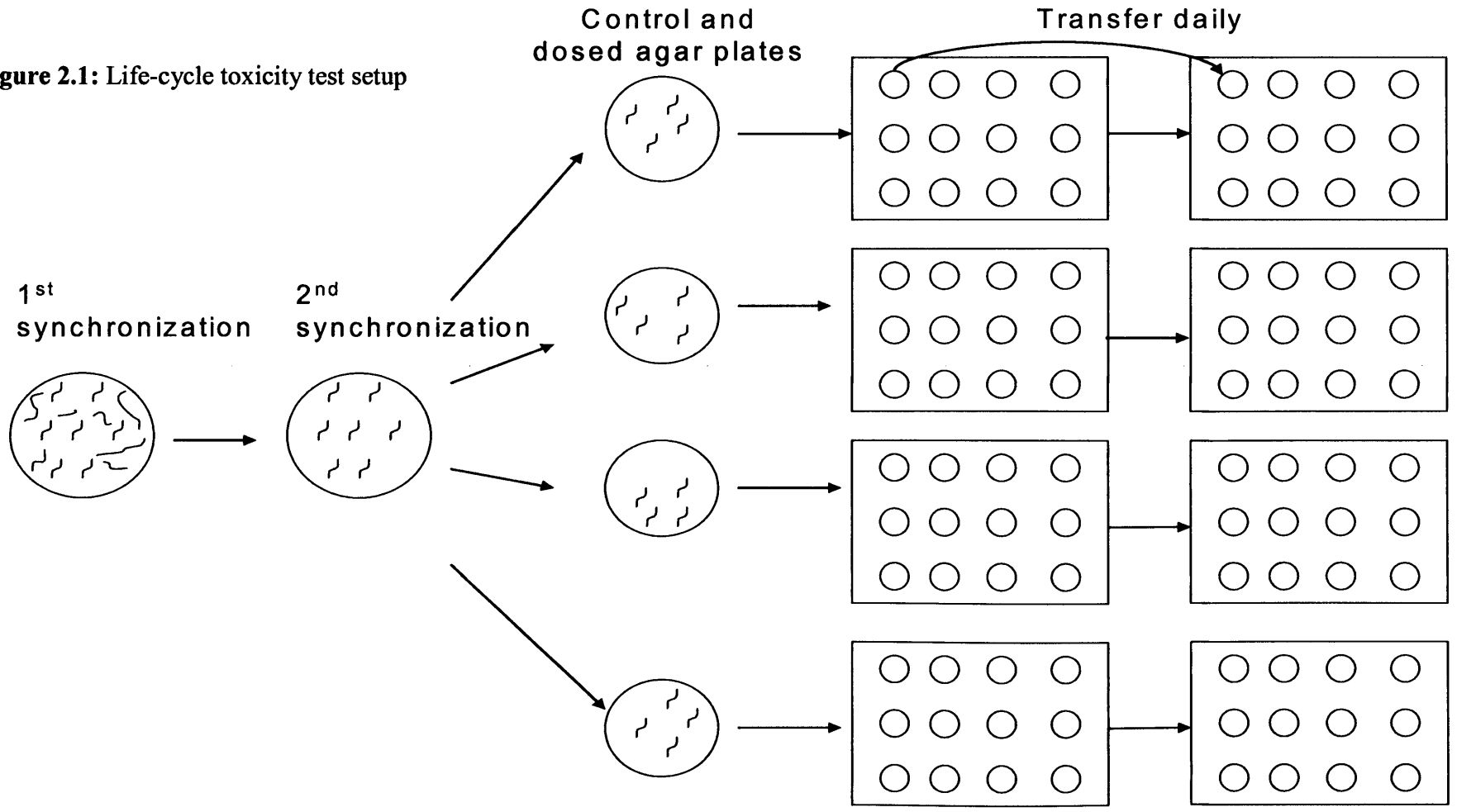


Figure 2.1: Life-cycle toxicity test setup

2.3.3 Image analysis

Nematode growth was measured by photographing worms at a series of time intervals over their entire growth period. Photographs of worms were taken using a Nikon Coolpix 4500 digital camera. The software Image Pro Express v 4.5.1.3 (Media Cybernetics) was used to measure length and area of the worms. The program was calibrated using a digital photograph of a micrometer taken at the same magnification as all photos. The program measures the perimeter of the worm, with the length taken as half of this value. Volumetric length (cubic root of body volume) were calculated using the following formula (2.1) that describes a cylinder with rounded ends (an approximation of the shape of a nematode):

$$\text{Volumetric length} = ((\tfrac{1}{8} * \pi * A^2) / (l))^{1/3} \qquad (2.1)$$

where A is the area of the nematode and l is the maximum length of the nematode (Alvarez et al., 2006).

Volumetric length is used in the dynamic energy budget toxicity (DEBtox) model to combat dimension problems (see Chapter 5).

2.4 Molecular biology methods

2.4.1 Isolating genomic DNA

Worms were washed off a NGM plate in 1ml M9 buffer and centrifuged at 2300 x g for 1 minute. The supernatant was discarded, and 1ml M9 buffer added to wash the worms. The tube was centrifuged at 2300 x g for 1 min again and the supernatant discarded. Genomic DNA was released by adding an equal amount of 2 x worm lysis buffer (2 x WLB) to the volume of the worm pellet and freezing at -80°C for one hour. This was followed with a 2 hour incubation at 65°C. Proteinase K was inactivated by incubation at 95°C for 20 min and the tube was kept on ice until use.

2.4.2 Isolating total RNA

Total RNA was extracted from nematodes using the TRI reagent method.
Worms were collected by washing off NGM plates in M9 buffer, centrifuging at 13,000 x g for 20 seconds and removing the supernatant and repeating once to remove bacteria. The worm pellet was immediately frozen in liquid nitrogen and stored at -80°C.

C. elegans samples were thawed on ice. The nematode pellet (100µl) was added to an equal volume of glass beads and 1ml TRI reagent and vortexed for 3-4 minutes (depending on the size of worms) to homogenise the worms. The supernatant was transferred to a fresh tube and incubated at room temperature for 5 minutes, to allow complete dissociation of nucleoprotein complexes. The phenol and guanidine thiocyanate in the TRI reagent separates the RNA from

DNA and inhibits RNAse activity. Chloroform (200µl) was added to the tube, vortexed, and incubated at room temperature for 10 minutes before being centrifuged at 12,000 x g at 4°C for 15 minutes. Chloroform separates out and precipitates proteins. The phases were separated by centrifugation at 12,000 x g at 4°C for 15 minutes and the upper, aqueous phase transferred to a new tube. RNA was precipitated out by adding 500µl isopropanol, incubating at room temperature for 10 minutes and then centrifuged at 12,000 x g at 4°C for 10 minutes. The supernatant was discarded and 1ml 75% ethanol was added and centrifuged at 12,000 x g at 4°C for 5 minutes to wash the RNA. All ethanol was then removed and the pellet left to dry at room temperature for 5 minutes before being resuspended in 100µl sterile HPLC water.

RNA samples were quantified and evaluated for purity as in Section 2.4.5. RNA integrity was analysed by agarose gel electrophoresis (Section 2.4.1). Samples were stored in water (short term) or ethanol (long term) at -80°C until required.

2.4.3 RNA Purification from Total RNA

RNeasy Mini Protocol for RNA cleanup was performed on total RNA as described by the manufacturer (Qiagen, 2001). Total RNA samples were made up to 100µl total volume with sterile HPLC water and 350µl buffer RLT was added and mixed. Absolute ethanol (250µl) was added and mixed thoroughly by pipetting. The high salt buffer system promotes selective binding of RNA to the RNeasy silica-gel membrane. The mixture was applied to an RNeasy column and centrifuged at 8000 x g at room temp for 15 seconds and the flow-through discarded. All RNA molecules longer than 200nc's were isolated which provided

enrichment for mRNAs. A wash buffer (manufactures code RPE; 500µl) was then applied to the column and centrifuged again under the same conditions as above. A further 500µl of wash buffer (RPE) was applied and the RNeasy column centrifuged for 2 minutes to dry the RNeasy membrane. RNA was eluted from the RNeasy column with the addition of RNase free water to the membrane and centrifuging for 1 minute, at 8000 x g and the amount and quality of RNA quantified as in Section 2.4.5.

2.4.4 Agarose gel electrophoresis

DNA and RNA samples were visualised by agarose gel electrophoresis (1% w/v agarose in 1 x TAE buffer). Ethidium bromide was added at a concentration of 400µg l^{-1} when the agarose was below 50°C. The DNA/RNA was mixed with 6 x blue loading dye and loaded onto the gel along with the appropriate molecular weight marker (ϕX174 digested with *Hae*III). Samples were run for 15-30 minutes at 150V in a gel tank containing 1 x TAE buffer. Nucleic acid bands were visualised under UV light and the size of each band was estimated relative to the migration of DNA markers of known size, as detailed in Section 2.1.6. Apparatus for visualising RNA bands was soaked overnight in 1% SDS solution before use to remove any possible RNAse contamination.

2.4.5 Assessment of quantity and purity of DNA

The concentration of DNA and RNA was estimated by spectrophotometry at a wavelength of 260nm. Absorbance readings at 230nm and 280nm gave an estimation of sample purity. Pure preparations of DNA and RNA will have an A_{260}/A_{280} ratio of approximately 1.8 - 2.0 respectively. Values lower than these

indicate protein or phenol contamination. An A_{260}/A_{230} ratio of less than 2 is indicative of ethanol or salt contamination (Sambrook et al., 1989) Optical density readings were performed on an ultraspec 2100pro UV/visible spectrophotometer (Amersham Pharmacia Biotech).

2.5 Microarray protocol

Global RNA transcripts of populations of nematodes were analysed using oligo microarrays (Figure 2.2).

2.5.1 Production of microarray slides

C. elegans microarray slides were printed in house using the *C. elegans* oligonucleotide set version 1.1 (Operon™), which represents every open reading frame in the genome. Oligonucleotides have been designed to the 3` most region and are 70bp in length (70mers), in total the set comprises 19,873 oligonucleotides. The oligonucleotides were printed in 3 x SSC buffer using a Perkin Elmer SpotArray 72 onto UltraGAPS™ (Corning) slides at a concentration of 15µM. Landmarks were printed in the top left hand corner of each sub-array for orientation. Lucidea universal scorecard (Amersham) was printed at a concentration of 25µM onto the slides. The Lucidea universal scorecard is a set of 23 unique microarray controls, consisting of 70-mer oligo dT and NH_2-oligonucleotide probes (TIB Biomol), 21 specific probes and two negative control sequences, calibration probes, ratio controls, utility probes and negative controls. Oligonucleotides were immobilised by baking at 80°C for 2 hours and UV cross linking in the UV Stratalinker™ 2400 bench top transilluminator (Stratagene

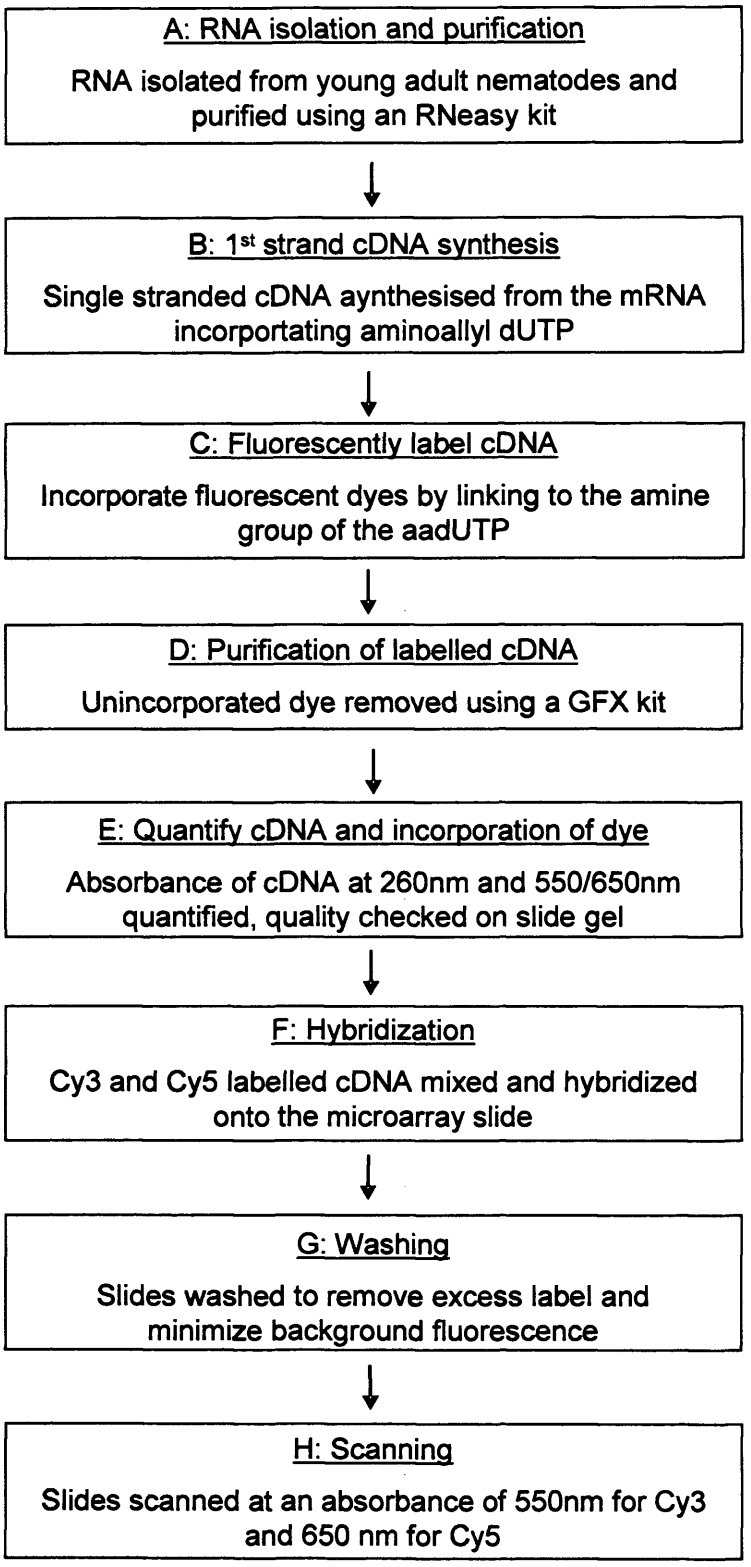


Figure 2.2: The processes involved in microarray preparation

Ltd.). Microarrays were stored desiccated at room temperature and protected from light.

2.5.2 Annotation of Microarray

Reporter annotation was performed by exploiting MegaBlast algorithm to identify the best match to the oligonucleotide sequences with cDNAs associated with release WS140 version of the *C. elegans* genome (Sanger Center release July 2005), the Refseq cDNA data set (NCBI release August 2005) and all deposited mRNAs/EST present in GenBank (August 2005) allowing a maximum of a 2 base-pair mismatches. Physical location and gene ontology assignment was assigned from genome release WS140 associated to the matching Wormbase gene identifier. Human homologues were identified using the Ensembl orthologue prediction tables (Sanger Center release July 2005).

2.5.3 Prevention of non-specific binding of target DNA to active amine groups

The Gamma Amino Propyl Silane (GAPS) coating of the Corning glass slides contains reactive amine groups which need to be blocked prior to DNA hybridisation. If the blocking is not performed, labelled DNA will bind non-specifically to the slide surface and cause excessive background fluorescence. Each microarray slide was blocked by placing the slide in a 50ml Coplin jar containing blocking solution (1% Bovine Serum Albumin [Fraction V], Sigma), 5 x SSC and 0.1% SDS) pre-warmed to 42°C – slides were incubated in a water bath at 42°C for 30 minutes. The slides were then dipped briefly five times in

sterile, filtered water. This was followed by 2 brief dips in isopropanol and dried using compressed air. The slides were stored in a light proof slide box and used within one hour.

2.5.4 Indirect labelling using amino allyl dUTP

Total RNA was extracted from nematode samples as described in Section 2.4.2. For use in microarray hybridisation, mRNA was purified from the total RNA (section 2.4.3). This purified RNA was then indirectly labelled with fluorescent dyes, Cy3 and Cy5 ready for array hybridisation. Indirect labelling involves two steps, the first (i) incorporates amino allyl dUTP bases during first strand synthesis of cDNA and the second step (ii) chemically couples the Cy dyes to the amino group on the aadUTPs.

(i) Reverse Transcription using amino allyl dUTP (aadUTP)

Purified RNA was used to generate a complementary DNA copy (cDNA) using reverse transcription enzyme. 15µg of purified RNA was added to 1µl control mRNA spike mix (Lucidea Universal Scorecard, Amersham Biosciences), 150pmol oligo dT and made up to 12µl with sterile HPLC water and incubated for 5 minutes at 70°C to remove secondary structure. The samples were then cooled to room temperature for 10 minutes to allow for optimal oligonucleotide primer annealing. The RNA was then reverse transcribed at 42°C for 3 hours in a reaction containing 500 units MMuLV (Moloney Murine Leukaemia Virus) reverse transcriptase, 0.2mM aadUTP, 1mM dATP; 1mM dCTP, 1mM dGTP, 0.4mM dTTP, mix in a 4µl 5 x RT buffer (ABgene, Surrey, UK).

After the reverse transcription the samples were brought to room temperature and any remaining RNA was hydrolysed by the addition of 2.5µl 2.5M NaOH which was incubated at 37°C for 15 minutes, followed by the addition of 10µl 2M HEPES. The cDNA was then precipitated by the addition of 3.6µl 3M NaOAC and 100µl absolute ethanol and incubated overnight at -20°C. The samples were centrifuged at 18,000 x g at 4°C for 22 minutes to pellet the precipitated cDNA. The pellet was then washed twice in 195µl 75% ethanol and air dried before being resuspended in 7.5µl 0.1M $NaHCO_3$ and incubated at room temperature for 15 minutes to make sure the pellet is fully resuspended.

(ii) Dye labelling

Cy3 and Cy5 dyes were used to label the cDNA ready for hybridisation onto the array slide. The dyes, which come in powder form, were resuspended to a concentration of 40000pmol by adding 0.1M NaHCO3 (pH9.0) to the dye tubes, for Cy3 14µl was added and for Cy5 7.5µl was added. The dyes were mixed thoroughly by vortexing. The dyes were added to the cDNA (8000pmol Cy3, 15000pmol Cy5), vortexed and centrifuged briefly and incubated at room temperature in the dark for 5 hours on a shaking platform (60rpm). 6µl 4M hydroxylamine was added and incubated at RT, 15 min in the dark; this quenches the reaction by inhibiting unincorporated dyes from binding to free amines.

74µl of water was added, followed by 10µl 3M NaOAC and 280µl absolute ethanol, vortexed and kept overnight at -20°C. The samples were centrifuged at 18,000 x g at 4°C for 22 minutes to pellet the precipitated cDNA. The pellet was then washed twice with 1ml 75% ethanol and air dried before being resuspended

in 50µl water and incubated at room temperature for 15 minutes to make sure the pellet is fully resuspended.

2.5.5 GFX purification

Cy3 and Cy5 labelled cDNA was purified using the CyScribe™ GFX Purification Kit (Amersham Biosciences) according to the manufacturer's instructions. This kit removes unincorporated dyes from the labelled cDNA.

500µl capture buffer was added to the GFX column, and the labelled cDNA added to the column. This was mixed by pipetting up and down 5 times. The column was centrifuged for 30 seconds at 13800 x g at room temperature and the flow through discarded. 600µl wash buffer was added to the column and centrifuged at 13800 x g for 30 seconds and the flow through discarded. This washing step was repeated 3 times. After the final wash step the flow through was discarded and the column replaced in the collection tube and centrifuged for a further 10 seconds to remove all wash buffer. The column was then placed in a fresh 1.5ml micro centrifuge tube and 60µl elution buffer added. The column was incubated at room temperature for 4 minutes and then centrifuged at 13800 x g for 1 minute to collect the purified labelled cDNA

2.5.6 Quantify cDNA and dyes.

The quality of cDNA labelling was assessed by running 1µl of sample on a slide gel. A 1.5% agarose gel (no ethidium bromide) was made and 1µl sample loaded with 1µl 60% glycerol and run at 130V for 30 minutes. The slide gel was scanned

on a GENETAC™ LS IV scanner, with a green laser (560BP filter) and red laser (675BP filter). The slide gel shows the size distribution of cDNA and degraded and unincorporated dyes.

Figure 2.3: An example of a slide gel, the left lane shows Cy3 labelled cDNA and the right shows Cy5 labelled cDNA.

The absorbance of the samples was measured at the following wavelengths to quantify amount of cDNA and dye incorporation:

260nm cDNA

550nm Cy3

650nm Cy5

The Frequency of Incorporation (FOI) of dyes was calculated using the following equation:

FOI = (Picomoles of incorporated dye * 324.5) / nanograms of cDNA

The FOI is derived from the following:

Absorbance of 1 at 260nm = 37µg/ml Cy-labelled ssDNA.

Average molecular weight of dNTPs = 324.5g/mole

Absorption coefficient of Cy3 = 0.15

Absorption coefficient of Cy5 = 0.25

Amount of labelled cDNA (ng) = A260 * 37 * volume of sample (µl)

For Cy3 incorporation: A_{550} * (Volume of sample (µl) / 0.15)

For Cy5 incorporation: A_{650} * (Volume of sample (µl) / 25)

For each hybridisation 20pmol of Cy dye molecules for each channel was required. The volume of each dye labelled cDNA sample needed for hybridisation was calculated using the following equations:

For Cy3 labelled cDNA: volume containing 20 pmol = $3/A_{550}$

For Cy5 labelled cDNA: volume containing 20 pmol = $5/A_{650}$

In samples where the volume for 20pmol label was more than 10µl, the required volume was transferred to a new tube and dried down in a Speed Vac, at 60°C, until the sample volume was less than 5µl and then resuspended to 10µl in water.

2.5.7 Hybridisation

20pmol of each Cy3 and Cy5 labelled cDNA were combined and denatured at 95°C for 3 minutes and then immediately centrifuged at 140000 x g for 30 seconds at room temperature. 20µl 2 x hybridisation buffer (50% formamide, 10 x SSC, 0.2% SDS) was added and briefly vortexed and centrifuged. The contents

of the tube were slowly pipetted down one side of a clean glass slide and the blocked microarray slide slowly lowered onto the slide face down, ensuring no air bubbles were present. The slides were quickly inverted so that the array slide was face up and the array was incubated in a hybridization chamber at 42°C for 24 hours.

2.5.8 Slide Washing

Slides were separated in 1 x SSC, 0.1% SDS buffer and washed for 10 min in the same buffer at 55°C. The slides were then washed for 10 min in a 0.1 x SSC, 0.1% SDS buffer at 55°C. This wash was repeated in new wash buffer at 55°C. The slides were then transferred to a 0.1 x SSC buffer for 1 min before a final wash step in 0.1 x SSC for 1 min. The slides were dried quickly using high compressed air and stored in a light proof slide box until scanned.

2.5.9 Signal detection

Hybridised microarray slides were stored in the dark until they were scanned (the fluorescence is light sensitive). The slides were scanned at wavelengths 633nm for the Cy5 channel and at 543nm for the Cy3 channel on the ScanArray™ Express HT microarray scanner (Perkin Elmer).

2.5.10 Image analysis

The scanned microarray image was analysed using the software package ImaGene™ (Biodiscovery Inc., CA, USA) microarray analysis package. This

software allowed for the identification of differentially expressed genes on the basis of the fluorescent intensity of each spot.

2.5.11 Microarray analysis

The software package GeneSpring GX 7.3 (Agilent Technologies, USA) was used to normalize and analyse the expression data. Specific details are given in Chapter 6.

Chapter 3

The effects of a model toxicant on a nematode: A comparison of temperature and strain influences

3.1 Introduction

Ecotoxicological testing can give valuable information on the toxicity of chemical pollutants on animals. It is important for ecotoxicological test methods to be reproducible, representative, responsive, robust, relevant and practical (Spurgeon et al., 2002).

The free-living bacterivorous nematode *Caenorhabditis elegans* proves to be an ideal organism for ecotoxicology testing to elucidate responses to stress. It is practical to use, is small in size (adults are approximately 1mm long) and its low cost and ease of culture (in the laboratory *C. elegans* is maintained on agar plates and fed with the bacteria *Escherichia coli*) makes it popular with users. It has a short life span with a generation time of 3.5 days at 20°C, which facilitates full life-cycle studies, and its transparency aids in the identification of specific life stages. It is found in almost every type of terrestrial environment (Wood, 1988) living in the interstitial spaces in soil. As most pollutants are transported by movement of water throughout the terrestrial environment it follows that these nematodes would be directly exposed to xenobiotics, giving an ecotoxicological perspective to the use of *C. elegans*, moreover, the amount of genomic

information known about this worm means that it can easily be used to try to identify mechanistic information about the effects of pollutants.

C. elegans has already been used by several research groups for studies in ecotoxicology. Although it is not the most sensitive nematode species (Kammenga et al., 1994) there are a number of advantages of using *C. elegans* as an ecotoxicology test species. The tests are rapid and can be performed in soil (Peredney and Williams, 2000), sediment (Hoss et al., 1999), agar (Popham and Webster, 1979) or liquid medium (Williams and Dusenbery, 1990). Also, the nematode can tolerate wide variations in salinity and pH without the stress response being affected (Khanna et al., 1997).

The effect of environmental pollutants on life cycle events has become an increasingly popular study area in recent years. Toxicants can have major impacts on populations by altering the life history traits of individuals. Life history patterns are relevant to ecotoxicological testing because population consequences of toxic action depend on the trade-offs between life-history components (Kammenga et al., 1996; Kammenga et al., 1997).

Theory behind natural selection leads to the assumption that in a favourable environment, life history traits will be modulated so as to give the individual highest fitness. Environmental conditions such as temperature and food availability can alter the length and timings of life cycle events. Pollution can also be viewed as simply another environmental variation so, when an organism encounters a polluted environment, as many do throughout their entire life cycle,

its normal functioning and life history may change. Lethal endpoints are frequently used in ecotoxicology but are less sensitive to toxicants than endpoints such as reproduction and growth that are also important for population dynamics. Sublethal toxic effects may seem to be small at the individual level, but may cause significant effects at the population level; alternatively the opposite may also occur. Elasticity analysis of population growth rates give the opportunity to examine which life history trait is the most important contributing factor to population growth rate (Hansen et al., 1999) (see Chapter 5).

For a species that allocates a great amount of energy for reproduction, such as *C. elegans*, reproductive rate is an important parameter. A number of life-cycle toxicity studies have been completed on nematodes, looking specifically at fitness and population dynamics (Jager et al., 2005; Kammenga et al., 1996; Kammenga et al., 1994; Kammenga et al., 1997) and the effects of multiple toxicants on life history traits and fitness in *C. elegans* has also been investigated by Jonker (2003). These studies have developed methods that allow the assessment of the effect of chemicals to be assessed for different species and strains and under different environmental conditions.

3.1.1 Factors influencing chemical effect

For the effective risk assessment of chemicals it is important in ecotoxicological testing performed throughout many different laboratories, to keep as many factors constant as possible to ensure uniformity of tests. This is important to ensure confidence in the toxicity data that are obtained. Two factors, amongst many, that have the potential to greatly influence an organism's response to toxicants are

temperature and genotype. In the study presented in this chapter the response of a single strain of *C. elegans* at two temperatures to the same chemical is examined to identify temperature effects on toxicity. A second experiment aimed to observe the differences in response to toxicants between strains from geographically distinct regions.

3.1.2 Temperature

In ectothermic animals, temperature has a major influence on many physiological and biochemical processes. An increase in temperature and thus metabolic rate leads to an increase in growth rate and alterations to the timing of life history events. Temperature may also affect the toxicity of chemicals as has been shown in a number of studies (Bat et al., 2000; Heugens et al., 2003; Rathore and Khangarot, 2002). An increase in ambient temperature will increase the metabolic and respiration rate of the organism, and can alter behaviours such as locomotion and feeding activity. These changes offer the opportunity to alter the uptake of a chemical, for example if feeding rate is increased then more of the toxicant will be ingested. It may also mean that detoxification and elimination rates may alter. Temperature can also have an effect on the organism's condition, making it more or less susceptible to toxicant induced stress.

3.1.3 Strains

The genotype of an organism can greatly influence response to stress. Each genotype will produce a range of phenotypes under different environmental conditions; this range is known as phenotypic plasticity of the genotype. Some

genotypes can deal better with stress, or respond in a greater number of ways. Calow and Forbes (1998) state that within any population there is likely to be variability between genotypes in terms of physiological responses to environmental variables that translates into variability in fitness responses. Differences in tolerance to lethal concentrations of cadmium within populations of *Daphnia magna* from geographically distinct regions have been reported (Barata et al., 2002). Baird et al. (1990) also found differences in body length and reproduction between genotypes under cadmium stress. In adapted populations it can be observed that sensitive genotypes may be removed and more tolerant genotypes may dominate. Thus, the range and sensitivity of genotypes present in a population can greatly influence its response to pollution.

C. elegans are self-fertilizing hermaphroditic nematodes and populations are clonal and therefore genetically homogeneous. *C. elegans* has been found and isolated in the field across the globe, and a large bank of cryo-preserved worm strains is available for use from the Caenorhabditis Genetics Centre (CGC), USA. There are more than 80 wild isolate strains currently available from the CGC. That *C. elegans* is a good organism to study genotype sensitivity is not only due to the number of strains with, by definition, different genotypes that are readily available, but also its short lifespan facilitates rapid assessment of genotype differences.

3.1.4 Experiment

In these experiments *C. elegans* were exposed to cadmium dosed agar, and their growth rate, total reproduction and lifespan were measured. Cadmium was

chosen as a model toxicant in these preliminary tests as its effects are well characterised and have been documented in other studies on *C. elegans* and other organisms.

Cadmium is a non-essential metal and is released into the environment by smelters and phosphate fertilizers applied directly to the soil. The main route of exposure to cadmium in nematodes is likely to be via direct absorption through the body wall. As a result of this exposure *C. elegans* has been seen to reduce pharyngeal pumping and thus reduce food intake, although a complete inhibition of pumping is not seen (Jones and Candido, 1999). A reduction in the proportion of feeding individuals that are exposed to cadmium has also been observed in another nematode species, *Panagrellus silusiae* (Mudry et al., 1982). Cadmium causes cellular lesions such as disrupted cytosomes and shortened microvilli (Popham and Webster, 1979). At the cellular level cadmium exposure leads to protein denaturation, DNA strand breaks and the formation of reactive oxygen species and lipid peroxidation (Al-Khedhairy et al., 2001).

3.1.5 Aims

The major aim of the experiments presented in this chapter was to determine a robust toxicological testing system with *C. elegans* and to understand how different parameters can affect toxicity.

The aim of the first of the two main experiments was to examine the life history response of *C. elegans* to the heavy metal cadmium, to temperature, and to the interaction between temperature and cadmium toxicity. The null hypothesis for

this experiment, on the effect of temperature on cadmium toxicity, was that there would be no influence of temperature on the doe-response effect of cadmium on *C. elegans.*

The aims of the second of the two main experiments described were firstly, to capture high quality data on the life history of three wild type isolates of *C. elegans,* to address the gap in current knowledge. Secondly, to examine whether life history differed between genotypes of *C. elegans* and thirdly, whether the toxic response to cadmium was altered between genotype. The null hypothesis for this experiment, on the effect of strain on cadmium toxicity, was that there would be no influence of strain on the dose response effects of cadmium on *C. elegans.*

The laboratory wildtype strain N2, has been investigated thoroughly although there has been very little characterisation of other wild isolates of *C. elegans*. The gap in current knowledge is addressed in this study.

The aim of the two experiments described in this chapter was to examine the life history response of *C. elegans* to the heavy metal cadmium, to temperature and to the interaction between temperature and cadmium toxicity. The null hypothesis for the first experiment presented, on the effect of temperature on cadmium toxicity, is that there is no influence of temperature on the dose-response effect of cadmium on *C. elegans*. The null hypothesis for the second experiment, on the effect of strain on cadmium toxicity, is that there is no influence of strain on the dose response effects of cadmium on *C. elegans*.

3.2 Approach

The initial part of this study on temperature was carried out on N2 strain nematodes at two temperatures, 15°C and 25°C. The second part of the study examining strain responses was carried out using three wild type isolates of *C. elegans*: N2, CB4856 and RW7000 (as described in Section 2.2.1). The N2 strain, otherwise known as 'Bristol' was isolated in Bristol, UK and is the reference wildtype worm used in *C. elegans* labs around the world. The RW7000 strain, referred to as 'Bergerac' from herein, was isolated in Bergerac, France. Strain CB4856, referred to as 'Hawaii' from herein, was isolated in Hawaii, USA. Nematodes were cultured as described in Section 2.2.4.

3.2.1 Chemicals

All chemicals were of the highest analytical grade available. Cadmium chloride (Sigma, UK) was used to make the stock solution ($CdCl_2$ 1 mg ml^{-1}). The solutions were made in sterile bidistilled water and then pushed through a 0.2μM filter to ensure sterility and kept at 4°C until use. After autoclaving the NGM agar and before solidification, cadmium was added at the required concentrations. Cadmium concentrations used were 4, 7, 10, 13 and 16 mg Cd L^{-1} agar.

3.2.2 Toxicity tests

Full life-cycle toxicity tests were performed as described in Section 2.3.2, with worms being age synchronised twice by transferring 15 gravid nematodes onto NGM plates for 4 hours, then removing the worms and letting the eggs develop

into adults. The second synchronisation step was carried out on Petri plates containing cadmium dosed agar corresponding with the test doses, again allowing 15 gravid adult worms to lay eggs for 4 hours. This ensured that there would be no more than 4 hours difference in the age of all worms used in the experiment and that maternal effects were limited as the environment that the mother experiences could affect the phenotype of her offspring independently from genotype.

3.2.3 Statistics

EC50 values for reproduction were calculated using the general form of the logistic model (Van Ewijk and Hoekstra, 1993) as below.

$$N = \frac{c}{\left(1 + \left(dose/EC50\right)^{b}\right)}$$

Where c is the maximal response (of controls), b is a slope parameter and EC_{50} is the concentration at which there is a 50% reduction in reproduction compared to that of controls.

Survival was analysed using the Weibull survival model (Weibull, 1951), as has been previously used to describe the survival curve of the nematodes *Plectus accuminatus* (Kammenga et al., 1996) and *C. elegans* (Vanfleteren et al., 1998). Percentage survival values at each time interval were subjected to least squared fitting of a Weibull curve according to:

$$L_t = \exp\left[-\ln 2 \cdot \left(\frac{t_a}{LT50}\right)^c\right]$$

Where L_t is the proportion of the population alive at time t, c is the slope of the Weibull curve, and LT50 is the estimated time for 50% mortality of the population.

Growth was modelled over time using a logistic adapted equation as below:

$$V_t = \frac{(Max_s \quad Min_s)}{1+\exp^{-b(\ln t - \ln LT50)}} + Min_s$$

Where V_t is the size at time t, Max_s is the maximum adult size, Min_s is the minimum size, LT50 is the time at which size is 50% that of Max_s (point of inflection of the curve) and b is a shape parameter. This logistic model has previously been used to describe body growth in other species (Hawthorne et al., 2004).

T-tests were used to test whether there were significant differences between the means of two sample groups. To compare survival and dose response curves between populations and treatments, F-tests were performed. Two-way ANOVA was used to explore interactions between strain and cadmium dose and temperature and cadmium dose, using general linear modelling (GLM) in Minitab (Version 14).

Censored individuals were either lost during the experiment as a result of the individual crawling up the side of the well and becoming desiccated or lost during transfer, these were not included in lifespan analysis, or reproduction if the nematode was lost prior to cessation of the reproductive period. Nematodes which exhibited 'bagging', where eggs hatch and the larvae grow inside the parent killing it, were only included in the assessment of time to maturity.

3.3 Results

3.3.1 Effect of the addition of cadmium to the food source

A preliminary experiment was undertaken to test whether dosing of the *E. coli* food source as well as the agar altered toxic response in reproduction and lifespan. Control and four increasing concentrations of cadmium dosed agar plates were used. One set of plates contained *E. coli* OP50 in which cadmium chloride solution was added, at the corresponding concentration to the agar, 1 hour prior to the *E. coli* being spread on the agar. A second set of agar plates contained *E. coli* OP50 which had not been treated with cadmium chloride prior to spreading. This would determine whether the food source needed to be dosed as well as the agar for cadmium to exert its full effect on *C. elegans*.

There were no significant differences in lifespan or brood size between plates containing OP50 which were dosed with cadmium prior to being spread on the agar and plates containing OP50 which had not been dosed with cadmium at any of the concentrations examined ($p > 0.05$ for all doses; see Table 3.1 for t-test results).

Cadmium was not added to the food source in the subsequent experiments in this chapter as it is assumed that the addition of cadmium causes minimal additional exposure. This result suggests either that exposure directly through the body wall is likely to be the dominant exposure route for these nematodes, or that the *E. coli* assimilates cadmium from the medium during the test.

Table 3.1: Mean reproduction (brood size) and lifespan of *C. elegans* exposed to cadmium via **i)** dosed agar and non-dosed food source or **ii)** dosed food alongside dosed agar. Values given in brackets are standard error of the mean and t-test results, sample size for each group was 16.

	Reproduction			**Lifespan**		
	Brood size			Mean (days)		
Cadmium in agar, Mg Cd L^{-1}	Non-dosed *E. coli* OP50	Cadmiun dosed *E. coli* OP50	*p* value (t, df)	Non-dosed *E. coli* OP50	Cadmiun dosed *E. coli* OP50	*p* value (t, df)
0	230 (± 21)	188 (± 13)	0.11 (1.64, 28)	15 (± 1)	14 (± 0.5)	0.47 (0.73, 28)
4	159 (± 10)	133 (± 10)	0.08 (1.81, 27)	13 (± 0.3)	13 (± 0.4)	0.24 (1.19, 27)
7	116 (± 5)	130 (± 5)	0.07 (-1.89, 30)	12 (± 0.4)	12 (± 0.2)	0.94 (-0.08, 30)
10	118 (± 6)	100 (± 8)	0.09 (1.77, 26)	11 (± 0.3)	12 (± 0.4)	0.52 (-0.65, 26)
16	61 (± 8)	64 (± 9)	0.82 (-0.23, 19)	10 (± 0.3)	11 (± 0.3)	0.16 (1.45, 19)

3.3.2 Temperature

3.3.2.1 Temperature effects on survival and longevity

The effect of temperature alone can be determined by analysing survival (Figure 3.1) and lifespan (Figure 3.2) in control worms. A clear difference between survival curves was seen between the two temperatures.

Survival was modelled using the Weibull survival curve, shown on Figure 3.1 alongside the actual data. The Weibull model gives an estimated LT50, which is the time at which there is 50% population mortality, and a slope parameter c. The r^2 values for the 15°C and 25°C Weibull curves were 0.98 and 0.99 respectively. LT_{50} for 25°C was more than 45% lower than that at 15°C. LT_{50} values were 15.1 days for 15°C and 7.7 days for 25°C. The slope of the curve was steeper at 25°C, with the c value at 6.25, compared the 5.83 at 15°C. The survival curves of the nematodes at 15°C and 25°C were significantly different to each other ($F = 430.7$, $df = 2, 36$, $p < 0.01$).

The mean lifespan of control worms, as shown in Figure 3.2 was significantly reduced at 25°C compared to 15°C (t-test, $t = 9.44$, $df = 44$, $p < 0.01$). Cadmium has a larger impact on mean lifespan at 15°C than at 25°C, as can be seen by the marked difference between dose responses (Figure 3.2). At 15°C lifespan at the highest dose tested, 16 mg Cd L^{-1}, was 40% shorter than that of controls, whereas at 25°C, lifespan at 16 mg Cd L^{-1} was only 26% shorter then controls, indicating that cadmium has a more limited knockdown effect at the higher temperature.

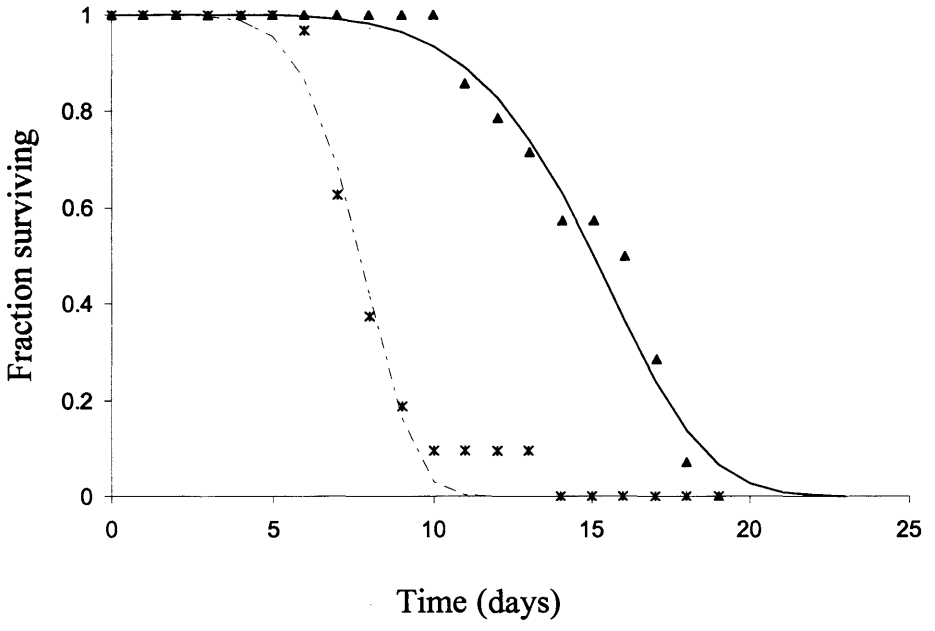


Figure 3.1: Survival for *C. elegans* strain N2 on control agar at two temperatures, ▲ 15°C data point's, —— 15°C Weibull curve; ✱ 25°C data point's, - - - - - 25°C Weibull curve.

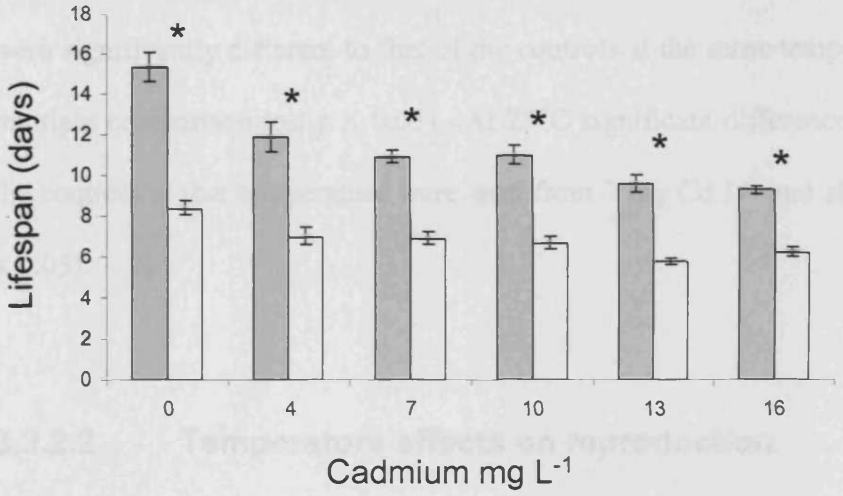


Figure 3.2: Mean lifespan of *C. elegans* strain N2 at ■ 15°C and □ 25°C at increasing concentrations of cadmium. Error bars represent standard error of the mean. Individual data points represent the mean values taken from 35 replicates (excluding individuals that were censored range 28-35). * are significantly different means at that concentration between temperatures (t-test, $p < 0.05$).

There were highly significant effects of temperature (2-way ANOVA (GLM): $F = 350.5$, $df = 1,5$, $p < 0.001$), cadmium concentration ($F = 29.64$, $df = 1,5$, $p < 0.001$) and the interaction between cadmium concentration and temperature ($F = 5.55$, $df = 1,5$, $p < 0.001$) on lifespan in *C. elegans*. At 15°C all lifespan responses were significantly different to that of the controls at the same temperature (Tukey multiple comparison test $p < 0.05$). At 25°C significant differences compared to the controls at that temperature were seen from 7 mg Cd L^{-1} and above (Tukey, $p < 0.05$).

3.3.2.2 Temperature effects on reproduction

No significant difference was seen in reproduction between nematodes grown at 15°C or 25°C on non-spiked agar (Tukey $p > 0.05$) (Figure 3.3). A clear reduction in reproduction was visible from 4 mg Cd L^{-1} and above at both temperatures. At 4 mg Cd L^{-1} reproduction was reduced by approximately 50% and 30% at 25°C and 15°C respectively and there was a 99.5% and 97% reduction in reproduction between control and 16 mg Cd L^{-1} worms at 25°C and 15°C, with nematodes at 25°C producing very few offspring above 10 mg Cd L^{-1}.

There was a significant effect of temperature (2-way ANOVA (GLM): $F = 64.19$, $df = 1,5$ $p < 0.001$), cadmium concentration ($F = 662.3$, $df = 1,5$, $p<0.001$), and the temperature and cadmium concentration interaction ($F = 14.93$, $df = 1,5$, $p<0.001$) on the total number of eggs laid. Significant differences were seen between the two temperatures at 4 and 7 mg Cd L^{-1} (Figure 3.3), no significant differences were found between temperatures at 10, 13 or 16 mg Cd L^{-1}.

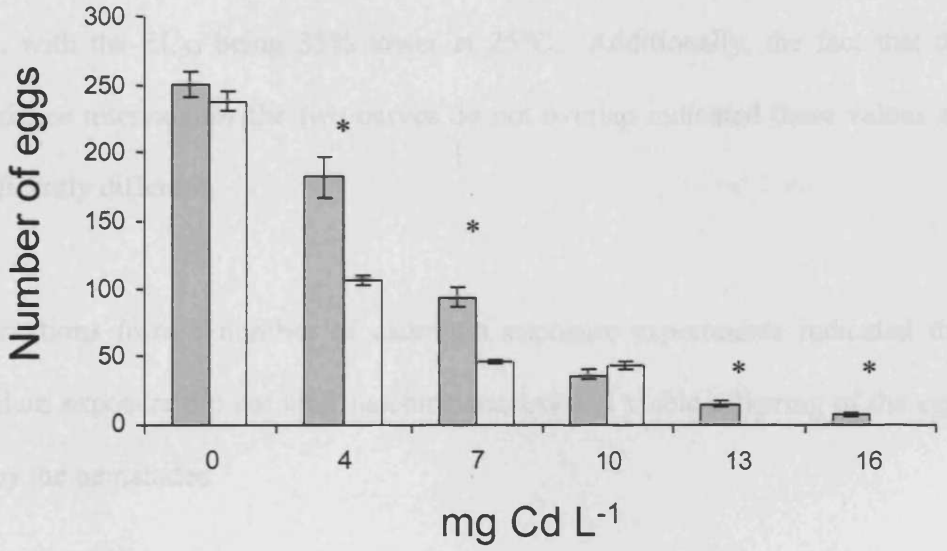


Figure 3.3: Mean total reproduction, measured as total number of eggs laid, of *C. elegans* strain N2 at ■ 15°C and □ 25°C. Error bars represent the standard error of the mean. Individual data points represent the mean values taken from 36 replicates (excluding individuals that were censored range 28-36). * are significantly different means of that concentration between temperatures (t-test, $p < 0.05$).

The dose response curve is steeper at 25°C than at 15°C. The dose response curves were significantly different to each other (F-test: $F = 39.0$, $df = 3, 267$, $p < 0.01$). Table 3.2 gives the $EC50_{reproduction}$ values and slope parameters of the logistic dose response to cadmium for both temperatures. Nematodes grown at the higher temperature of 25°C were more sensitive to cadmium than those at 15°C, with the EC_{50} being 35% lower at 25°C. Additionally, the fact that the confidence intervals for the two curves do not overlap indicated these values are significantly different.

Observations from a number of cadmium exposure experiments indicated that cadmium exposure did not alter hatching success and viable offspring of the eggs laid by the nematodes.

3.3.2.3 Temperature effects on growth

Nematode growth was measured from the L1 stage to reproducing adults over the first seven days of life. Body length and area were measured; volume was calculated as described in Section 2.3.3, and volumetric length shown in Figure 3.4 is the cube root of the volume.

Growth was modelled using the logistic adapted equation described in Section 3.2.3. Figure 3.4 shows the fit of the model to the mean growth data in control worms at control and a representative mid and high cadmium dose. The good fit of the data to the model ($r^2 = 0.98$ for both controls and mid, $r^2 = 0.8$ for the highest dose) indicates that the model is suitable for the description of nematode

Table 3.2: Parameter values (± 95% confidence intervals) and coefficient of determination of the logistic concentration response curve for reproduction in *C. elegans* strain N2 exposed to cadmium. EC_{50} is the concentration at which total number of offspring is 50% that of controls *c*; *b* is the slope of the dose response curve.

	15°C	25°C
EC_{50} (mg L^{-1})	5.75	3.73
	(5.31-6.20)	(3.47-3.99)
b	3.06	2.26
	(2.54-3.58)	(1.97-2.55)
c	250	238
	(236-263)	(230-246)
r^2	0.93	0.93

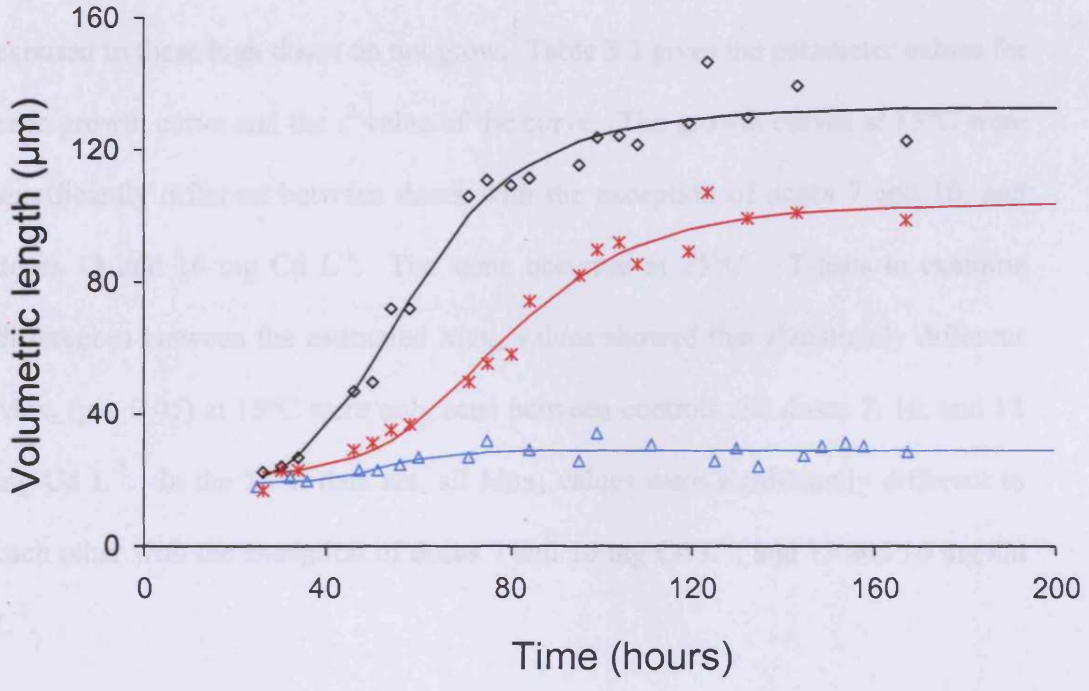


Figure 3.4: Nematode growth data and fit of the growth curve modelled using a logistic adapted model. Mean size is shown as points, and the model as an unbroken line. Data shown is growth of *C. elegans* strain N2 at 25°C, under — control, — 7 mg Cd L^{-1}, and — 16 mg Cd L^{-1} cadmium exposure.

growth.

Growth over time for each temperature was modelled (logistic adapted model) at each treatment and shown in Figure 3.5. This clearly shows the increase in growth rate at 25°C compared to 15°C, for control treatments. The two highest doses, 13 mg Cd L^{-1} and 16 mg Cd L^{-1} at 25°C, showed vastly different response to cadmium than the corresponding doses at 15°C. At 25°C the nematodes exposed to these high doses do not grow. Table 3.3 gives the parameter values for each growth curve and the r^2 value of the curve. The growth curves at 15°C were significantly different between doses with the exception of doses 7 and 10, and doses 13 and 16 mg Cd L^{-1}. The same occurred at 25°C. T-tests to examine differences between the estimated Max_s values showed that statistically different Max_s ($p < 0.05$) at 15°C were only seen between controls and doses 7, 10, and 13 mg Cd L^{-1}. In the 25°C data set, all Max_s values were significantly different to each other with the exception of doses 7 and 10 mg Cd L^{-1}, and 13 and 16 mg Cd L^{-1}.

Exposure to cadmium in the nematodes environment evidently slows down growth, development, maturity, and reproduction. This could reduce the ability of the nematodes to complete their entire reproductive period before dying, which would impact on fitness. Under control conditions the proportion of the lifespan spent pre- or post- reproductive was found to be similar between temperatures (Table 3.4). The developmental time and reproductive period were in the same proportions of overall lifespan at each temperature, and the senescent period at

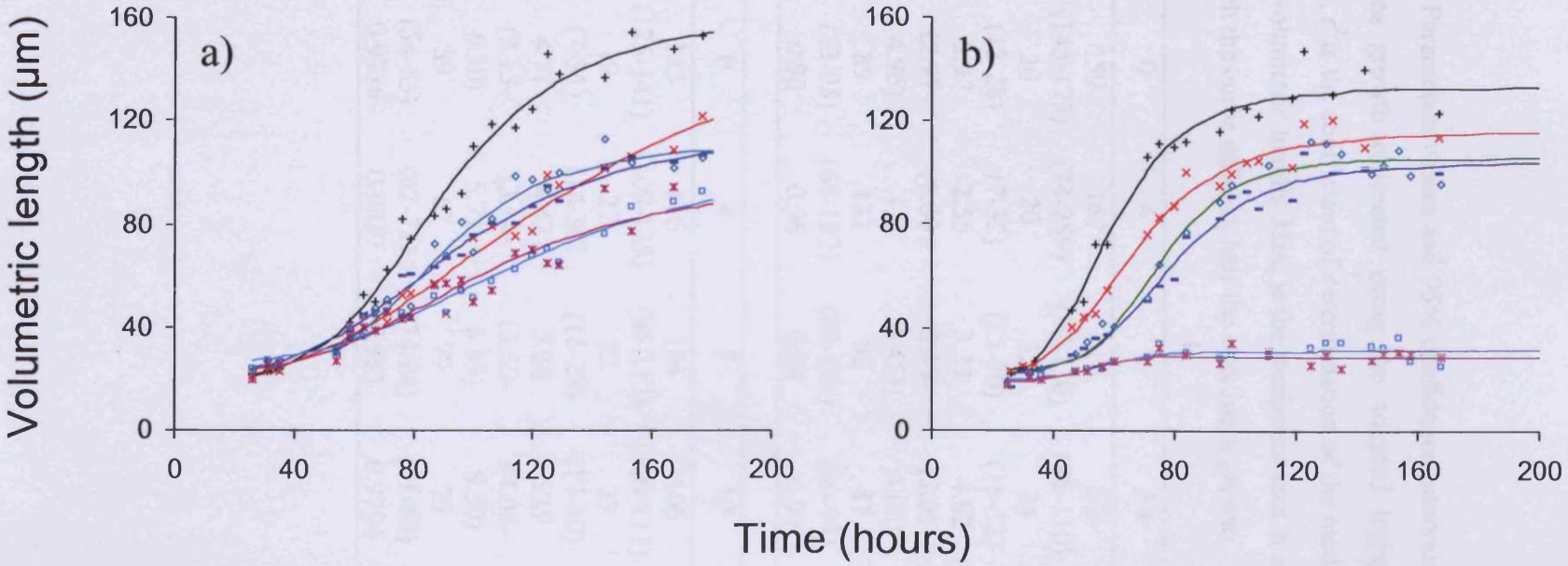


Figure 3.5: Growth, measured as volumetric length, of control and cadmium exposed *C. elegans* strain N2 at two temperatures a) 15°C and b) 25°C under + Control, × 4, - 7, ◇ 10, □ 13 and * 16 mgL^{-1} cadmium treatment. Unbroken lines in the corresponding colour as the data points for each dose show the growth curve fitted with the adapted logistic growth model.

Table 3.3: Parameters values and 95% confidence intervals of control and cadmium dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, Min_s is the minimum size, b is a constant and LT50 the time at which the curve inflects, half the maximum growth.

15°C	0	4	7	10	13	16
Max_s	159 (145-173)	167 (74-259)	114 (99-130)	106 (96-116)	101 (97-105)	117 (32-201)
Min_s	20 (12-28)	20 (7-32)	22 (15-30)	24 (16-32)	25 (19-30)	18 (2-34)
b	3.97 (2.97-4.96)	2.55 (1.00 - .11)	3.32 (2.19-4.45)	4.82 (3.05-6.60)	2.79 (2.15-3.44)	2.14 (0.08-4.21)
LT_{50}	89 (83-95)	132 (68-197)	92 (82-102)	87 (80-94)	114 (105-122)	121 (30-212)
r^2	0.98	0.96	0.98	0.96	0.96	0.93

25°C	0	4	7	10	13	16
Max_s	133 (125-141)	116 (109-123)	104 (96-112)	106 (100-111)	31 (29-33)	29 (27-30)
Min_s	19 (7-31)	22 (14-30)	22 (16-28)	23 (17-30)	19 (15-24)	19 (15-23)
b	4.71 (3.13-6.30)	4.42 (3.14-5.71)	5.20 (3.50-6.89)	6.19 (4.08-8.30)	6.34 (-2.12-14.80)	8.00 (-4.47-20.46)
LT_{50}	59 (54-63)	67 (62-71)	79 (74-84)	75 (71-80)	57 (44-70)	52 (43-62)
r^2	0.9786	0.9837	0.981	0.9796	0.7329	0.7212

Table 3.4: Average time (± standard deviations) for life cycle parameters of *C. elegans* strain N2 worms at two temperatures 15°C and 25°C. Tj is the pre-reproductive period, Tr the reproductive period and Tpr, the post-reproductive (or senescent) period, and lifespan

	15 °C				25°C			
Dose (mg Cd L^{-1})	**Tj (days)**	**Tr (days)**	**Tpr (days)**	**Lifespan (days)**	**Tj (days)**	**Tr (days)**	**Tpr (days)**	**Lifespan (days)**
0	4.1 (± 0.3)	4.9 (± 0.9)	6.4 (± 2.8)	15.3 (± 2.8)	2.0 (± 0.01)	3.1 (± 0.8)	3.0 (± 2.2)	8.2 (± 2.0)
4	4.2 (± 0.4)	7.1 (± 1.4)	1.0 (± 1.1)	11.9 (± 2.4)	2.3 (± 0.1)	3.2 (± 0.6)	1.8 (± 3.0)	7.0 (± 2.5)
7	5.4 (± 0.5)	5.3 (± 0.8)	0.3 (± 0.5)	10.9 (± 1.1)	3.0 (± 0.2)	2.8 (± 0.3)	1.1 (± 1.6)	7.0 (± 1.7)
10	5.4 (± 0.7)	5.2 (± 1.0)	0.5 (± 0.8)	11.0 (± 1.7)	2.8 (± 0.2)	2.6 (± 0.5)	1.5 (± 1.6)	6.7 (± 2.0)
13	5.8 (± 0.4)	4.2 (± 1.0)	0.2 (± 0.4)	9.6 (± 1.4)	5.8 (± 1.1)	0.0 (± 0.0)	0.0 (± 0.0)	5.8 (± 1.1)
16	6.5 (± 0.7)	3.0 (± 0.9)	0.2 (± 0.6)	9.3 (± 0.7)	6.2 (± 1.3)	0.0 (± 0.0)	0.0 (± 0.0)	6.2 (± 1.3)

15°C was slightly longer in proportion to lifespan compared to that at 25°C. However, from a population fitness point of view, the senescent period is not important, unless it impacts on food availability or density dependence in the population. The senescent period is very short in comparison to that of controls at the lower doses of cadmium for both temperatures and is minimal if at all from 10 mg Cd L^{-1} and above at both temperatures. There were no obvious differences between the proportions of lifespan spent developing and reproducing in dosed worms between the temperatures.

3.3.3 Comparison of strains

Life-cycle toxicity tests with cadmium were performed with strains N2, CB4856 (Hawaii) and RW7000 (Bergerac) at 15°C. The 2^{nd} generation nematodes of the Bergerac strain were not able to grow to maturity and produce any offspring at the concentrations 13 and 16 mg Cd L^{-1}. Therefore the lifespan data for Bergerac comes from controls and 3 doses of cadmium. Growth and reproduction were able to be measured in all doses.

3.3.3.1 Strains effects on survival

The differences in lifespan and survival of the three strains of worms were determined by analysing survival (Figure 3.6) and lifespan (Figure 3.7) in control worms. Survival was modelled using the Weibull survival curve (Figure 3.6). The r^2 values for the Weibull curves for N2, Hawaii, and Bergerac were 0.99, 0.99 and 0.89 respectively, indicating the excellent fit for the first two strains, but a weaker fit for

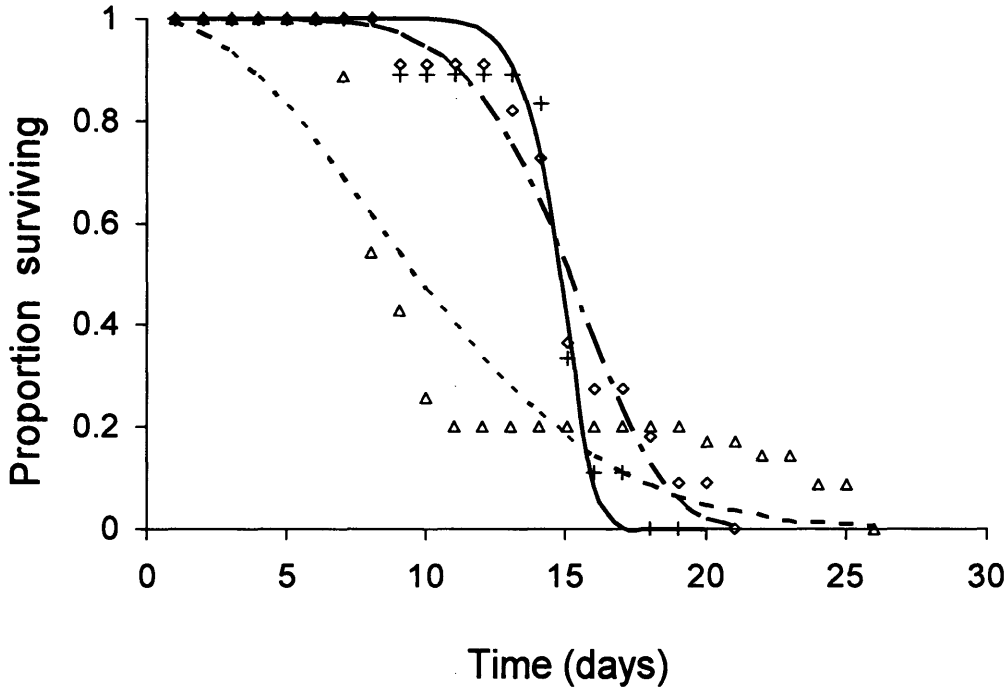


Figure 3.6: Survival of three strains of *C. elegans* under control conditions.

+ N2 data points, ——— N2 Weibull curve; ◇ Hawaii data points, — - – Hawaii Weibull curve; △ Bergerac data points, - - - - Bergerac Weibull curve.

Bergerac, which showed high initial mortality and an extended survival tail. The LT_{50} value for Bergerac strain was approximately 40% lower than that of N2 and Hawaii strains being 14.7 days for N2, 15.1 days for Hawaii and 9.6 days for Bergerac. The slope of the curve was steeper for N2 (15.6) than either Hawaii (6.1) or Bergerac (2.0), indicating that the majority of nematodes in the N2 population died within a short time frame, compared to a much larger dispersal of deaths over time particularly in Bergerac. Significant differences were seen between the Weibull survival curves of the three strains (F-tests: N2-Hawaii, $F = 12.4$, $df = 2, 48$, $p < 0.05$, N2-Bergerac, $F = 61.1$, $df = 2, 48$, $p < 0.05$, Bergerac – Hawaii, $F = 53.5$, $df = 2, 48$, $p < 0.05$).

The mean lifespan in days of the nematodes over the dose range is shown in Figure 3.7. There was a significant difference in mean lifespan between strains under control conditions (Kruskal-Wallis nonparametric test as variances were unequal: $H = 14.1$, $df = 3$, $p < 0.05$). Dunn's multiple comparison test showed that mean lifespan was not significantly different between the N2 and Hawaii populations ($p > 0.05$), but were significantly different for N2 and Bergerac ($p < 0.01$) and Hawaii and Bergerac ($p < 0.05$).

As mortality data was unavailable for the Bergerac strain above 10 mg Cd L^{-1}, 2-way ANOVA (GLM) was performed on N2, Hawaii and Bergerac data up to 10 mg Cd L^{-1} only to examine whether there was a significant interaction between strain and cadmium ($F = 4.46$, $df = 6$, $p < 0.05$). There were significant differences in lifespan in 10 mg Cd L^{-1} in N2 worms compared to N2 controls (Tukey multiple comparison test $p < 0.05$), in 4 and 7 mg Cd L^{-1} Hawaii compared to Hawaii controls (Tukey multiple

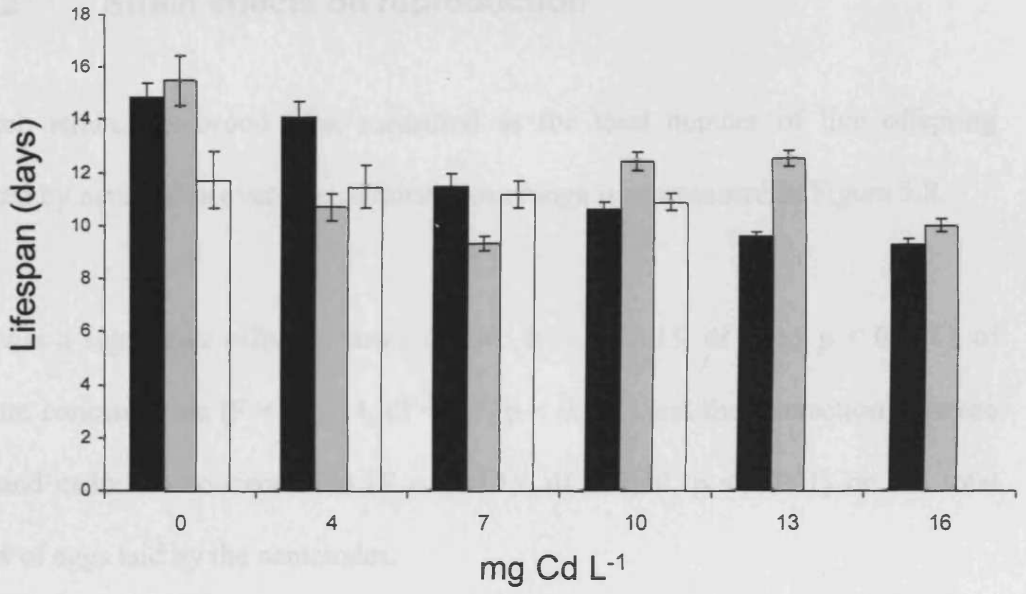


Figure 3.7: Mean lifespan in days of nematodes of three wild type strains (■ N2 ▣ Hawaii and □ Bergerac) exposed to a series of cadmium concentrations. Error bars represent standard error of the mean. Mean N2 values were obtained from 23 replicates (excluding individuals that were censored range 18-23), mean Hawaii values were obtained from 19 replicates (excluding individuals that were censored range 11-19), and mean Bergerac values were obtained from 45 replicates (excluding individuals that were censored range 29-45). Compared to controls, N2 lifespan at 10 mg L^{-1} was significantly lower, Hawaii lifespan at both 4 and 7 mg L^{-1} were significantly lower. There were no significant differences to controls in Bergerac lifespan.

comparison test $p < 0.05$), and no significant difference in lifespan over the range of cadmium concentrations compared to controls in the Bergerac data set ($p > 0.05$).

3.3.3.2 Strain effects on reproduction

For each strain, the brood size, measured as the total number of live offspring produced by nematodes over the cadmium dose range is represented in Figure 3.8.

There was a significant effect of strain (GLM: $F = 1118.15$, $df = 2,5$ $p < 0.001$), of cadmium concentration ($F = 451.14$, $df = 2,5$, $p < 0.001$) and the interaction between strain and cadmium concentration ($F = 106.15$, $df = 2,10$, $p < 0.001$) on the total number of eggs laid by the nematodes.

Significant reductions in reproduction from the 4 mg Cd L^{-1} and above were seen within each strain compared to that of the relevant control (1-way ANOVA: N2, $F=118.44$, $df = 5, 118$, $p<0.05$; Hawaii, $F = 190.68$, $df = 6, 138$, $p<0.05$; Bergerac, $F = 59.98$, $df = 5, 222$, $p<0.05$). Reproduction was clearly different in strain Bergerac, with a complete knockdown of reproduction in 100% of the nematodes at 10 mg Cd L^{-1} cadmium and above. This explains the earlier inability to produce the F2 worms required for use in the test.

Reproduction of N2 and Hawaii worms grown on un-spiked agar were not significantly different (Tukey multiple comparison test, $p>0.05$), significant effects in reproductive output between these two strains were seen only at 7 mg Cd L^{-1}. Significant differences in reproduction were seen in all treatments between strains N2

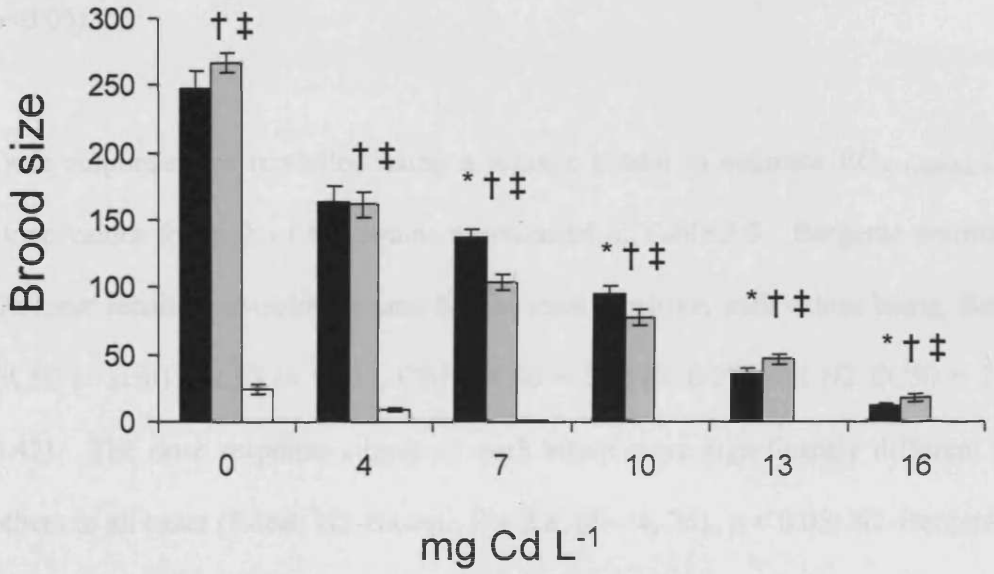


Figure 3.8: Mean brood size of three wild type strains of *Caenorhabditis elegans*, ■ N2, ▣ Hawaii and □ Bergerac exposed to a range of concentrations of cadmium. Mean values were obtained from 23 replicates of the N2 strain (excluding individuals that were censored, range 18-23), mean Hawaii values were obtained from 24 replicates (no individuals required censoring), and mean Bergerac values were obtained from 45 replicates (excluding individuals that were censored range 29-45). Error bars represent standard error of the mean. Significant differences ($p < 0.05$) between strains within a dose are denoted by * N2 and Hawaii, † N2 and Bergerac, and ‡ Hawaii and Bergerac. Significant differences were seen in strains N2 and Hawaii at all doses compared to controls, in Bergerac significant differences were seen in all doses except for 4 mg L^{-1} compared to controls.

and Bergerac (Tukey, $p < 0.05$), total reproduction in control Bergerac worms was just 10% that of either the Hawaii or N2 strains, and in control and all doses of cadmium except for 16 mg Cd L^{-1} between strains Hawaii and Bergerac (Tukey, $p<0.05$).

Dose response was modelled using a logistic model to estimate $EC_{50\text{-reproduction}}$ and slope values for each of the strains represented in Table 3.5. Bergerac worms were the most sensitive to cadmium and N2 the least sensitive, with values being, Bergerac EC50 (± sem) = 2.93 (± 0.91), CBH EC50 = 5.32 (± 0.27) and N2 EC50 = 7.00 (± 0.42). The dose response curves of each strain were significantly different to the others in all cases (F-test: N2-Hawaii, $F = 2.8$, $df = 4, 261$, $p < 0.05$; N2-Bergerac, $F = 16.9$, $df = 93, 257$, $p < 0.001$; Hawaii-Bergerac, $F = 25.7$, $df = 94, 276$, $p < 0.001$). EC50's were significantly different to each other between strains (Unpaired t-test: N2-Hawaii: $t = 3.352$, $df = 210$, $p<0.001$, N2-Bergerac: $t = 4.047$, $df = 190$, $p < 0.001$, Hawaii-Bergerac: $t = 2.509$, $df = 159$, $p < 0.05$).

3.3.3.3 Strain effects on growth

Growth was measured over the first 7 days of the nematode lifespan, covering the 4 larval stages and young to mid aged adults. Worm length and area were measured; volume and volumetric length were calculated as described in Section 2.3.3.

There was no significant difference in body length at 26 hours (larval stage 1) between control worms of N2 and Hawaii strains (Figure 3.9), however Bergerac worms were significantly longer than both N2 and Hawaii worms (1-way ANOVA: F

Table 3.5: Parameter values (± 95% confidence intervals) and coefficient of determination (r^2) of the logistic concentration response curve for *C. elegans* strains exposed to cadmium. EC_{50} is the concentration at which total number of offspring is 50% that of controls, *c*, and *b* is the slope of the dose response curve.

	N2	Hawaii	Bergerac
EC_{50} (mg L^{-1})	7.00 (6.17-7.82)	5.32 (4.79-5.85)	2.93 (1.15-4.72)
b	2.20 (1.76-2.63)	1.76 (1.49-2.02)	3.77 (-2.66-10.20)
c	239 (221-257)	266 (253-279)	24 (21 - 27)
r^2	0.7905	0.8647	0.5244

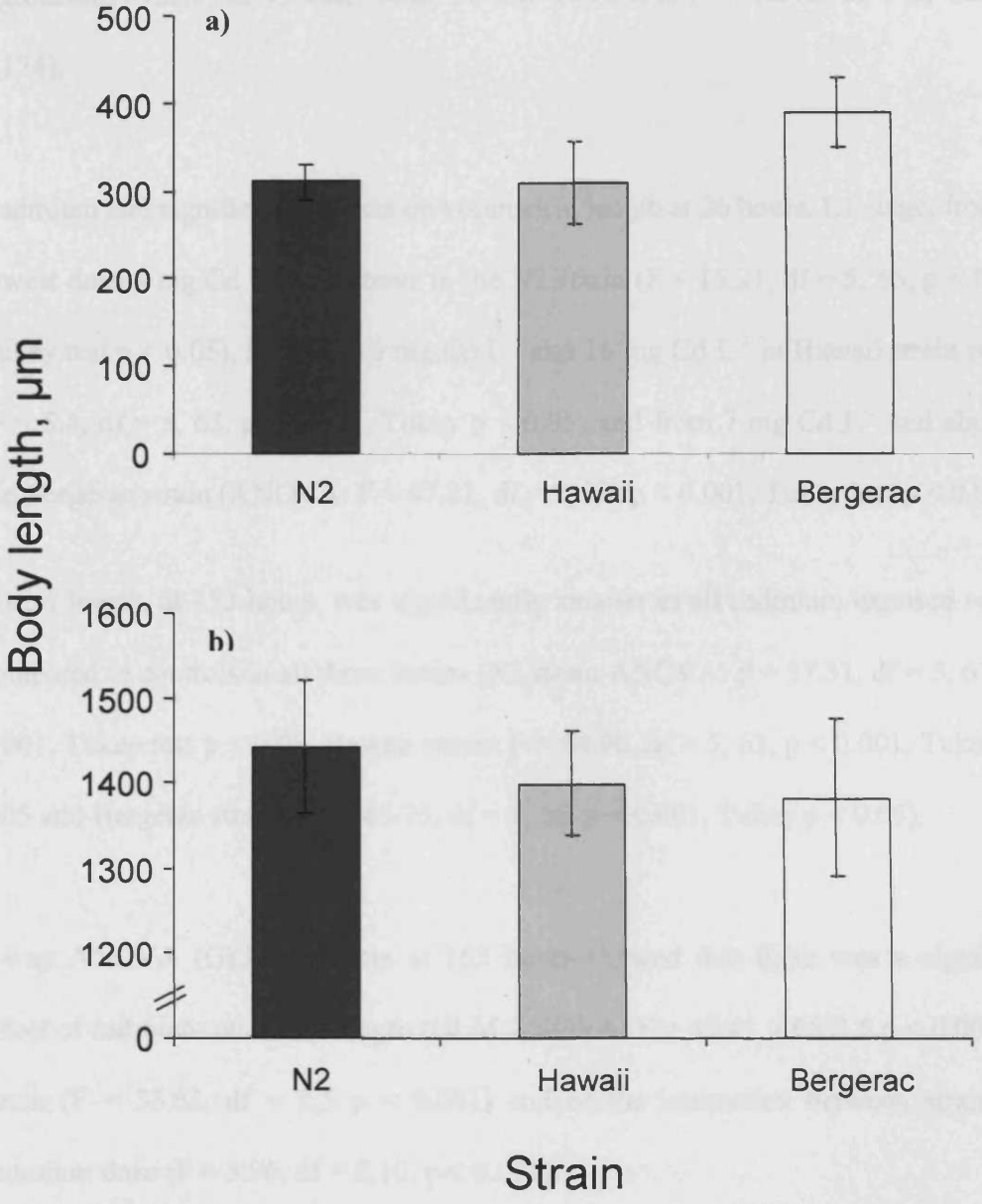


Figure 3.9: Mean nematode body length at **a)** 26 (larval stage 1) and **b)** 153 hours (reproducing adult stage) old. Error bars represent standard deviations. The only statistically significant difference in body size is that Bergerac at 26 hours old was longer than either N2 or Hawaii strains. There were no differences at 153 hours.

=19.30, df = 2, 37, $p < 0.001$, Tukey multiple comparison test $p < 0.05$). At 153 hours (reproductive adult stage) the length of the worms from each strain were not significantly different to each other (1-way ANOVA: $F = 1.848$, df = 2, 32, $p = 0.174$).

Cadmium had significant impacts on volumetric length at 26 hours, L1 stage, from the lowest dose 4 mg Cd L^{-1} and above in the N2 strain ($F = 15.21$, df = 5, 66, $p < 0.001$, Tukey test $p < 0.05$), at doses 10 mg Cd L^{-1} and 16 mg Cd L^{-1} in Hawaii strain worms ($F = 5.4$, df = 5, 63, $p < 0.001$, Tukey $p < 0.05$) and from 7 mg Cd L^{-1} and above in the Bergerac strain (ANOVA: $F = 47.21$, df = 5, 80, $p < 0.001$, Tukey test $p < 0.05$).

Worm length, at 153 hours, was significantly smaller in all cadmium exposed worms compared to controls in all three strains (N2 strain ANOVA: $F = 57.31$, df = 5, 61, $p < 0.001$, Tukey test $p < 0.05$, Hawaii strain: $F = 84.96$, df = 5, 62, $p < 0.001$, Tukey $p < 0.05$ and Bergerac strain: $F = 145.75$, df = 5, 56, $p < 0.001$, Tukey $p < 0.05$).

2-way ANOVA (GLM) on data at 153 hours showed that there was a significant effect of cadmium on worm length (GLM ANOVA: $F = 47.41$, df = 2,5 $p < 0.001$), of strain ($F = 33.62$, df = 2,5 $p < 0.001$) and of the interaction between strain and cadmium dose ($F = 5.96$, df = 2,10, $p < 0.001$).

Volumetric length was modelled using the adapted logistic equation described in Section 3.1.6. The fit of the model to the data from control worms of each strain is shown in Figure 3.10. The correlation coefficients for N2, Hawaii and Bergerac were 0.98, 0.98 and 0.97 respectively. Figure 3.11 illustrates the mean volumetric length over time for each strain at increasing cadmium concentrations. This data was

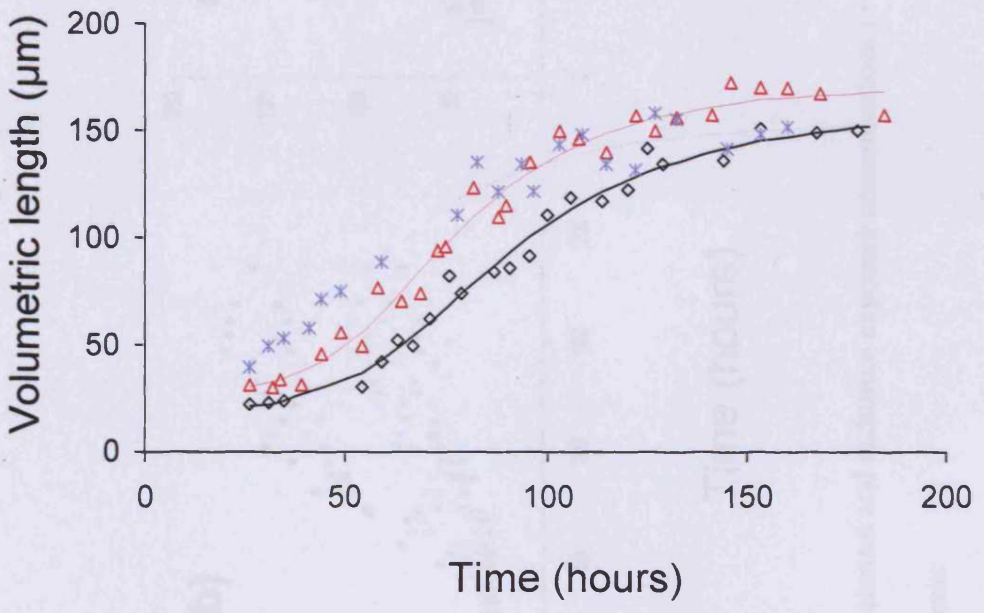


Figure 3.10: Growth of control nematodes of strains (◇ N2, △ Hawaii, and ✳ Bergerac). Data points represent actual measurements; unbroken lines show the growth curve modelled using a logistic adapted equation.

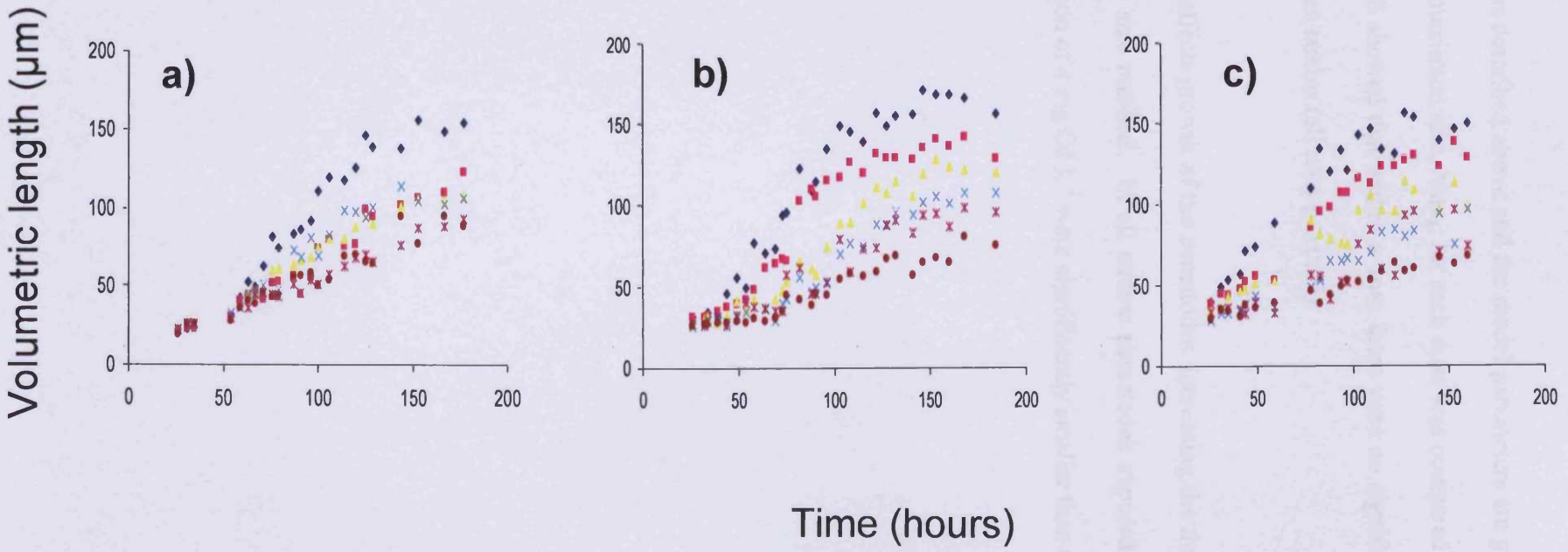


Figure 3.11: Growth over time of the nematode *C. elegans* under control and cadmium exposure concentrations (♦ 0 ■ 4 ▲ 7 ✕ 10 ● 13 ✕ 16 mg L^{-1}) of three wild isolate strains; a) N2, b) Hawaii, c) Bergerac

modelled as described above and the model parameters are given in Table 3.6. The estimated maximum size, Max_s, for each dose was compared between strains, using a t-test which showed that within a dose there were no significant differences in body size between strains (all tests $p > 0.05$).

Cadmium affects growth of the nematodes, increasing the time taken to grow and the final body size reached. In all strains nematodes exposed to the lowest cadmium concentration of 4 mg Cd L^{-1} were significantly smaller than their respective controls.

Table 3.6: Parameters values and 95% confidence intervals of control and cadmium dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, Min_s is the minimum size, b is a constant and LT_{50} the time at which the curve inflects, half the maximum growth.

N2	0	4	7	10	13	16
Max_s	159 (145-173	167 (74-259)	114 (99-130)	106 (96-116)	101 (97-105)	117 (32-201)
Min_s	20 (12-28)	20 (7-32)	22 (15-30)	24 (16-32)	25 (19-30)	18 (2-34)
b	3.97 (2.97-4.96)	2.55 (1.00-4.11)	3.32 (2.19-4.45)	4.82 (3.05-6.60)	2.79 (2.15-3.44)	2.14 (0.08-4.21)
LT_{50}	89 (83-95)	132 (68-197)	92 (82-102)	87 (80-94)	114 (105-122)	121 (30-212)
r^2	0.98	0.96	0.98	0.96	0.96	0.93

Hawaii	0	4	7	10	13	16
Max_s	171 (160-183)	136 (131-140)	128 (119-137)	113 (103-122)	105 (83-127)	78 (66-89)
Min_s	29 (18-39)	33 (28-38)	31 (27-36)	29 (25-32)	28 (21-36)	26 (22-30)
b	4.09 (2.93-5.25)	6.38 (2.98-7.78)	5.42 (4.03-6.81)	5.35 (3.97-6.73)	4.11 (1.76-6.46)	4.18 (2.27-6.10)
LT_{50}	77 (72-82)	78 (75-81)	99 (93-104)	107 (100-113)	105 (87-123)	103 (89-117)
r^2	0.98	0.99	0.98	0.98	0.93	0.95

Bergerac	0	4	7	10	13	16
Max_s	156 (139-173)	137 (87-115)	116 (87-145)	95 (78-113)	87 (63-112)	75 (56-93)
Min_s	34 (9-60)	43 (38-48)	33 (11-39)	33 (26-39)	35 (24-45)	33 (29-37)
b	3.14 (1.44-4.83)	4.68 (3.28-6.08)	3.02 (0.16-5.88)	4.57 (1.46-7.69)	6.28 (-2.05-14.6)	3.89 (1.12-6.65)
LT_{50}	62 (51-72)	81 (75-85)	76 (56-96)	94 (81-108)	97 (76-119)	106 (80-132)
r^2	0.97	0.99	0.92	0.95	0.77	0.95

3.4 Discussion

Cadmium is a non-essential heavy metal that is highly toxic to organisms. The effects of cadmium on life history parameters were examined with the interaction of temperature and cadmium, and genotype and cadmium, by performing toxicity tests with sub-lethal levels of cadmium on *C. elegans* over their entire lifespan.

In all tests reproduction was the most sensitive parameter measured, followed by growth, and lifespan being the least sensitive trait to cadmium. Significant effects on reproduction were seen at the lowest dose tested, 4 mg Cd L^{-1}. Significant growth effects occurred from 4 mg Cd L^{-1} in most cases and significant lifespan reductions varied from 4 mg Cd L^{-1} in the N2 strain at 15°C to totally unaffected by cadmium in the Hawaii strain.

3.4.1 Cadmium In food

The presence or absence of cadmium in the *E. coli* OP50 food source did not significantly effect either reproduction or survival rate. For this reason it can be assumed either that exposure is likely to be predominantly through the body wall or that the *E. coli* OP50 assimilates cadmium from the agar over time. This would mean the nematodes would be exposed via feeding on the bacteria whether or not cadmium has been added to the food source.

3.4.2 Temperature

Temperature proved to be a major factor in the timing of life cycle events and in influencing response to cadmium. The average lifespan of control worms at 15°C and 25°C were 15 and 8 days correspondingly. The average N2 lifespan most often quoted, found by Johnson and Wood (1982), is 18 days at 20°C. Although this temperature is not used in this experiment this value does appear anomalous to the data measured here, since it would be anticipated that the value would be intermediate between the 15 and 25°C values. Possible reasons for the shorter lifespan found in this experiment are currently not clear, although there are potential influences of culture conditions and handling. The 15 day lifespan at 15°C agrees with data published by Swain et al. (2004) who found that worms under control conditions had an average lifespan of 14.5-15 days, these data corroborating our observations.

The difference in survival curves of the controls at 15°C and 25°C can be readily explained by the factor 'rate of living' at a higher temperature (Pearl, 1928; Walker et al., 2005). It is well known in poikilothermic organisms, such as nematodes, an increase in ambient temperature will increase a range of physiological processes, including respiration rate and metabolic rate which in turn increases growth rate and alters the timing and consequences of life history traits. It has been shown previously that elevated temperatures decreases lifespan in *C. elegans* (Klass, 1977). The rate of living theory of aging proposed that rate of living effects could be attributed to changes in respiration rate. Developing on this theme, the oxidative damage theory of aging, suggests that reactive oxygen species, by-products of oxidative metabolism, cause damage to cell components which accumulate in the cell, leading to the ageing

phenotype. It follows then that an increase in metabolism would increase ageing. This was clearly seen in the much shorter post-reproductive period seen at 25°C.

The resulting increase in metabolism that can be assumed at the higher temperature leads to a faster growth rate. It is thought that before an organism can begin to reproduce it has to reach a specific size, at which point the switch between growth and reproduction can occur (Kooijman, 2000) With an increased growth rate age at maturity will be lower which gives fitness advantages in terms of population growth rate (Kammenga et al., 1996).

Contrary to the findings of others, the number of offspring was unaffected under control conditions between temperatures. Byerly et al. (1976) found that the number of eggs laid by N2 worms at 16°C and 25°C went from 275 to 170 eggs respectively. A similar result was found by Jager et al. (2005) who found little difference between the total number of eggs laid by N2 worms at 16°C and 20°C (average brood size of 265), but a large reduction in reproduction at 25°C (average brood size of 165). The result found here, however, suggest that the differences seen in these previous studies may not be entirely temperature mediated.

Cadmium has a substantial impact on reproduction, with significant effects seen from the lowest dose tested, 4 mg Cd L^{-1}, at both temperatures. Reproductive rate is an especially important parameter for a species that allocates a great amount of resources to reproduction. A reduction in reproduction is often linked to a decrease in body size, as was seen in this study, therefore making growth an ecologically relevant parameter.

Cadmium reduced growth rate and maximum body size in the nematodes. This could be the result of direct toxic effects of cadmium, or indirect effects such as reduced energy intake. On exposure to cadmium, *C. elegans* reduces its feeding rate (Jones and Candido, 1999), which is observable in the rate of pharyngeal pumping. Whilst feeding rate was not a measured parameter in this experiment, pumping rate was noticeably less frequent at the highest cadmium dose than in control worms. This effect of cadmium has also been observed in another free living species of nematode *Panagrellus silusiae* (Mudry et al., 1982) and in a further study on *C. elegans* (Alvarez et al., 2005).

It has been speculated that feeding cessation could be caused by damage to proteins essential to pharyngeal function, or activation or synthesis of proteins that inhibit feeding (Jones et al., 1996). Reduced feeding rate is one mechanism that can potentially explain reductions in survival, growth and reproduction occurring in toxicant-exposed organisms (Allen et al., 1995). Decreases in reproduction and lifespan are also attributable to toxic effects due to the cadmium. Although the worms are capable of expressing stress proteins and metallothioneins (MTL-1 and MTL-2) (Liao and Freedman, 1998) to detoxify and repair damage caused by cadmium, long-term exposure resulted in early death and reduced fecundity. In *C. elegans*, the use of metallothionein GFP (green fluorescent protein) linked mutants has shown that while *mtl-1* is constitutively expressed in the pharynx, *mtl-2* is induced upon cadmium exposure in the intestine (Swain et al., 2004). Cadmium detoxification is thought to involve cadmium binding to metallothionein; however, at high concentrations overflow of this system can lead to cadmium association with other proteins leading to cellular dysfunction and ultimately to toxic effects.

Cadmium has major impacts on life history parameters. Growth rate has been studied as an endpoint in ecotoxicology, not only in *C. elegans* (Anderson et al., 2001; Hoss et al., 1999; Hoss et al., 2002), but in other species such as the earthworm *Eisenia fetida* (Spurgeon and Hopkin, 1996), the isopod *Porcellio scaber* (Abdel-Lateif et al., 1998) and other soil dwelling invertebrates (FolkerHansen et al., 1996). Results from the experiment presented in this chapter indicate that growth as an endpoint is more sensitive to cadmium than mortality, although reproduction was the most sensitive parameter.

For lifespan there appeared to be a more severe reduction relative to controls in cadmium exposed worms at 15°C than at 25°C. However, whilst the response differs, it is important to consider that the worms in both temperatures had finished reproducing and as the senescence period has little or no impact on population fitness, longevity past the end of reproduction is not considered particularly relevant. As lifespan is least sensitive to cadmium, it would not be all that useful to use this as a toxicity endpoint alone. A better approach would be to look for sub-lethal effects of chemicals, which come into play at a much earlier time, or at lower dosage, which could then be used to indicate toxic effects earlier. Sub-lethal toxic effects on the individual which appear small may cause large consequences at the population level.

While some studies have found strong correlations of temperature and toxicity parameters others have found inconsistent or no effects. Heugens et al. (2003) found that at elevated temperatures cadmium toxicity was higher in the freshwater amphipod *Daphnia magna* in 48 hour acute toxicity tests. By investigating tissue concentrations they found that the uptake rate of cadmium increased at higher temperatures,

suggesting an increase in metabolic rate could have resulted in higher active transport across the membrane. Lethal toxicity of copper, zinc and lead were found to be higher at higher temperature to the freshwater amphipod *Gammarus pulex* by Bat et al. (2000). Not all studies however have found correlations between higher temperature and higher metal toxicities. A study on the springtail *Folsomia candida* by Sandifer and Hopkin (1997) found that EC_{50}-reproduction values for cadmium, copper and zinc to be similar at 15 and 20°C, however lead was more toxic at 15°C than 20°C.

The findings of this experiment indicate *C. elegans* is more sensitive to cadmium at a higher temperature in terms of effects on reproduction and growth, but not on lifespan. The different effect of temperature on the sensitivity of different traits may thus account for the different effects of temperature on sensitivities observed in previous studies. Higher temperature caused a greater reduction in reproduction at low doses of cadmium; however at high cadmium dose the decrease in reproduction was equal between temperatures. At 25°C the decrease in reproduction at 4 mg Cd L^{-1} was almost double that which occurred at 15°C. At the highest cadmium dose tested, 16mg Cd L^{-1}, similar reductions in reproduction of 97% and 99% that of controls were seen at 15°C and 25°C respectively. Temperature also had implications on cadmium toxicity in terms of lifespan. Whilst the higher temperature reduced the lifespan of the nematodes, lifespan was more sensitive to cadmium at the lower temperature, with significant reductions measured at 4 mg Cd L^{-1} and above, with dose dependent decreases. At 25°C significant lifespan reduction was not seen until 7 mg Cd L^{-1}, and no clear dose dependent reduction was observed. Temperature affected growth of the nematodes under cadmium exposure; higher temperature had a

severe impact on growth at the two highest cadmium treatments in comparison with the lower temperature.

At 25°C egg production was more sensitive to cadmium but lifespan was less sensitive compared to the 15°C measurements. The opposite was seen at 15°C, with cadmium having less impact on egg production but more impact on lifespan. This could be explained as a trade-off in energy used for reproduction and energy used for maintenance, repair and defence against cadmium, to survive. A major tenet in trade-off theory is the idea that there is a limited energy source for somatic maintenance, growth and reproduction, and that these characteristics trade-off against each other. Trade-offs between survival and reproduction in a number of vertebrate and invertebrate species have been measured, including in another model organism, *Drosophila* (Partridge and Farquhar, 1981). Stearns (1992) also documented twenty cases in arthropods and nematodes where virgins and individuals that mate less live longer, associating reproduction rate with survival probability. The results observed in this experiment suggest that at the lower temperature more energy was invested in reproduction than in repair and defence against cadmium, and at the higher temperature more energy was used in defending the organism and so less energy was available for egg production. In the case of the higher temperature, it is known that the higher metabolic rate due to increased temperature also increases the amount of reactive oxygen species (ROS) and thus a higher level of detoxification mechanisms is needed to deal with this. In this context, cadmium can be seen as an additional stressor which requires detoxification. Consistent with the idea of a link between metabolism and stress is a body of evidence that suggests that chronic exposure to the upper level of organism's normal level of heat tolerance (25°C for *C. elegans*) can

confer cross-tolerance to other stressors, such a metal exposure of hypoxia. Treinin et al. (2003) demonstrated that acclimating wildtype worms at 25°C prior to exposing them to increasing concentrations of cadmium increased cadmium tolerance, and that the cross-tolerance require the gene *hif-1*. The increased temperature may also have induced the expression of heat shock proteins (HSPs) which could confer cross-tolerance to cadmium. Cadmium alone is capable of inducing HSPs (Kammenga et al., 1998). The major HSPs function mainly in degradation and/or salvage pathways for dealing with damages proteins. It is thus consistent that HSP induction by heat can lead to greater protection of cells against the toxic effects of cadmium.

3.4.3 Strains

A comparative experiment was performed to gain baseline data on the key life history parameters; (lifespan, reproduction, and growth) of three wild isolate strains of *C. elegans*; (N2, Bergerac, and Hawaii), and to investigate their response to increasing concentrations of cadmium. Across populations of organisms there are a range of genotypes and genotype responses (phenotypic plasticity) which can offer the potential for adaptation of populations.

Under control conditions there was an obvious difference seen in all parameters tested between the Bergerac strain and the other two strains investigated. Bergerac was far less fit, with reduced lifespan and reproduction and lower growth rate. The *C. elegans* genome contains transposable elements, of which Tc1 is an example. Strains N2 and Hawaii have relatively low Tc1 copy number, about 30, which are dormant. The Bergerac strain however is undergoing active transposition of Tc1 (Emmons et al., 1983) and has a very high copy number of up to 500 of these jumping elements in

its genome. It has been suggested that it is probably the cumulative deleterious effect of these several hundred additional transposons in the genome that make strains with high copy number of Tc1 elements generally have low fitness (Hodgkin and Doniach, 1997). Bergerac is also phenotypically different to N2 and Hawaii strains, in that it is an uncoordinated worm, with very limited movement, and is unable to move with the sinusoidal movement characteristic of *C. elegans* indicating that the effects of the Tc1 element may extend beyond the observed effects on life-history.

Mean control lifespan of N2 and Hawaii were not significantly different from each other, however, the difference in slope parameter c of the survival curve indicates that lifespan was more variable in the Hawaii strain than in the N2 population. Mean control lifespan of the Bergerac population was significantly shorter than either N2 or Hawaii; however, the longest living worm in the experiment came from the Bergerac population, which is reflected in the spread of variance around the median lifespan. The mean lifespan for N2 at 15°C of 15 days in this experiment compares well with the mean lifespan of 14.5-15 days measured by Swain et al. (2004). The mean lifespan for Hawaii at 20°C obtained by Raices et al. (2005) was 18 days. Whilst this cannot be directly compared with the 15 day lifespan at 15°C measured in this experiment, it gives an indication of expected lifespan. Bergerac lifespan of 18 days at 20°C (Johnson and Wood, 1982) does not closely match the 12 days lifespan observed at 15°C in this chapter.

Brood size was not significantly different between Hawaii and N2 worms under control conditions. Bergerac had very few offspring. Under control conditions the brood size of N2 was similar to that reported by other authors, Hodgkin and Barnes

reported a brood size of 327 (1991), Byerly et al. reported a brood size of 275 (1976), and Alvarez et al. reported a brood size of 269 for nematodes kept at 15°C and moved daily (2005) which compared well with the mean brood size from this experiment (247 eggs). The small reduction in brood size may be because the worms in this experiment were transferred daily through their reproductive period which could affect their behaviour. Hawaii brood size obtained in this experiment (267) matched well with the 281 measured by Hodgkin and Barnes (1991). There was a large difference between the brood size of Bergerac worms as obtained from this experiment (24) and that found in the literature by Hodgkin and Barnes (1991) totalling 218. One possible explanation, although unlikely, of this is the difference in temperature that the measurements took place at, as the Hodgkin and Barnes experiment took place at 20°C. Alternatively it could be the result of culturing and different genotypes which may arise during the continuous culture of these nematodes in different laboratories.

A study on the divergence of *C. elegans* wild type isolates showed that 70% of single nucleotide polymorphisms (SNP's) found in the Hawaii strain were not found in 9 other tested strains, including N2, indicating that this strain has significantly diverged (Koch et al., 2000). Given that brood size under control conditions was not significantly different between N2 and Hawaii populations, that they come from geographically distinct regions, and that the strains have genetically diverged, this suggests that there is an optimal reproductive strategy, which both strains have arrived at in their respective localities.

Baird et al. (1990) investigated the differences different populations of *D. magna* have on toxicity tests, using cadmium amongst other chemicals. As *Daphnia* forms clonal populations, similar in this respect to *C. elegans*, the genotypic effects could cause significant differences in tolerance or sensitivity to chemicals. Baird et al. (1990) found that clones do not respond to chemicals in the same manner or even different chemicals in the same way. Two clones with an order of magnitude difference in acute response to cadmium were identified. On testing a further chemical 3, 4-dichloroaniline (DCA) they found that the clonal population that was more tolerant to cadmium was intolerant to DCA, and the opposite effect in the second clonal population. As *D. magna* is a recognised and used ecotoxicological test organism, these genotypic differences could make a large difference in ecological risk assessment.

In this experiment strain did have an impact on sensitivity to cadmium although the most sensitive strain for each measured parameter was inconsistent. EC_{50}-reproduction values indicated that the most sensitive strain to cadmium was Bergerac, with no reproductive output from 10 mg Cd L^{-1} cadmium and above, then Hawaii with N2 being the least sensitive, however all EC_{50}'s were within an order of magnitude of each other. There appeared to be more impact on lifespan in N2 worms, a variable response in Hawaii and little overall effect on lifespan of the Bergerac worms. The N2 strain was most sensitive in its growth response at the first larval stage, with significant effects of cadmium seen at 4 mg Cd L^{-1}, Bergerac at 7 mg Cd L^{-1} and Hawaii from 10 mg Cd L^{-1}. However, as adults all strains were significantly smaller at the 4 mg Cd L^{-1} exposure.

3.4.4 Summary

The results presented in this chapter demonstrate that it is not necessary to dose the *E. coli* OP50 food source for the nematodes, as well as the agar it grows on, with cadmium. There were no significant differences in reproductive output or lifespan of nematodes tested on two sets of cadmium doses agar plates, one set contained agar and OP50 which had cadmium added to both, the other set contained cadmium dosed agar and non-dosed OP50.

Temperature has a large influence on the timing of life-cycle events and in the response of life history parameters to cadmium exposure. At higher temperatures lifespan is decreased and the length of the developmental period was shortened. However, contrary to other published data there was no evidence for a reduction in the brood size of the N2 strain at 25°C.

Differences in life history were observed between three wild-type isolate strains of *C. elegans*. Strain Bergerac (originated in France) was quickly established as being less fit that strains N2 (Bristol) and Hawaii (Hawaii), due to its very low reproductive rate and slower developmental and growth rates. Strains Hawaii and N2, although originally from very distinct geographical regions, had similar life-history schedules. No significant differences were seen in their lifespan or reproductive output under control conditions. There were however, differences in the sensitivity of strains to cadmium exposure, Bergerac was the most sensitive strain, then Hawaii, with N2 being the least sensitive. However, the $EC_{50\text{-reproduction}}$ values for each strain were within 1 order of magnitude of each other.

Chapter 4

Life-history consequences of environmental chemicals on the nematode *Caenorhabditis elegans*

4.1 Introduction

An increase in environmental pollution has lead to a need to understand how pollutants challenge and change the environment. Ecotoxicology undertakes this in an attempt to understand the physiological and molecular effects of xenobiotics, and forms the basis of regulatory testing and identification of polluted areas.

In ecotoxicology single life-cycle endpoints are often used in acute or chronic exposures, with the primary aim of these toxicity tests to estimate critical effect levels (ECx, LCx) from concentration response relationships for single life-cycle variables such as mortality, growth and reproduction (Kammenga et al., 1997). There is however a need for full life cycle toxicity tests to discover the sublethal impacts of toxicants on an organism's life cycle. The most sensitive trait measured may not always have the most impact in terms of population fitness (Kammenga et al., 1996). Life cycle toxicity tests have added value in that the data collected can be used to predict toxicant consequences on population fitness. Population fitness is a better metric for making predictions about long-term impacts on populations and is useful in ecological risk assessment (Calow et al., 1997).

The effect of environmental pollutants on life cycle events has become an increasingly popular study area in recent years (Alvarez et al., 2005; Kammenga et al., 1994; Sibly and Calow, 1989; Spurgeon et al., 2005; Stark and Vargas, 2003; Widarto et al., 2004). Toxicants can have major impacts on populations by altering the life history traits of individuals. Life history patterns are relevant to ecotoxicological testing because population consequences of toxic action depend on the trade-offs between life-history components.

Theory behind natural selection leads to the assumption that in a favourable environment life history traits will be modulated so as to give the individual highest fitness. Environmental conditions such as temperature and food availability can alter the length and timings of life cycle events. Pollution can be viewed simply as another environmental variation and so when an organism enters a polluted environment, as many do throughout their entire life cycle, its normal functioning and life history may change. Understanding the physiological responses of individuals to stress is important (Maltby, 1999), firstly to provide insight into the development of stress tolerance and secondly for predicting population level effects. Toxic substances can have multiple toxicological effects, so looking at more than just one end point can give insight into the dominant ecotoxicological mode of action.

Many organisms experience a lifetime exposure to chemicals, which induces changes in development rate and timings of important life cycle events. There are trade-offs between life-cycle traits in organisms exposed to environmental stress, which determine ultimate response to stress. Data on life-cycle changes in individuals can be integrated to predict effects on population growth rate (Calow et al., 1997) and

energy usage (Kooijman and Metz, 1984) using mathematical models. Alterations to life cycle parameters may be viewed as a consequence of changes in energy allocation to parameters such as growth and reproduction. One model, DEBtox, bases itself on changes in energy allocations, but is also able to predict the mode of action of the toxicant (Kooijman and Bedaux, 1996a). These models rely on having good data available.

Toxic chemicals may be grouped into numerous chemical classes, each with its own general mode of action. Toxicants with differing modes of action may bring about different physiological responses in life history. Barata and Baird (2000) demonstrated three distinct toxicological responses to three chemicals from different classes using *Daphnia magna* in a short ecotoxicological test. Differences were observed in egg and adult survival and production rates. The most sensitive life history trait to different chemicals is not always the same. In ecotoxicology experiments using the nematode *Plectus acuminatus* the most sensitive trait to cadmium exposure was the reproductive period (Kammenga et al., 1996); however, upon exposure to pentachlorophenol the most sensitive trait was egg survival (Kammenga et al., 2001). This illustrates the difficulty of establishing generalised rules concerning the effects of toxicants on life history. Clearly, data regarding the effect of diverse chemicals are needed to understand the variety.

In the study presented in this chapter, three chemicals (cadmium, fluoranthene and atrazine) were selected for their differing eco-toxic modes of action. This was done to assess the variations of life cycle responses to 'typical' members of three major groups of chemicals, a metal (cadmium), an inorganic chemical with a putative non-

specific mode of action (fluoranthene), and an organic chemical with a putative specific mode of action (atrazine). A short summary of current knowledge concerning the toxicity of these compounds is presented below.

Cadmium, a non-essential heavy metal, is highly toxic. Previous experiments have shown that cadmium affects all life history parameters studied. It has also been shown that nematodes under cadmium exposure reduce feeding, alter locomotion and produce a genetic stress response. *C. elegans* has a suite of genes which are induced in response to cadmium exposure; one of these detoxification pathways is the metallothionein pathway where MTL-1 and MTL-2 bind cadmium. Swain et al. (2004) have show that mtls are induced at 2.5 μM cadmium in a 24 hour exposure, and at 20 μM induction is within 1 hour. *mtl-1* is constitutively expressed in the second pharyngeal bulb but induced in the pharynx and intestine, *mtl-2* when induced is expressed in the intestine. Cadmium causes cellular lesions such as disrupted cytosomes and shortened microvilli (Popham and Webster, 1979).

Fluoranthene, a polycyclic aromatic hydrocarbon (PAH) is formed as a result of incomplete combustion of fossil fuels. Based on the structure of PAHs (no functional groups), its toxic mode of action is likely to be an interference with membrane fluidity, a phenomenon termed nonpolar narcosis. Fluoranthene is the most ubiquitous and abundant of the pyrogenic PAH pollutants. It has been identified in cigarette smoke, char-broiled foods, drinking water, lake sediments and ambient air. It is thought to interact with DNA, cause heritable genetic disorders and has been associated with lung, intestinal and pharyngeal cancer. PAHs are inert; the narcotic toxicity effects are thought to arise from accumulation within biological membranes.

This affects the membrane structure and processes like osmoregulation and neurotransmission. Microarray studies using *C. elegans* exposed to fluoranthene have shown a number of known stress responsive genes to be upregulated, including the cytochrome P450, CYP35 and GSTs (Menzel et al., 2005; Reichert and Menzel, 2005). Cytochrome P450 enzymes have been implicated in the bioactivation or detoxification of many hydrophobic drugs and xenobiotics.

Atrazine (2-chloro, 4-ethylamino, 6-isopropylamino-s-triazine) is a systemic herbicide used for pre and post- emergence control of annual and broad-leaved weeds and perennial grasses. Its mode of action on its target organisms is to inhibit photosynthesis and it also interferes with other enzymic processes. It has been shown to be of low toxicity to bees (contact $LD_{50} > 100$ µg / bee), and to birds (no mortality seen at 10,000 mg / kg diet in adult Peking ducks). It is however moderately toxic to aquatic organisms, with a *Daphnia* 48hr EC_{50} of 6.9 mg L^{-1} (Tomlin, 1994). A microarray study using *C. elegans* exposed to atrazine has shown a number of genes to be over-expressed, including the cytochrome P450 enzymes, GSTs, UDP-glucoronosyltransferases, collagens and c-type lectins (Reichert and Menzel, 2005).

This study aimed to determine the sub-lethal effects of three common pollutants, from differing chemical classes, and to examine whether different chemical classes elicit a specific response in individuals. This aim was met using the free-living nematode *C. elegans*, a fully sequenced model organism with a short developmental period and lifespan. The demographic data presented here were obtained from large experiments which simultaneously produced samples for microarray analysis at 3 different life stages of the nematode. For this reason, GE31 was selected as a test strain, to be able

to harvest single stage nematode samples. GE31 is wild-type at 15°C, but when grown at 25°C is embryonic lethal temperature sensitive. Eggs are laid, but these do not hatch. Life history parameters measured were time to maturity, defined as the time at which the nematode first reproduces reproductive rate (per 12 hours), total reproduction, reproductive period, survival and growth.

4.2 Approach

Nematodes were cultured as described in section 2.2.4.

This experiments carried out in this chapter were performed on strain GE31, a temperature sensitive mutant. Below 25°C the nematodes are wild-type, above 25°C GE31 is an embryonic lethal strain. The adults lay eggs, but the eggs do not hatch.

4.2.1 Chemicals

All chemicals were of the highest analytical grade available. Cadmium chloride (Sigma, UK), Fluoranthene (Fisher, UK) and atrazine (Sigma, UK) were used to make the stock solutions. Cadmium chloride was made in sterile bidistilled water, fluoranthene in ethanol, atrazine in DMSO. Final % of solvent in the agar for ethanol was 0.04% and for DMSO 0.25%. Equal amounts of solvent were added to control agar. The solutions were filter sterilized through a 0.2μM filter prior to use to ensure sterility. The agar was cooled to 55°C and then the chemicals were added at the concentrations required. Chemicals were also added to OP50 two hours prior to the bacteria being spread on the agar at the equivalent concentration, and solvents were added to control OP50 also.

4.2.2 Life-cycle toxicity tests

Full life-cycle toxicity tests were performed as described in section 2.3.2. Worms used in the tests were age-synchronised by large-scale egg prep, as described in section 2.2.5 ii) at 15°C. L1 larvae were put onto dosed agar plates and the temperature switched to 25°C.

Endpoints measured were time to first reproduction, total reproduction, brood period, lifespan, growth over time. Image analysis was performed as in 2.3.3 to obtain a growth curve.

4.2.3 Statistics

EC50 values for reproduction were calculated using the general form of the logistic model (Van Ewijk and Hoekstra, 1993) as below.

$$N = \frac{c}{\left(1+\left(dose/EC50\right)^{b}\right)}$$

Where c is the maximal response (of controls), b is a slope parameter and EC_{50} is the concentration at which there is a 50% reduction in reproduction compared to that of controls.

Survival was analysed using the Weibull survival model (Weibull, 1951), as has been previously used to describe the survival curve of the nematodes *Plectus acuminatus* (Kammenga *et al.*, 1996) and *C. elegans* (Vanfleteren *et al.*, 1998). Percentage survival values at each time interval were subjected to least squared fitting of a Weibull curve according to:

$$L_t = \exp\left[-\ln 2 \cdot \left(\frac{t_a}{LT50}\right)^{c}\right]$$

Where L_t is the proportion of the population alive at time t, c is the slope of the Weibull curve, and LT50 is the estimated time for 50% mortality of the population.

Growth was modelled over time using a logistic adapted equation as below:

$$V_t = \frac{(Max_s - Min_s)}{1 + \exp^{-b(\ln t - \ln LT50)}} + Min_s$$

Where V_t is the size at time t, Max_s is the maximum adult size, Min_s is the minimum size, LT_{50} is the time at which size is 50% that of Max_s (point of inflection of the curve) and b is a shape parameter. This logistic model has previously been used to describe body growth in other species (Hawthorne *et al.*, 2004).

T-tests were used to test whether there were significant differences between the means of two sample groups. To compare survival and dose response curves between populations and treatments, F-tests were performed. Two-way ANOVA was used to explore interactions between strain and cadmium dose, and temperature and cadmium dose, using GLM in Minitab (Version 14).

Censored individuals were either lost during the experiments as a result of the individuals crawling up the side of the well and becoming desiccated or lost during transfer, these were not included in lifespan analysis, or reproduction analysis if the nematode was lost prior to cessation of the reproductive period. Nematodes which exhibited 'bagging', where eggs hatch and the larvae grow inside the parent killing it, were only included in the assessment of time to maturity.

4.3 Results

4.3.1 Difference between GE31 and N2 wildtype

Prior to strain GE31 being used, its life history characteristics were assessed to compare it against the wild type strain N2. Life-history parameters measured were brood size, brood period, time to first reproduction, final body size and lifespan. Table 4.1 gives the mean values for each parameter and t-test summary statistics. The only significantly different life history parameter measured was the time to reproduction ($p < 0.001$), with GE31 having a slightly longer time to maturity than N2 at 25°C. There was a slightly lower brood size in GE31 and increased variability which may limit the ability to detect a change so NOECs could be higher for GE31.

4.3.2 Assay reproducibility

To confirm the repeatability of the assay, lifespan and brood size of the control nematodes from each of the three life cycle tests were compared. Lifespan was not significantly different between the three controls (ANOVA: $F = 0.89$, $df = 2, 131$, $p = 0.412$). Brood size was also not significantly different between the control groups (ANOVA: $F = 0.07$, $df = 2, 101$, $p = 0.937$).

Table 4.1: Mean values for life–history parameters measured under control conditions for both strains N2 and GE-31. 2-way t-tests were performed to examine whether the means between the strains were significantly different. Values shown are means (± standard deviations).

Life-history parameter	N2	GE-31	T	df	p
Time to first reproduction, hours	49 (± 0.2)	56 (± 6.5)	7.64	41	<0.001
Brood size, n	238 (± 44)	201 (± 106)	1.70	38	0.098
Brood period, days	3.2 (± 0.6)	3.0 (± 0.9)	1.13	51	0.263
Volumetric length at 147hrs, μm^3	122 (± 21)	123 (± 16)	0.96	18	0.352
Lifespan, days	8.4 (± 2.1)	9.0 (± 3.1)	1.01	74	0.314

4.3.3 Life-history effects of Cadmium

4.3.3.1 Lifespan and survival

Mean lifespan for control nematodes was 9.0 days. Lifespan (Figure 4.1) was increased compared to controls at all doses up to and including 8 mg L^{-1}, but decreased to approximately 80% that of controls in the highest dose tested, 16 mg L^{-1}. Lifespan was significantly increased at 3 mg L^{-1} compared to controls, but all other doses were not significantly different to controls (ANOVA: $F = 13.01$, $df = 12, 504$, $p < 0.001$, Tukey, multiple comparison test $p < 0.05$).

Survival was modeled using the Weibull survival curve alongside the actual data (Figure 4.2). The Weibull model gives an estimated LT_{50}, which is the time at which there is 50% population mortality, and a slope parameter *c*, these values are given in Table 4.2 along with the coefficient of determination (r^2) for each curve. F-tests were performed to assess whether differences in the survival curves compared to the control survival curve. All curves were significantly different from the control survival curve ($p < 0.001$). The largest difference from the control curve was the survival curve of treatment group 3 mg L^{-1}, which exhibited a higher proportion of nematodes surviving for a longer period of time followed by a shorter period of time where the majority of nematodes died. Doses 10 – 16 mg L^{-1} all had curves lower than the control curve. The survival curve of 12 mg L^{-1} had a similar slope to controls; however, the 10, 14 and 16 mg L^{-1} curves had much steeper slopes as the variance in lifespan of the nematodes within these treatments was lower.

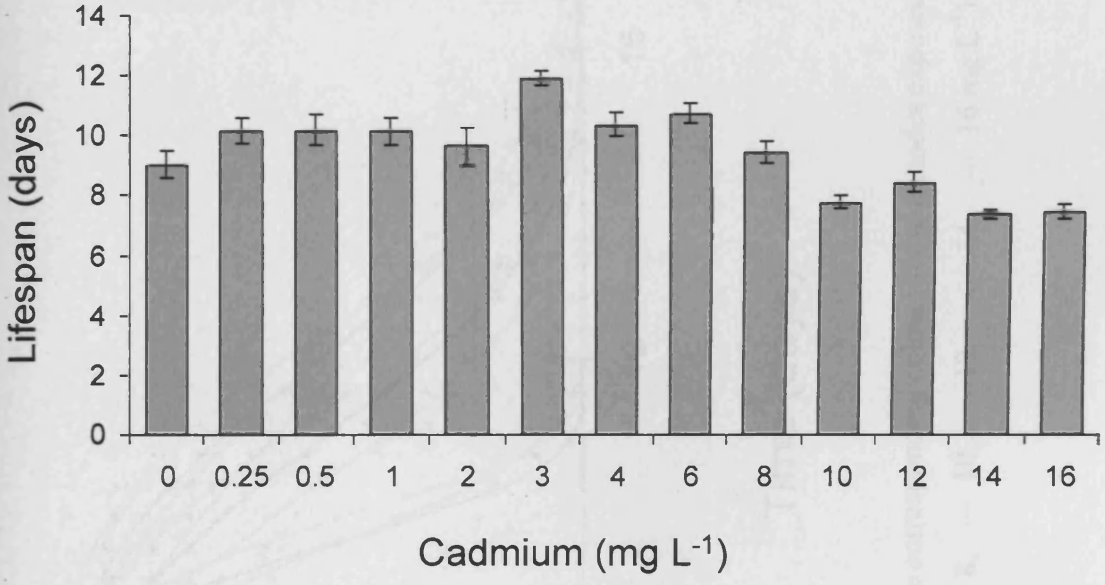


Figure 4.1: Mean lifespan of *C. elegans* strain GE31 at increasing concentrations of cadmium. Error bars represent standard error of the mean. Values are mean of 46 replicates excluding any individuals that were censored (range 40-46). No significant differences compared to the control were found for any treatment.

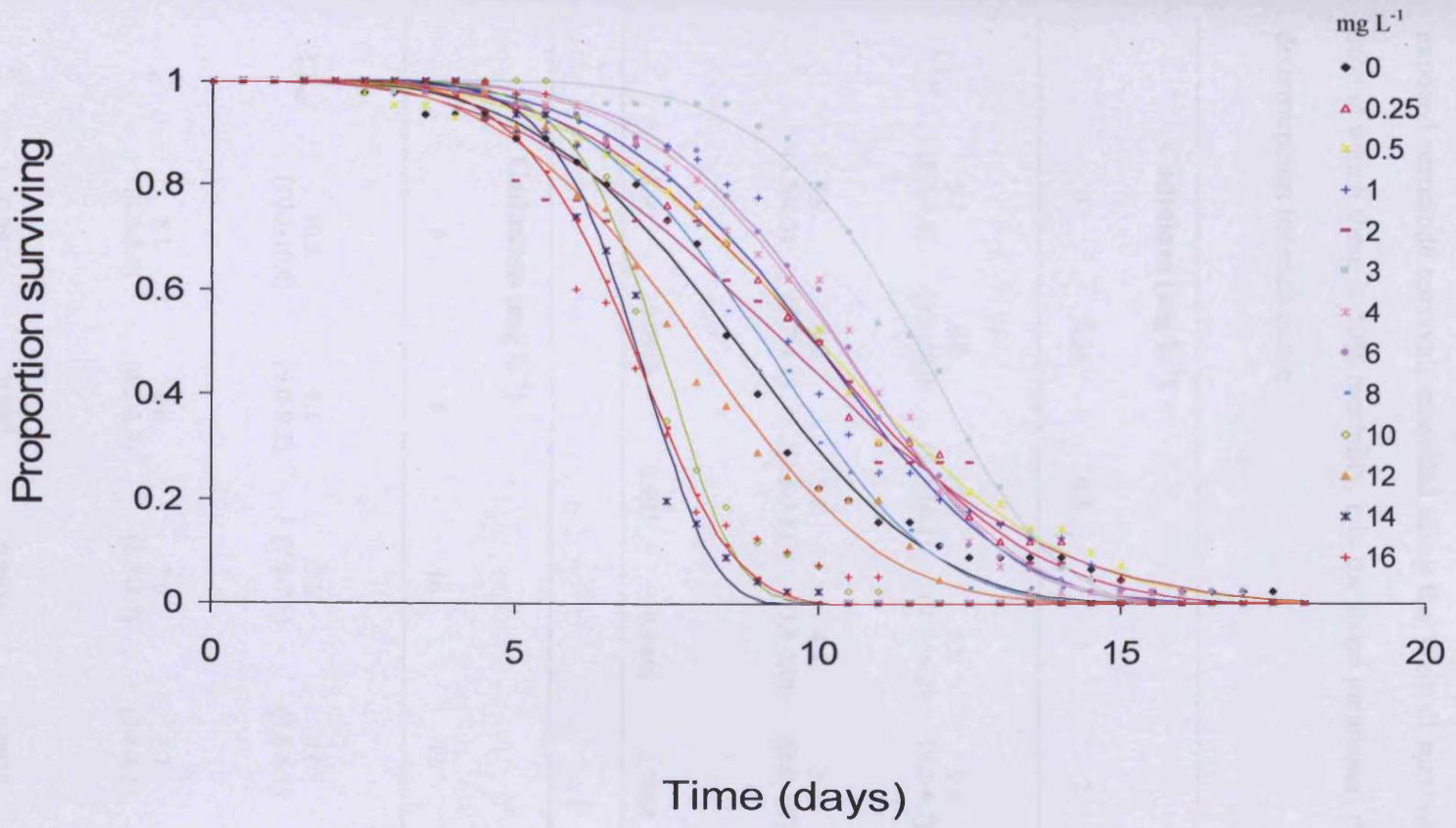


Figure 4.2: Survival curves (unbroken lines) and data points (in the corresponding colour) for nematodes exposed to cadmium, (— 0, — 0.25, — 0.5, — 1, — 2, — 3, — 4, — 6, — 8, — 10, — 12, — 14, — 16 mg L^{-1}).

Table 4.2: Parameter values and 95% confidence intervals of control and cadmium exposed nematode survival, modelled using the Weibull survival model. LT_{50} is the time at which there is 50% mortality, c is the slope parameter, r^2 is the coefficient of determination for each curve.

	Cadmium (mg L^{-1})						
	0	0.25	0.5	1	2	3	4
LT_{50}	8.7 (8.5-8.8)	9.9 (9.8-10.0)	10.0 (9.9-10.2)	9.9 (9.7-10.2)	9.4 (9.2-9.7)	11.6 (11.5-11.7)	10.3 (10.2-10.4)
c	3.9 (3.5-4.3)	3.9 (3.7-4.1)	2.6 (3.3-3.8)	4.4 (3.8-5.0)	3.1 (2.8-3.4)	7.0 (6.5-7.5)	4.9 (4.5-5.2)
r^2	0.99	0.9968	0.995	0.9846	0.9864	0.9966	0.9956

	Cadmium (mg L^{-1})					
	6	8	10	12	14	16
LT_{50}	10.3 (10.3-10.4)	9.1 (9.0-9.2)	7.3 (7.2-7.5)	8.0 (7.8-8.1)	7.0 (6.9-7.1)	6.9 (6.8-7.1)
c	5.1 (4.8-5.4)	4.6 (4.3-4.9)	7.6 (6.5-8.7)	3.7 (3.4-4.1)	7.5 (6.6-8.4)	5.6 (4.7-6.5)
r^2	0.9974	0.997	0.9934	0.9937	0.9957	0.989

4.3.3.2 Time to first reproduction

Cadmium had a significant impact on the time taken for the first egg to be laid (ANOVA: $F = 121.15$, $df = 12, 529$, $p < 0.001$) (Figure 4.3). Doses 1 mg L^{-1} and 4 mg L^{-1} and above had significantly longer developmental times compared to controls (Tukey multiple comparison test $p < 0.05$). The NOEC concentration was found to be 0.5 mg L^{-1}. Time to reproduction at 16 mg L^{-1} was 80% longer than that of controls.

4.3.3.3 Brood size

Cadmium had a large significant impact on the reproductive output of *C. elegans* (Figure 4.4). Significant decreases in reproduction were found in all doses except for 0.25 mg L^{-1} and 1 mg L^{-1}. As cadmium concentration increased the mean brood size was reduced, showing a clear dose response pattern. Total reproduction in the highest dose tested, 16 mg L^{-1} was reduced by 93% compared to control reproduction. The dose response was modelled using a logistic dose response equation to give an estimated $EC_{50\text{-reproduction}}$ value and a slope parameter *b*. EC_{50} (95% confidence limits) was 3.6 (2.8-4.4) mg L^{-1}.

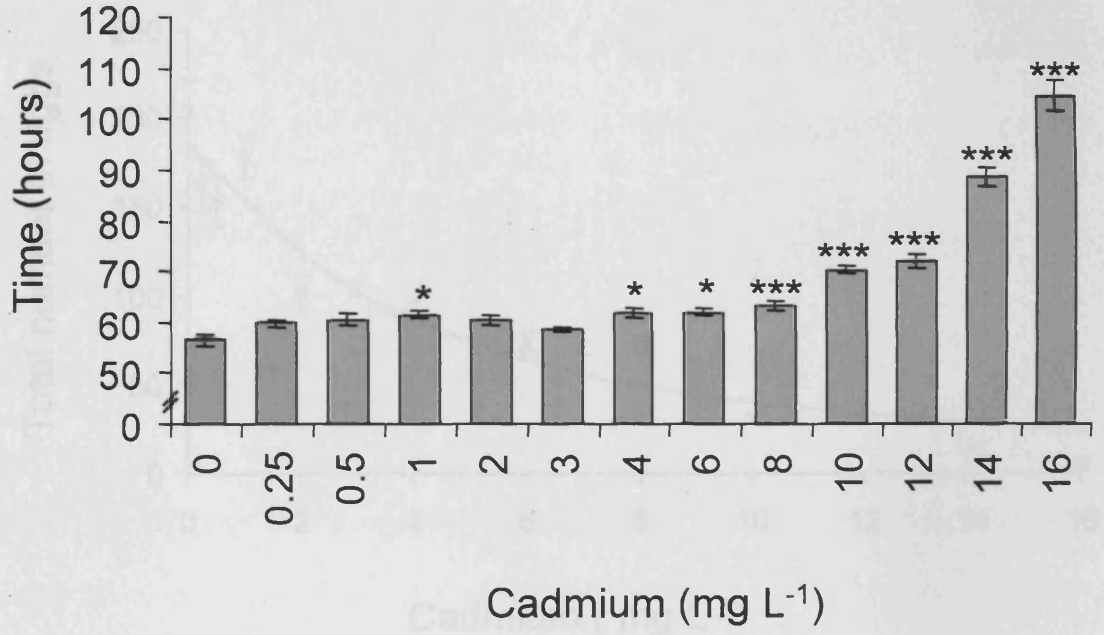


Figure 4.3: Mean time to first reproduction of control and cadmium exposed *C. elegans* strain GE31. Error bars represent standard error of the mean. Values are the mean of 48 replicates excluding any individuals that were censored (range 18-48). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

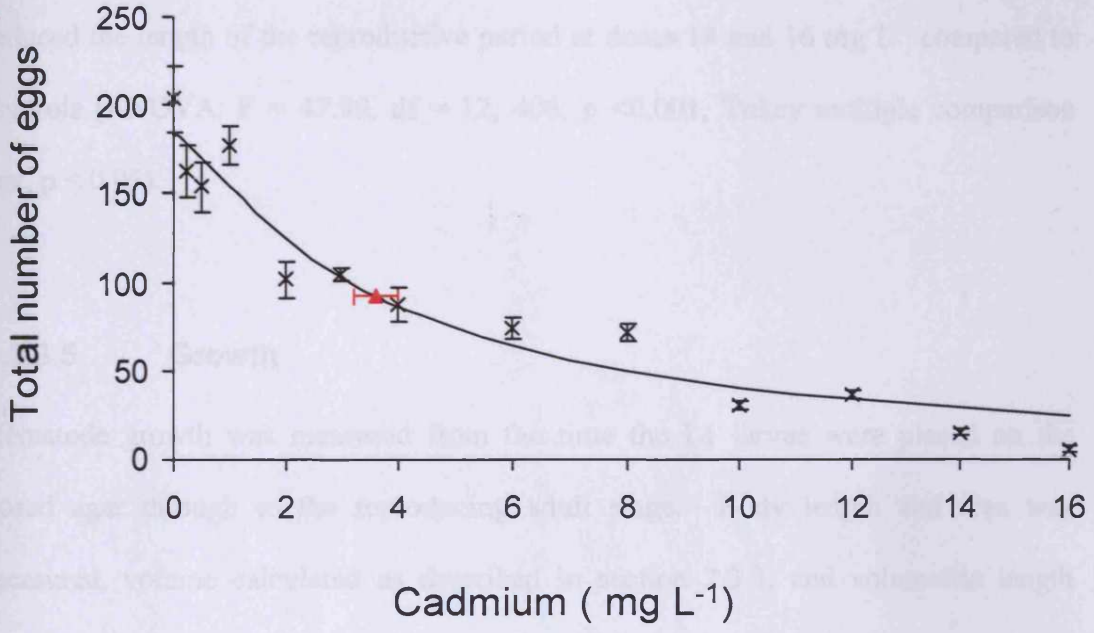


Figure 4.4: Mean total reproduction measured as total number of eggs laid, of *C. elegans* strain GE31 under control and cadmium exposure. Error bars represent the standard error of the mean. Individual data points represent the mean values taken from 41 replicates (excluding individuals that were censored range 18-41). The unbroken line is the modelled logistic dose response. The $EC_{50\text{-reproduction}}$ is shown in red.

4.3.3.4 Brood period

Mean brood period length increased at the lower doses of cadmium and decreased at the higher end of the dose range (Figure 4.5). Cadmium significantly increased the length of the reproductive period at doses 2, 3, 4 and 6 mg L^{-1}, and significantly reduced the length of the reproductive period at doses 14 and 16 mg L^{-1} compared to controls (ANOVA: $F = 47.99$, $df = 12, 406$, $p < 0.001$, Tukey multiple comparison test, $p < 0.05$).

4.3.3.5 Growth

Nematode growth was measured from the time the L1 larvae were placed on the dosed agar through to the reproducing adult stage. Body length and area was measured, volume calculated as described in section 2.3.3, and volumetric length (Figure 4.6), is the cube root of the volume.

Growth was modelled using the logistic adapted equation described in Section 4.2.3 and shown on Figure 4.6 alongside the mean volumetric length at each time point for each treatment. This clearly shows the impact that cadmium has on growth rate and final size for the nematodes. Table 4.3 contains the parameter data for each growth curve. The growth model estimates four parameters, maximum size, minimum size, LT50, the time taken for the nematodes to reach 50% of maximum size and a slope parameter, *c*. This model gives an excellent fit to the data, as shown by the r^2 value for all curves being above 0.95.

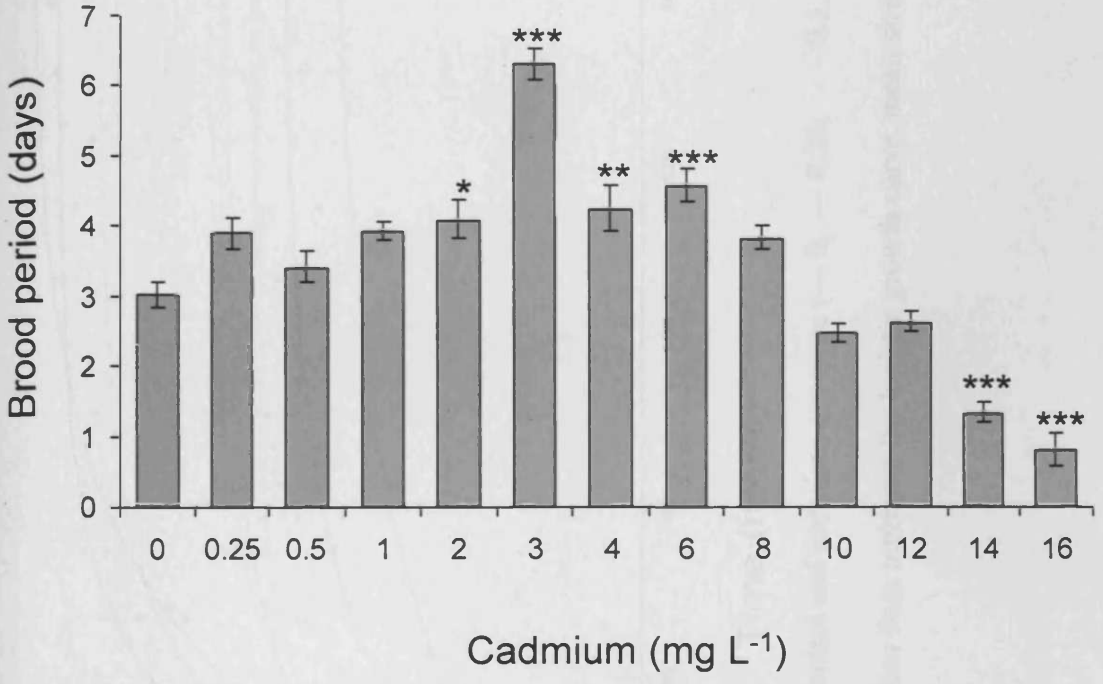


Figure 4.5: Mean reproductive period of control and cadmium exposed *C. elegans* strain GE31. Error bars represent the standard error of the mean. Values are the mean of 41 replicates, excluding any individuals that were censored (range 18-41). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

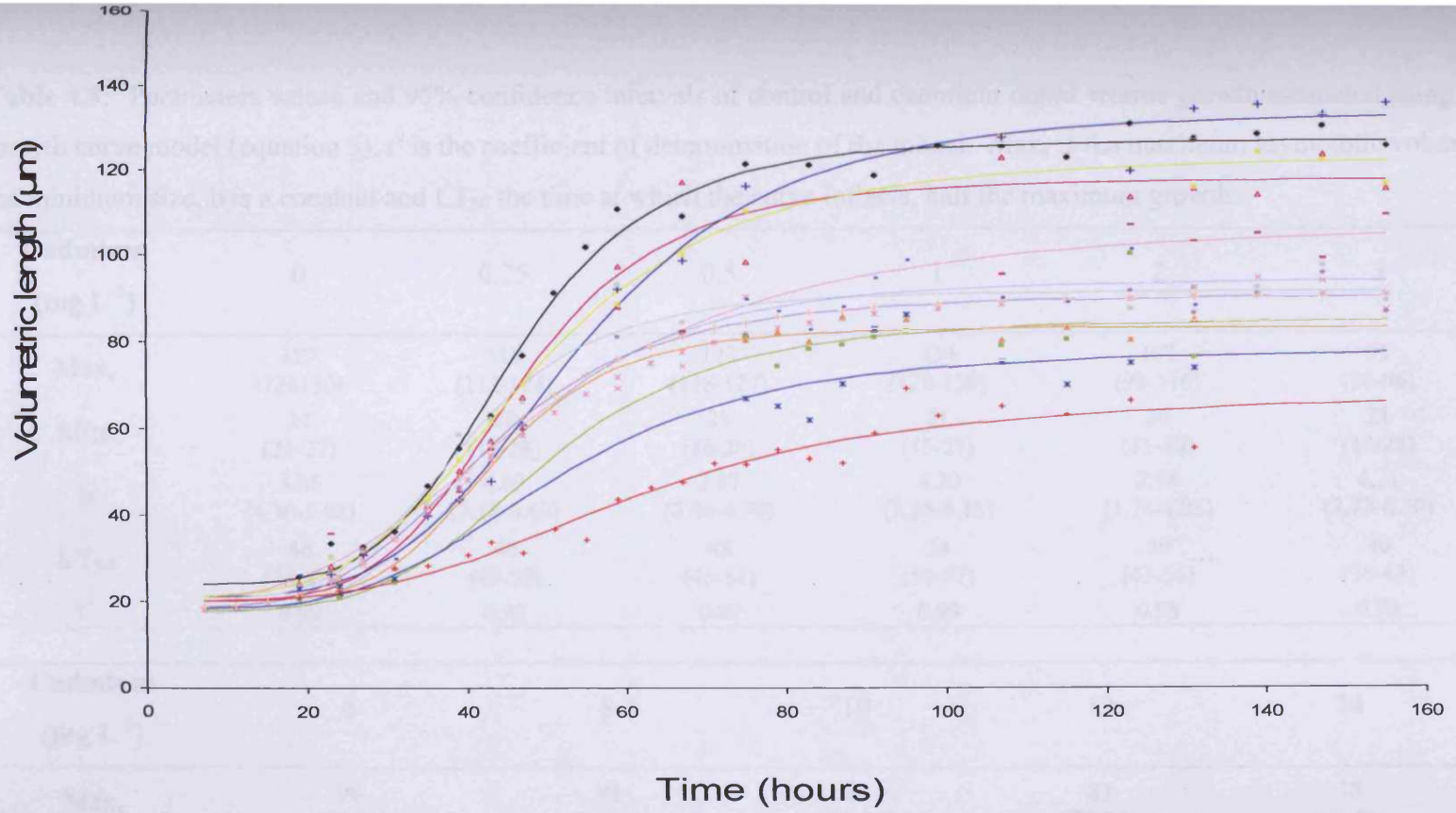


Figure 4.6: Volumetric growth of nematodes under control and cadmium exposure (— 0, — 0.25, — 0.5, — 1, — 2, — 3, — 4, — 6, — 8, — 10, — 12, — 14, — 16 mg L^{-1}). Unbroken lines represent the logistic growth curve, mean size at each time point is shown in the corresponding colour for each treatment.

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Table 4.3: Parameters values and 95% confidence intervals of control and cadmium dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, min_s is the minimum size, b is a constant and LT_{50} the time at which the curve inflects, half the maximum growth.

Cadmium (mg L^{-1})	0	0.25	0.5	1	2	3	4
Max_s	127 (124130)	118 (112-124)	123 (118-127)	134 (128-139)	107 (98-116)	91 (86-96)	91 (85-97)
Min_s	24 (21-27)	20 (12-28)	21 (16-26)	21 (15-27)	20 (11-30)	21 (13-28)	18 (12-26)
b	5.06 (4.30-5.82)	4.93 (3.16-6.69)	3.87 (3.04-4.70)	4.20 (3.25-5.15)	2.98 (1.74-4.23)	4.71 (2.72-6.70)	3.48 (2.13-4.84)
LT_{50}	46 (44-47)	46 (43-50)	48 (45-51)	54 (51-57)	50 (43-56)	40 (36-43)	44 (40-49)
r^2	0.99	0.98	0.99	0.99	0.98	0.98	0.98

Cadmium (mg L^{-1})	6	8	10	12	14	16
Max_s	90 (87-94)	94 (86-102)	85 (76-94)	83 (80-86)	78 (66-89)	67 (59076)
Min_s	19 (13-24)	17 (11-23)	18 (11-25)	20 (17-23)	18 (11-26)	20 (16-24)
B	4.03 (2.91-5.16)	4.22 (2.09-6.34)	4.43 (1.38-7.47)	5.56 (3.18-7.93)	3.82 (0.86-6.78)	3.43 (1.97-4.89)
LT_{50}	42 (39-45)	45 (34-56)	50 (34-65)	43 (36-50)	51 (33-69)	50 (52-69)
r^2	0.99	0.98	0.97	0.99	0.96	0.95

Maximum size, Max_s, was compared to controls at each dose and within doses using t-tests. All doses except for 0.5 mg L^{-1} had a significantly different Max_s compared to control ($p < 0.05$). There was a stepped dose response to cadmium for Max_s which is illustrated in Figure 4.7. Treatment groups which were not statistically different to each other have been indicated with a letter above them. The maximum size of nematodes at 16 mg L^{-1}, was approximately 50% that of control nematodes.

4.3.3.6 Size at first reproduction

Size at first reproduction was estimated from the growth curve models (Figure 4.8). It was not possible to photograph and measure each worm as it began reproducing, so the size was estimated in the following way. The time to reproduction of each individual was used to obtain a value for body size using the growth curve parameters which had been estimated in Section 4.3.2.5. Cadmium significantly reduced mean size at first reproduction (ANOVA: $F = 188.85$, $df = 12, 529$, $p < 0.001$). All doses were significantly smaller than control size at first reproduction (Tukey, $p < 0.05$). The mean size at first reproduction followed a dose dependent response, with size decreasing as cadmium concentration increased. A 40% reduction in size at first reproduction was observed between controls and dose 16 mg Cd L^{-1}.

Cadmium had detrimental effects on every life-cycle parameter measured. Lifespan, whilst not statistically significantly different at the highest concentration examined, was nevertheless 20% shorter than controls, there was also evidence of a hormetic effect of cadmium in that low doses extended the lifespan. Cadmium slowed growth rate and reduced the size of adult nematodes with effects at very low doses. Developmental time was increased in response to cadmium, with very large effects on

time to maturity at the highest concentration. Reproductive output was much reduced by cadmium exposure, and followed a clear dose dependent reduction; the length of the reproductive period was initially extended at low dose and reduced at higher doses.

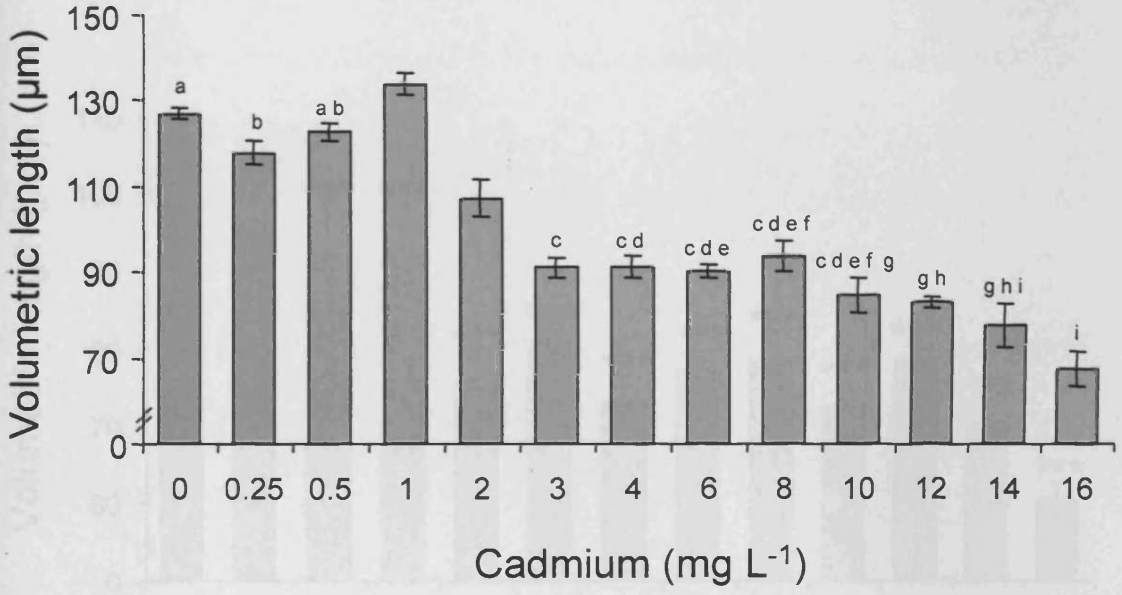


Figure 4.7: Maximum body size (estimated from the logistic growth model) in control and cadmium exposed nematodes. Error bars represent standard error of the mean. Where columns share the same lower case letter no statistically significant difference was found between them (t-test $p > 0.05$).

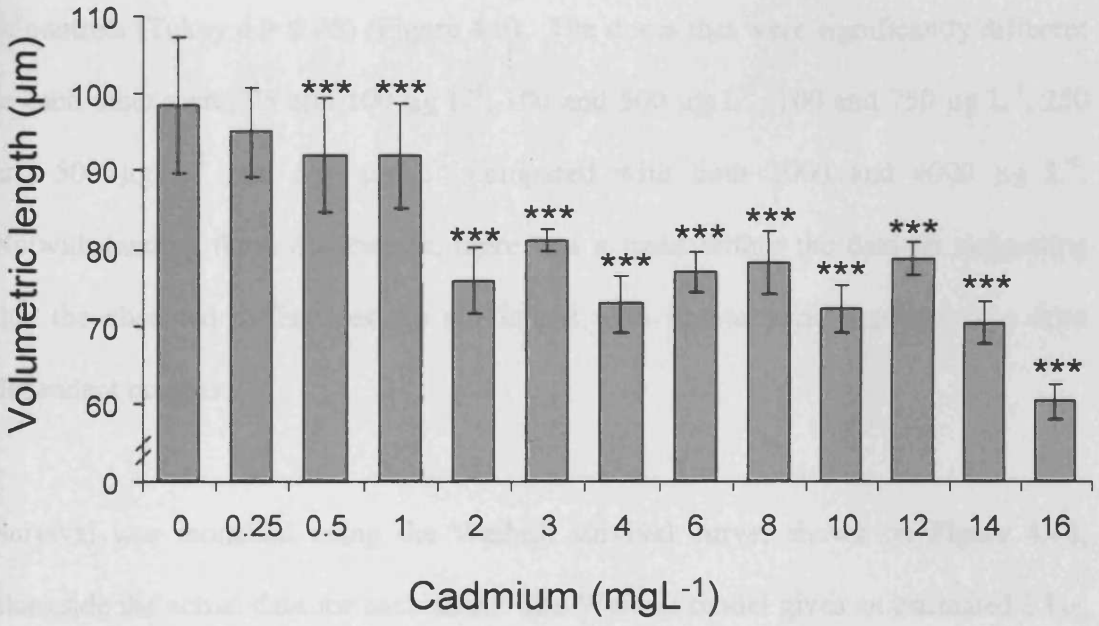


Figure 4.8: Mean estimated size at first reproduction. For control and cadmium exposed *C. elegans* strain GE31. Error bars represent the standard deviations. Values are means of 48 replicates, excluding censored individuals (range 18-48). *** $p < 0.001$ compared to control.

4.3.4 Life-history effects of Fluoranthene

4.3.4.1 Survival and longevity

Mean lifespan for control nematodes was 9.7 days. There was a significant impact of fluoranthene on lifespan (ANOVA: $F = 3.65$, $df = 12, 589$, $p < 0.001$) between specific doses outlined below (Tukey multiple comparison test, $p < 0.05$). However there were no significant differences in lifespan of dosed nematodes compared to that of controls (Tukey $p > 0.05$) (Figure 4.9). The doses that were significantly different to each other were; 75 and 100 $\mu g\ L^{-1}$, 100 and 500 $\mu g\ L^{-1}$, 100 and 750 $\mu g\ L^{-1}$, 250 and 500 $\mu g\ L^{-1}$ and 500 $\mu g\ L^{-1}$ compared with both 2000 and 4000 $\mu g\ L^{-1}$. Notwithstanding these differences, there was a trend within the data-set suggesting that the observed differences are not linked with fluoranthene exposure in a dose dependent manner.

Survival was modelled using the Weibull survival curve, shown on Figure 4.10, alongside the actual data for each dose. The Weibull model gives an estimated LT_{50}, which is the time at which there is 50% population mortality, and a slope parameter c, given in Table 4.4 alongside the r^2 value for each curve. Overall there was a good fit of the data to the Weibull model. F-tests were performed to assess whether differences in the survival curves compared to the control survival curve. All curves were significantly different to the control survival curve ($p < 0.001$). There did not appear to be a dose dependent pattern in the survival curves.

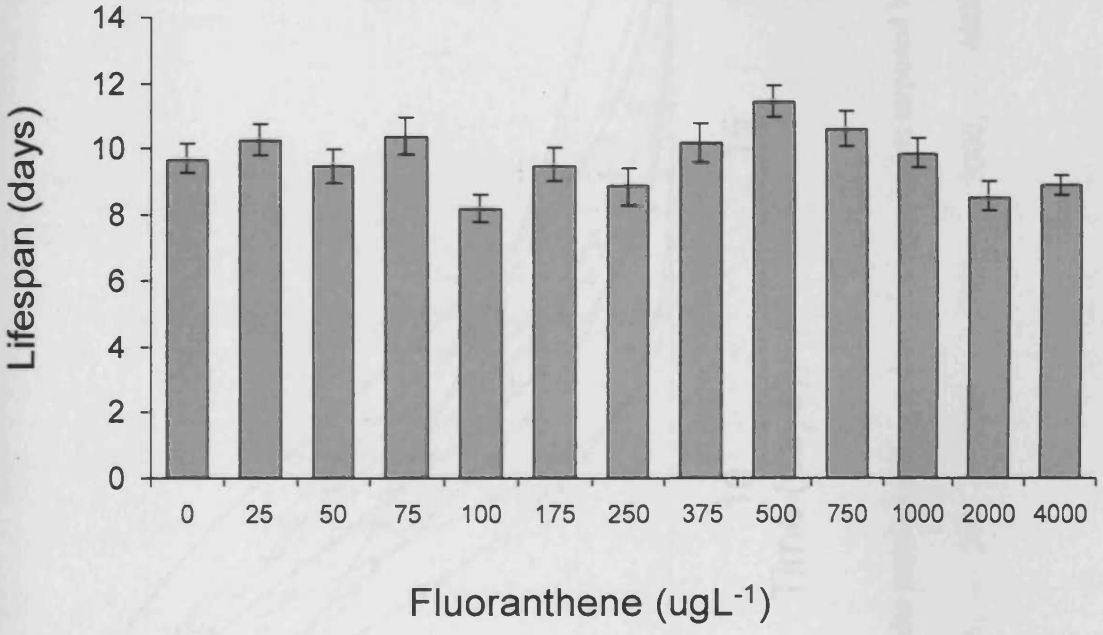


Figure 4.9: Mean lifespan of *C. elegans* strain GE31 at increasing concentrations of fluoranthene. Error bars represent standard error of the mean. Values are means of between 42-48 replicates. No significant differences compared to the control were found for any treatment.

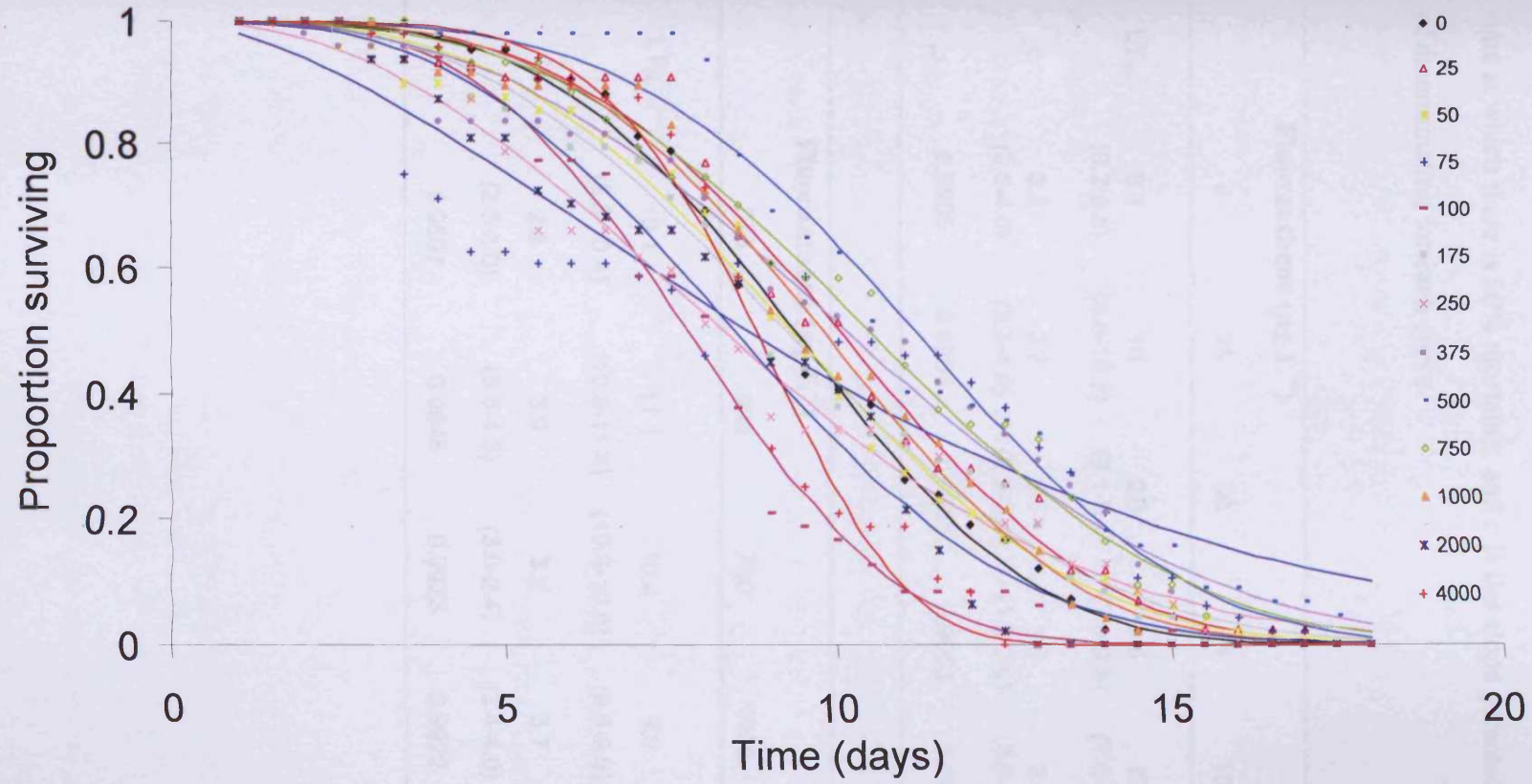


Figure 4.10: Survival curves (unbroken lines) and data points (in the corresponding colour) for nematodes exposed to fluoranthene (— 0, — 25, — 50, — 75, — 100, — 175, — 250, — 375, — 500, — 750, — 1000, — 2000, — 4000 μg L^{-1}).

Table 4.4 Parameter values and 95% confidence intervals of control and fluoranthene exposed nematode survival, modelled using the Weibull survival model. LT_{50} is the time at which there is 50% mortality, and c is the slope parameter, r^2 is the coefficient of determination for each curve.

	Fluoranthene (µg L^{-1})						
	0	25	50	75	100	175	250
LT_{50}	9.3 (9.2-9.5)	10 (9.8-10.2)	9.3 (9.1-9.4)	8.6 (7.9-9.3)	7.7 (7.6-7.9)	9.2 (9.0-9.4)	8.3 (8.0-8.5)
c	3.8 (3.5-4.0)	3.7 (3.3-4.0)	3.1 (2.9-3.4)	1.6 (1.3-2.0)	3.6 (3.2-3.9)	3 (2.7-3.1)	2.2 (2.0-2.4)
r^2	0.9955	0.9904	0.9935	0.9053	0.9926	0.9934	0.988

	Fluoranthene (µg L^{-1})					
	375	500	750	1000	2000	4000
LT_{50}	10.1 (9.8-10.4)	11.1 (10.9-11.4)	10.4 (10.3-10.6)	9.8 (9.6-9.9)	8.2 (8.2-8.7)	8.8 (8.6-8.9)
c	2.8 (2.5-3.0)	3.9 (3.5-4.3)	3.2 (3.0-2.4)	3.7 (3.4-4.0)	3 (2.6-3.3)	5.5 (4.8-6.2)
r^2	0.9807	0.9848	0.9923	0.9922	0.9811	0.9904

4.3.4.2 Time to first reproduction

Fluoranthene significantly increased the amount of time taken for nematodes to begin reproducing (ANOVA: $F = 94.88$, $df = 12, 438$, $p<0.001$) (Figure 4.11). Tukey multiple comparison tests showed that doses 500 μg L^{-1} and above significantly increased the time taken to reproduce ($p < 0.05$). Time to reproduction was increased by a third compared to controls at the highest dose tested 4 mg L^{-1}.

4.3.4.3 Brood size

Fluoranthene significantly reduced brood size compared to controls in all doses except for 50 and 75 μg L^{-1}. (ANOVA: $F = 7.33$, $df = 12, 505$, $p < 0.001$, Tukey multiple comparison test, $p < 0.05$) (Figure 4.12). $EC_{50\text{-reproduction}}$ value could not be calculated using the logistic model because a 50% reduction in reproduction was not achieved in the dose range tested. An initial drop in reproductive output, of approximately 24%, was seen at the lowest dose 25 μg L^{-1}. This reduction in reproduction was maintained throughout the dosed nematodes and at the highest dose tested, 4 mg L^{-1}, there was a 42% reduction in reproduction compared to the controls.

4.3.4.4 Reproductive period

Fluoranthene significantly altered the time taken to reproduce (ANOVA: $F = 2.23$, $df = 12, 504$, $p < 0.05$) (Figure 4.13). The only significant difference in the length of the reproductive period was between doses 100 and 750 μg L^{-1} (Tukey $p <0.05$). There were no significant differences in the reproductive period in any dose compared to controls (Tukey multiple comparison test, $p > 0.05$).

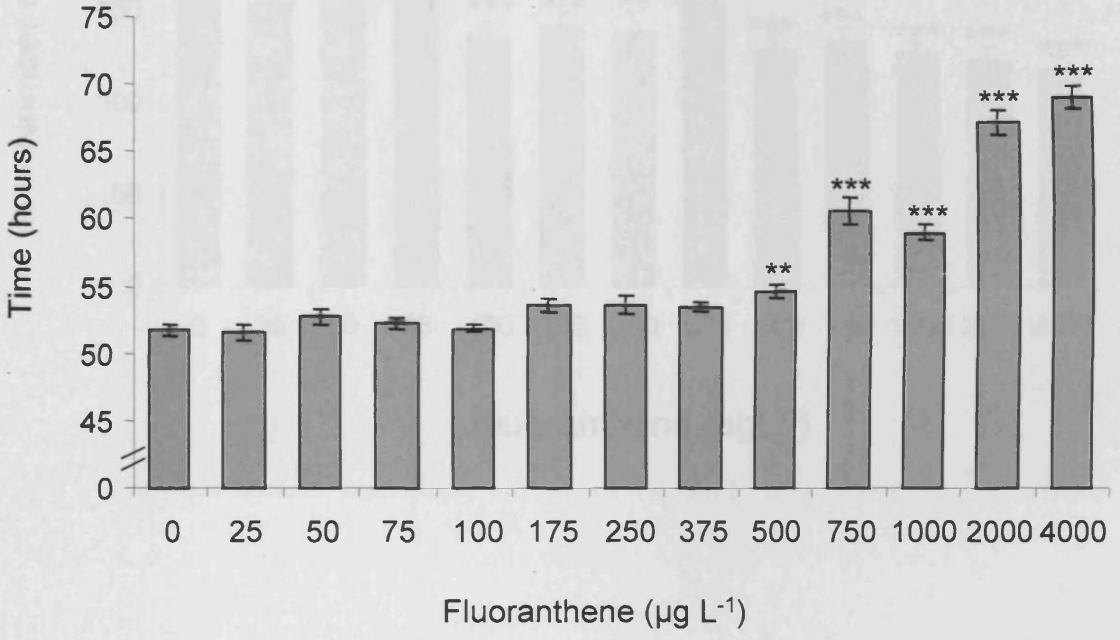


Figure 4.11 Mean time to first reproduction of control and fluoranthene exposed *C. elegans* strain GE31. Error bars represent standard error of the mean. Values are means of 46 replicates, excluding individuals that were censored (range 28-46). ** $p < 0.01$, *** $p < 0.001$.

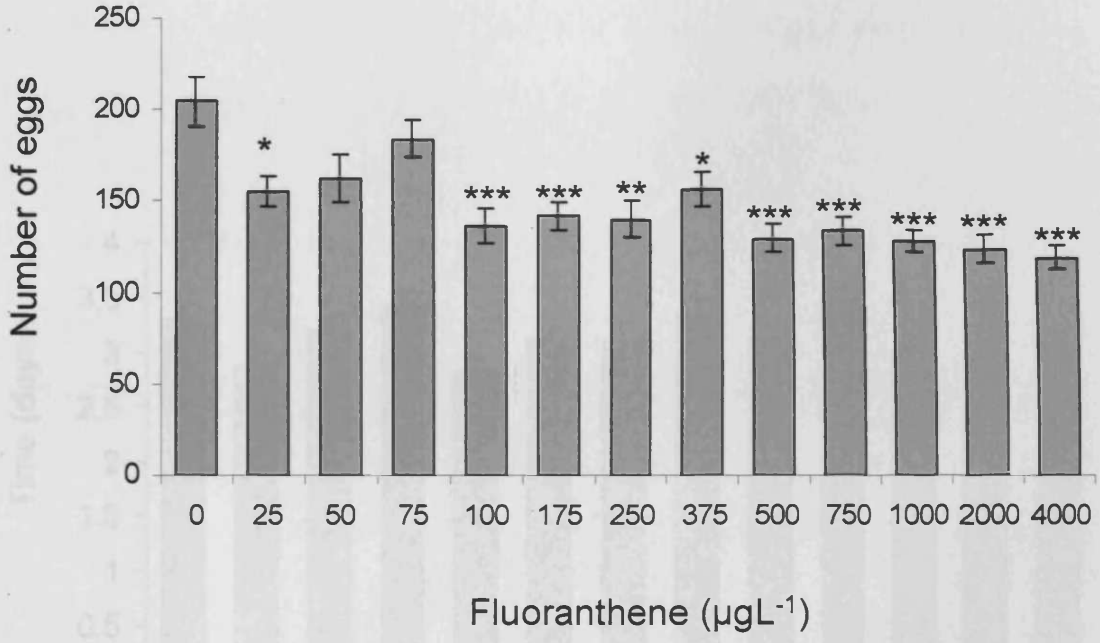


Figure 4.12: Mean total reproduction, measured as the total number of eggs laid by *C. elegans* strain GE31. Error bars represent the standard error of the mean. Values are means of 47 replicates, excluding individuals that were censored (range 27-47). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls.

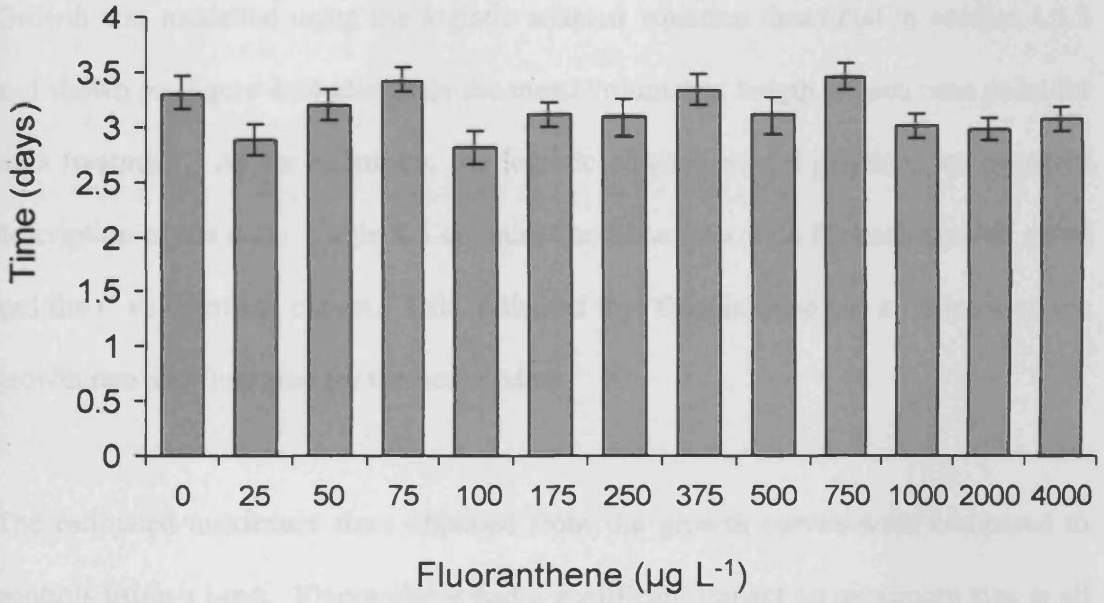


Figure 4.13: Mean reproductive period of control and fluoranthene exposed *C. elegans* strain GE31. Error bars represent the standard error of the mean. Values are means of 46 replicated, excluding individuals that were censored (range 28-46). No significant differences compared to the control were found for any treatment.

4.3.4.5 Growth

Growth was measured from L1 stage larvae through to reproducing adults for 7 days. Body length and area was measured, and volume calculated as described in section 2.3.3 and volumetric length (Figure 4.14) is the cube root of the volume.

Growth was modelled using the logistic adapted equation described in section 4.2.3 and shown on Figure 4.14 alongside the mean volumetric length at each time point for each treatment. As for cadmium, the logistic adapted model provided an excellent description of the data. Table 4.5 contains the parameter data for each growth curve and the r^2 value of the curves. This indicated that fluoranthene has an impact on the growth rate and final size for the nematodes.

The estimated maximum sizes obtained from the growth curves were compared to controls using a t-test. Fluoranthene had a significant impact on maximum size at all doses compared to controls ($p < 0.05$) (Figure 4.15). The largest difference in size from controls was a 10% reduction at the highest dose tested 4000 $\mu g\ L^{-1}$. Although in the majority of treatments fluoranthene reduced maximum size, the reductions were not as large as those seen with the cadmium treatments.

4.3.4.6 Size at first reproduction

Size at first reproduction was estimated from the fitted growth curve for each treatment, as described previously for cadmium (Figure 4.16). Fluoranthene significantly impacted the nematode size at first reproduction (ANOVA: $F = 21.89$, df = 12, 438, $p < 0.001$). Doses 100 $\mu g\ L^{-1}$ and above, with the exceptions of 500 and 1000 $\mu g\ L^{-1}$ were all significantly smaller than controls at first reproduction (Tukey p

<0.05). Size at first reproduction in the highest dose tested 4000 μg L^{-1} was 10% smaller than controls.

4.3.4.7 Slippery pharynx phenotype

An observation made during this study was that after approximately 6 or 7 days exposure to fluoranthene at doses 2 and 4 mg L^{-1} a high proportion of nematodes showed a black nose, or slippery pharynx phenotype (Figure 4.17). The procorpus region of the pharynx became black, which increased as exposure time increased.

Fluoranthene had significant impacts on several life history parameters. Exposure to fluoranthene reduced growth rate and thus increased developmental time. Final adult body size was reduced along with the number of eggs laid by the nematodes. Fluoranthene did not cause any significant impacts on lifespan nor the length of the reproductive period. A phenomenon which was seen after approximately 6 days exposure to fluoranthene in a large number of nematodes at 2 and 4 mg L^{-1} was a darkening of the procorpus region of the pharynx.

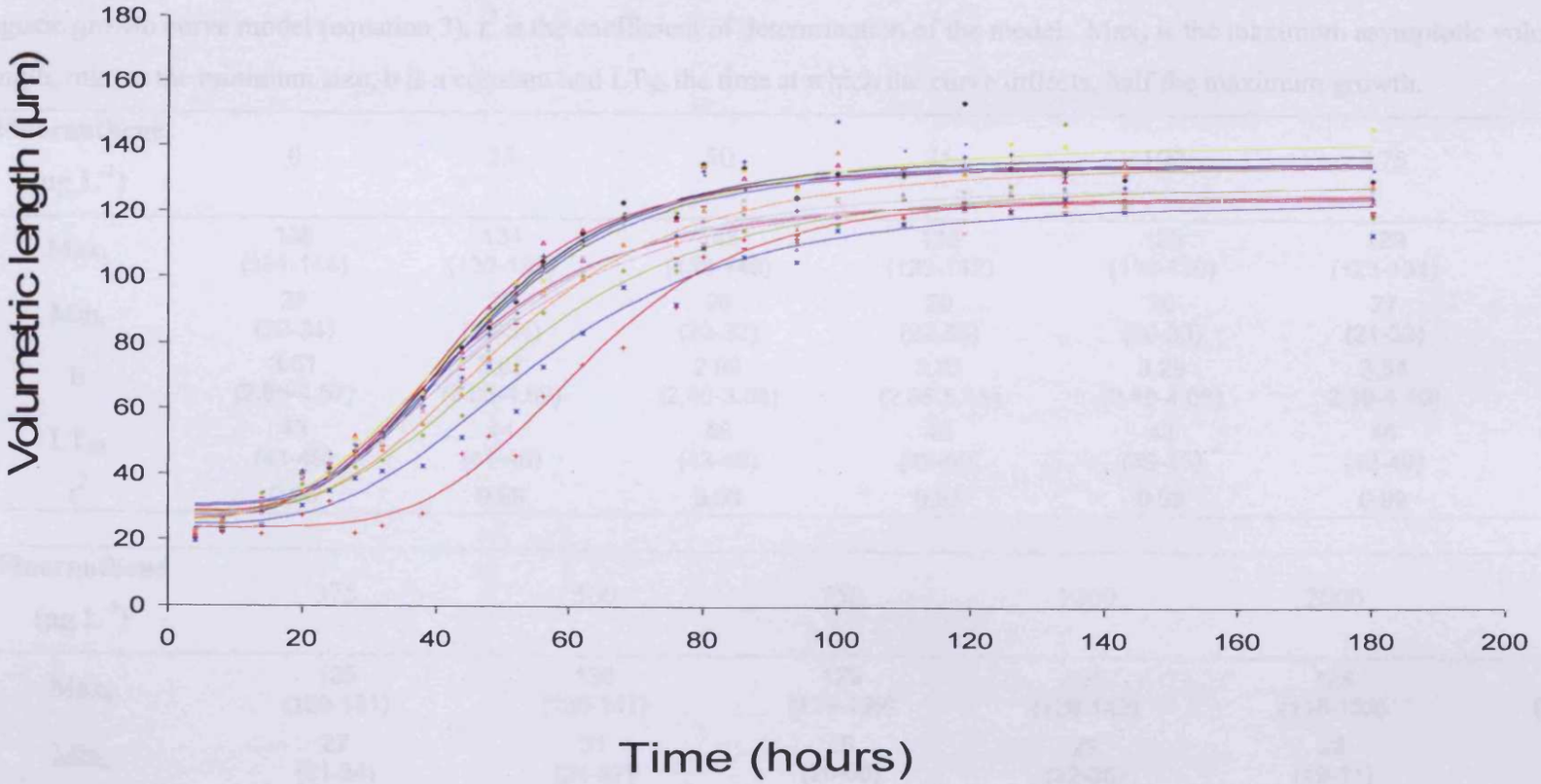


Figure 4.14: Volumetric growth of nematodes under control and cadmium exposure (— 0, — 25, — 50, — 75, — 100, — 175, — 250, — 375, — 500, — 750, — 1000, — 2000, — 4000 µg L^{-1}). Unbroken lines represent the logistic growth curve, mean size at each time point is shown in the corresponding colour for each treatment.

Table 4.5: Parameters values and 95% confidence intervals of control and fluoranthene dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, min_s is the minimum size, b is a constant and LT_{50} the time at which the curve inflects, half the maximum growth.

Fluoranthene ($\mu g\ L^{-1}$)	0	25	50	75	100	175	250
Max_s	138 (131-144)	134 (130-139)	143 (136-149)	135 (128-142)	125 (119-130)	129 (123-134)	127 (120-133)
Min_s	27 (20-34)	29 (23-34)	26 (20-32)	30 (22-38)	26 (20-33)	27 (21-33)	27 (21-34)
b	3.61 (2.65-4.57)	3.87 (3.05-4.69)	2.99 (2.40-3.58)	3.93 (2.68-5.18)	3.28 (2.48-4.08)	3.34 2.58-4.10)	3.18 (2.37-4.00)
LT_{50}	45 (41-49)	44 (41-46)	46 (43-49)	46 (42-50)	43 (39-46)	46 (42-49)	46 (42-50)
r^2	0.98	0.99	0.99	0.97	0.98	0.99	0.98

Fluoranthene ($\mu g\ L^{-1}$)	375	500	750	1000	2000	4000
Max_s	125 (120-131)	135 (130-141)	129 (119-139)	135 (128-142)	124 (116-132)	125 (120-131)
Min_s	27 (21-34)	31 (24-37)	28 (20-36)	29 (22-35)	25 (19-31)	24 (19-28)
b	3.40 (2.52-4.28)	3.73 (2.80-4.65)	3.28 (2.12-4.42)	3.52 (2.58-4.46)	3.37 (2.45-4.28)	4.95 (2.83-6.07)
LT_{50}	44 (40-48)	46 (43-50)	53 (47-59)	51 (47-55)	55 (51-60)	63 (59-66)
r^2	0.98	0.98	0.97	0.98	0.98	0.99

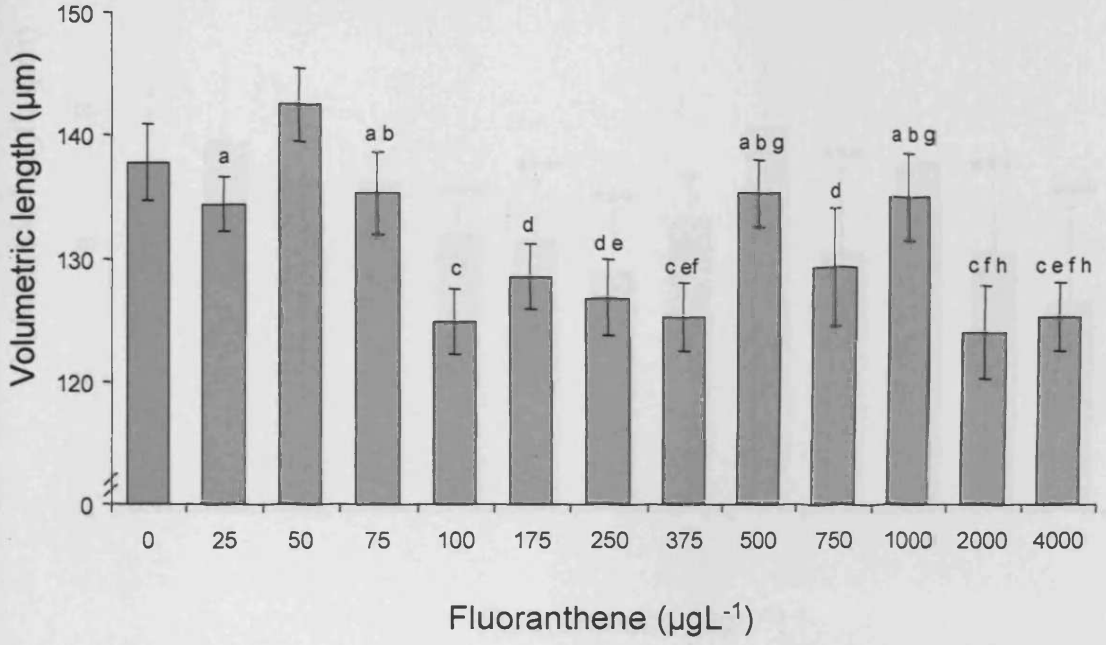


Figure 4.15: Maximum body size (estimated from the logistic growth model) in control and fluoranthene exposed nematodes. Error bars represent standard error of the mean. Columns which share the same letter (a-h) indicate that no statistically significant differences were found between these groups (t-test $p > 0.05$).

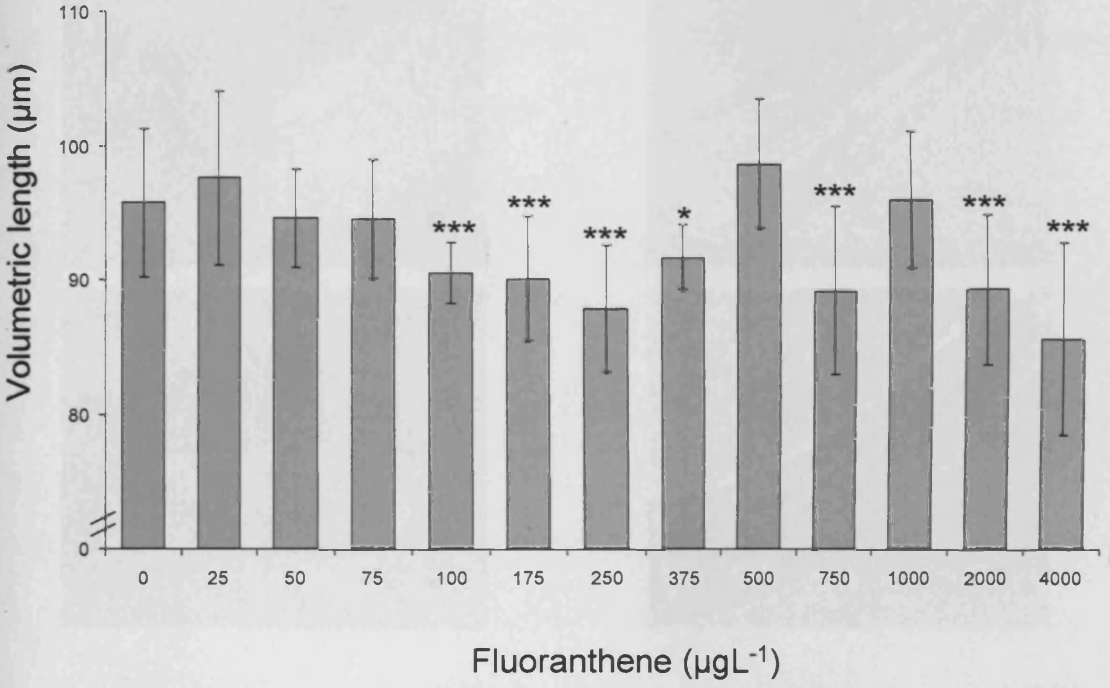


Figure 4.16: Mean estimated size at first reproduction. For control and fluoranthene exposed *C. elegans* strain GE31. Error bars represent the standard deviations. Values are means of between 28-46 replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls.

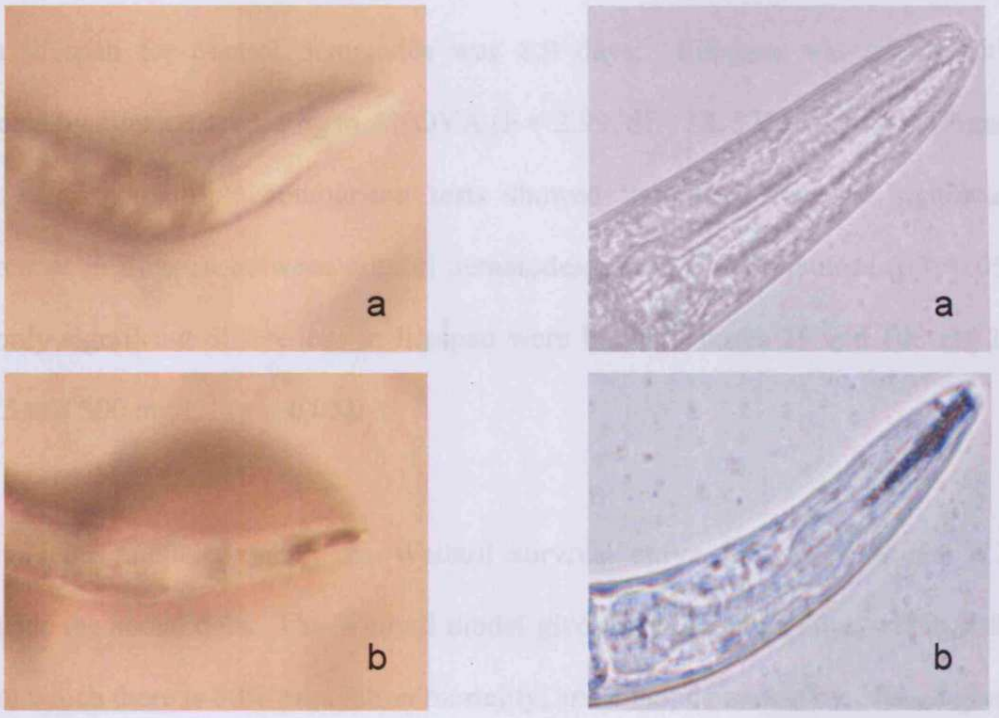


Figure 4.17: After approximately 7 days of exposure to fluoranthene a slippery pharynx phenotype was observed at the two highest doses tested, 2 and 4 mg L^{-1}. The photographs show the pharynx region of the nematodes, with a dark area in the procorpus: a) control and b) 2 mg L^{-1}.

4.3.5 Life-history effects of Atrazine

4.3.5.1 Survival and longevity

Mean lifespan for control nematodes was 8.9 days. Lifespan was significantly impacted by atrazine according to ANOVA ($F = 2.99$, $df = 12, 575$, $p < 0.001$) (Figure 4.18). Tukey multiple comparison tests showed that there were no significant differences in lifespan between control nematodes and any atrazine dose ($p > 0.05$). The only significant differences in lifespan were between doses 25 and 100 mg L^{-1} and 25 and 300 mg L^{-1} ($p < 0.05$).

Survival was modeled using the Weibull survival curve, shown on Figure 4.19 alongside the actual data. The Weibull model gives and estimated LT_{50}, which is the time at which there is 50% population mortality, and a slope parameter c, values given in Table 4.6. F-tests were performed to assess differences in the survival curves compared to the control survival curve. All curves were significantly different to the control survival curve ($p < 0.05$). With the exception of the two highest doses there did not appear to be a clear dose dependent response pattern.

4.3.5.2 Time to first reproduction

Atrazine significantly increased the length of time taken to begin reproducing compared with control nematodes at dose 50 mg L^{-1} and above (ANOVA: $F = 33.12$, $df = 12, 544$, $p < 0.001$) (Figure 4.20). Tukey multiple comparison test ($p < 0.05$) shows that there is a significant increase in steps, with lower doses significantly impacted at 50 mg L^{-1}, and a significant difference between the 50 mg L^{-1} dose and doses above 150 mg L^{-1} (with the exception of 250 mg L^{-1}). There was a 12%

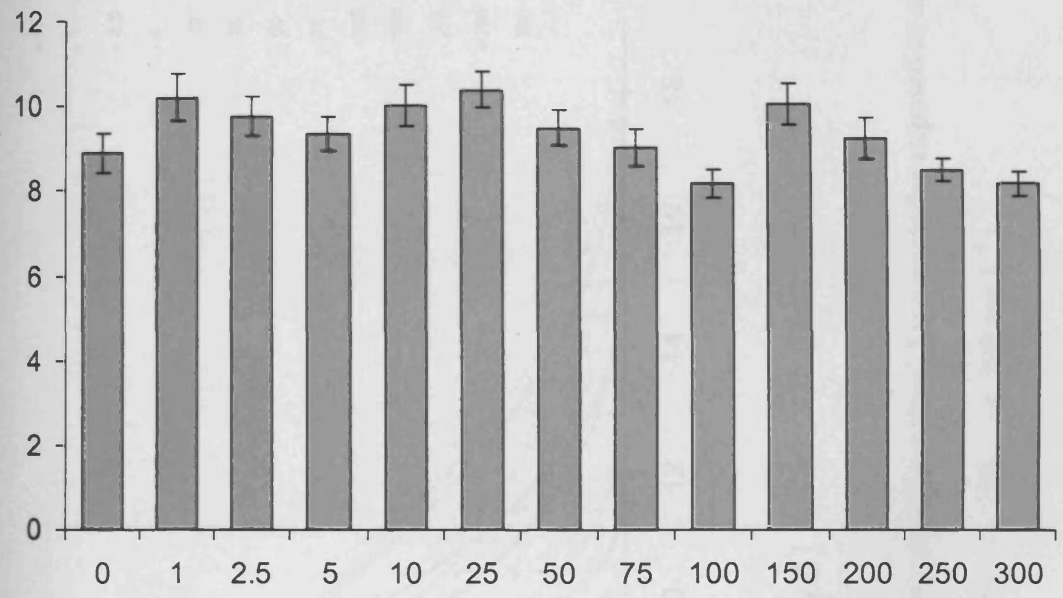


Figure 4.18: Mean lifespan of *C. elegans* strain GE31 at increasing concentrations of atrazine. Error bars represent standard error of the mean. Values are the means of between 44-47 replicates. No significant differences compared to the control were found for any treatment.

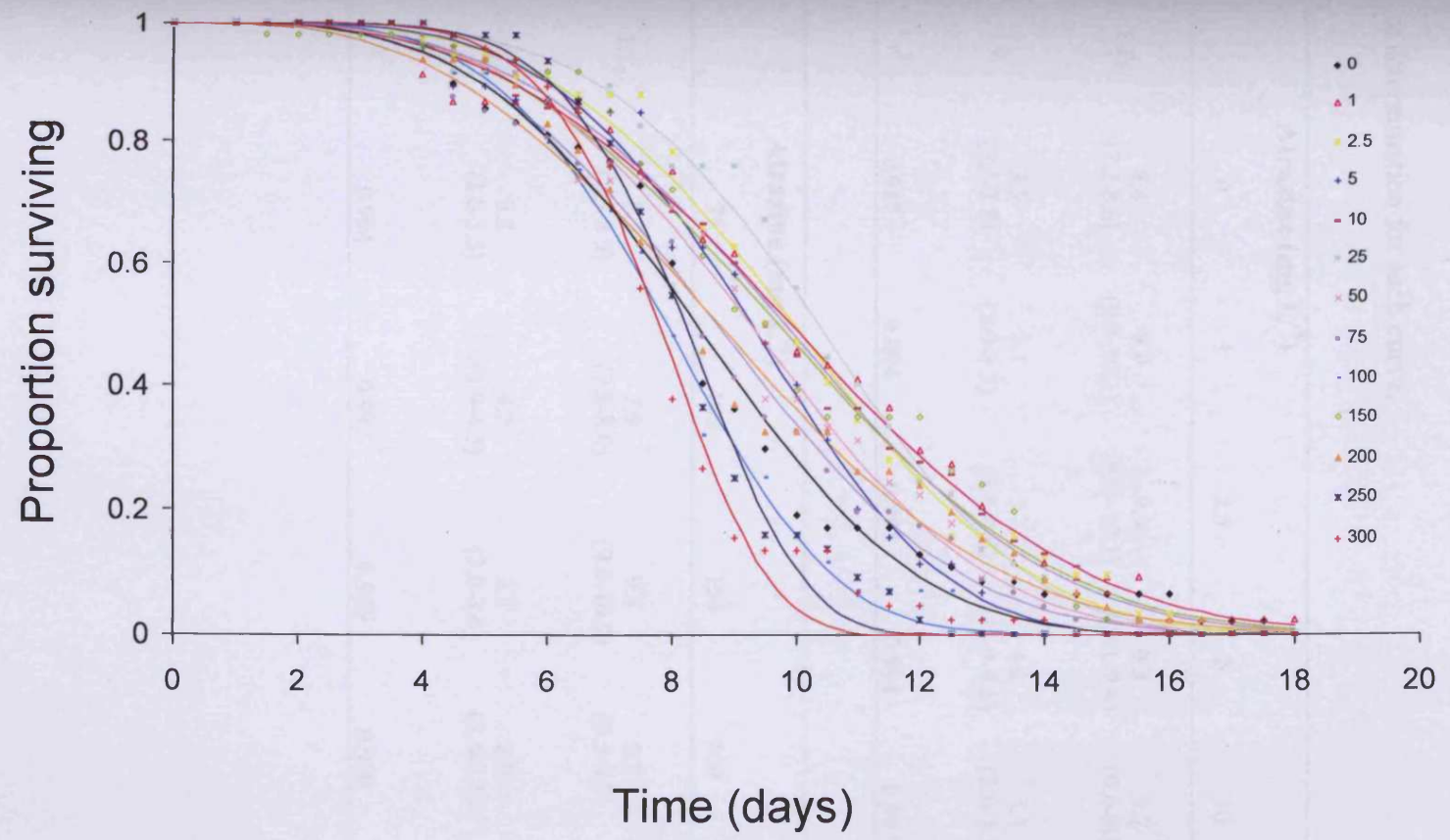


Figure 4.19: Survival curves (unbroken lines) and data points (in the corresponding colour) for nematodes exposed to atrazine (— 0, — 1, — 2.5, — 5, — 10, — 25, — 50, — 75, — 100, — 150, — 200, — 250, — 300 mg L^{-1}).

Table 4.6: Parameter values and 95% confidence intervals of control and atrazine exposed nematode survival, modelled using the Weibull survival model. LT_{50} is the time at which there is 50% mortality, and c is the slope parameter, r^2 is the coefficient of determination for each curve.

	Atrazine (mg L^{-1})						
	0	1	2.5	5	10	25	50
LT_{50}	8.4 (7.2-8.6)	9.9 (9.7-10.1)	9.9 (9.9-10.1)	9.3 (9.1-9.4)	9.7 (9.6-9.8)	10.3 (10.2-10.5)	9.3 (9.1-9.4)
c	3.5 (3.1-3.8)	3.1 (2.9-3.3)	3.7 (3.5-4.0)	4.2 (3.9-4.6)	3.1 (3.0-3.3)	4.3 (4.0-4.6)	3.5 (3.2-3.7)
r^2	0.987	0.994	0.995	0.994	0.997	0.996	0.994

	Atrazine (mg L^{-1})					
	75	100	150	200	250	300
LT_{50}	8.7 (8.5-8.9)	7.9 (7.8-8.0)	9.8 (9.6-10.0)	8.7 (9.5-9.0)	8.2 (8.1-8.3)	7.8 (7.7-7.9)
c	3.2 (3.0-3.5)	4.2 (3.9-4.5)	3.3 (3.0-3.6)	2.9 (2.6-3.1)	6.0 (5.4-6.6)	6.1 (5.4-6.9)
r^2	0.994	0.997	0.989	0.990	0.995	0.993

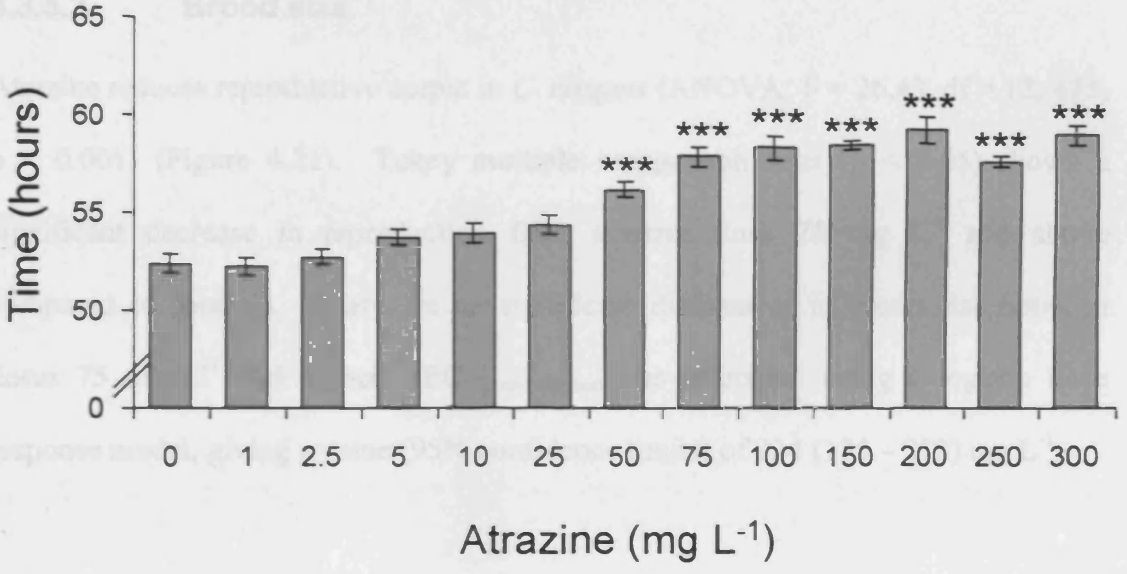


Figure 4.20: Mean time to first reproduction of control and atrazine exposed *C. elegans* strain GE31. Error bars represent standard error of the mean. Values are means of between 38-47 replicates. *** $p < 0.001$ compared to controls.

increase in time to first reproduction at the highest dose tested 300 mg L^{-1} compared to controls. There were no significant differences between the time taken to become reproductive at the higher doses from 75 mg L^{-1} and above.

4.3.5.3 Brood size

Atrazine reduces reproductive output in *C. elegans* (ANOVA: $F = 26.43$, $df = 12, 475$, $p < 0.001$) (Figure 4.21). Tukey multiple comparison tests ($p < 0.05$) shows a significant decrease in reproduction from atrazine dose 75 mg L^{-1} and above compared to controls. There are no significant differences in brood size between doses 75 mg L^{-1} and higher. $EC_{50\text{-reproduction}}$ was estimated using a logistic dose response model, giving a value (95% confidence limits) of 224 (175 – 273) mg L^{-1}.

4.3.5.4 Brood period

Brood period was significantly impacted by atrazine exposure (ANOVA: $F = 7.73$, $df = 12, 476$, $p < 0.001$) (Figure 4.22). Tukey multiple comparison test ($p < 0.05$) show significant differences between control and dose 25 mg L^{-1} only. Trends in the data appear to suggest an increase in brood period at doses from 0 – 25 mg L^{-1}, followed by a reduction to the highest dose. These trends are however not significant.

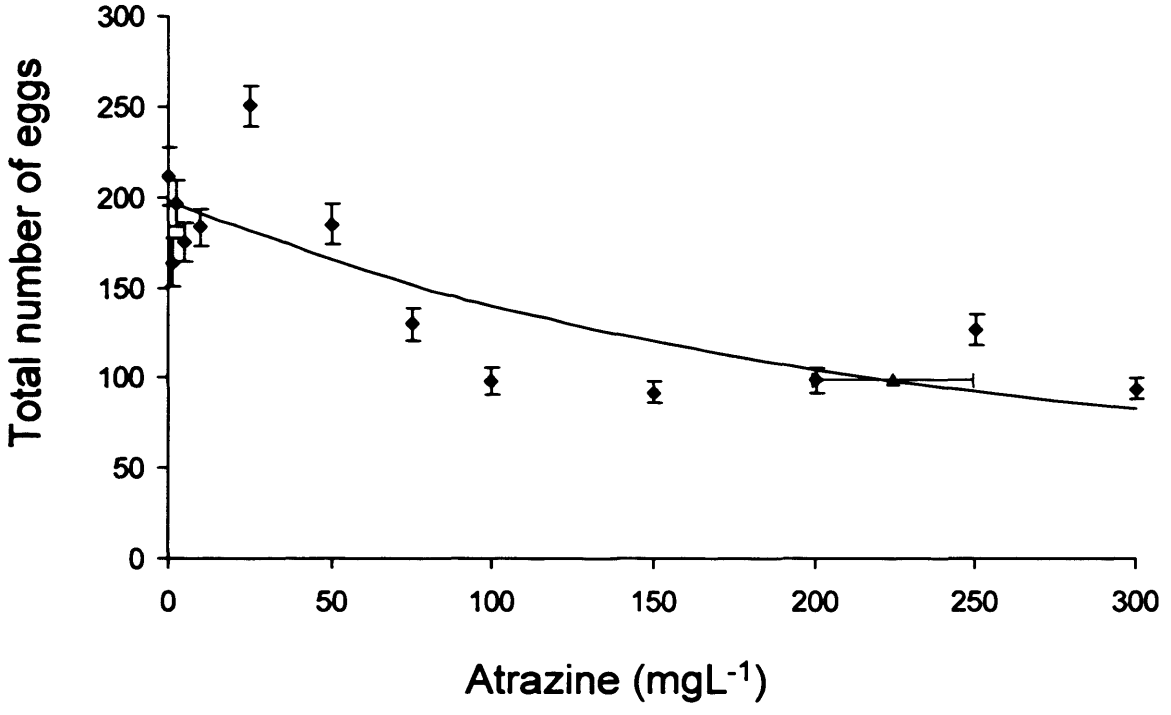


Figure 4.21: Mean total reproduction measured as total number of eggs laid, of *C. elegans* strain GE31 under control and atrazine exposure. Error bars represent the standard error of the mean. Individual data points represent the mean values. The unbroken line is the modelled logistic dose response. The $EC_{50\text{-reproduction}}$ is shown in red.

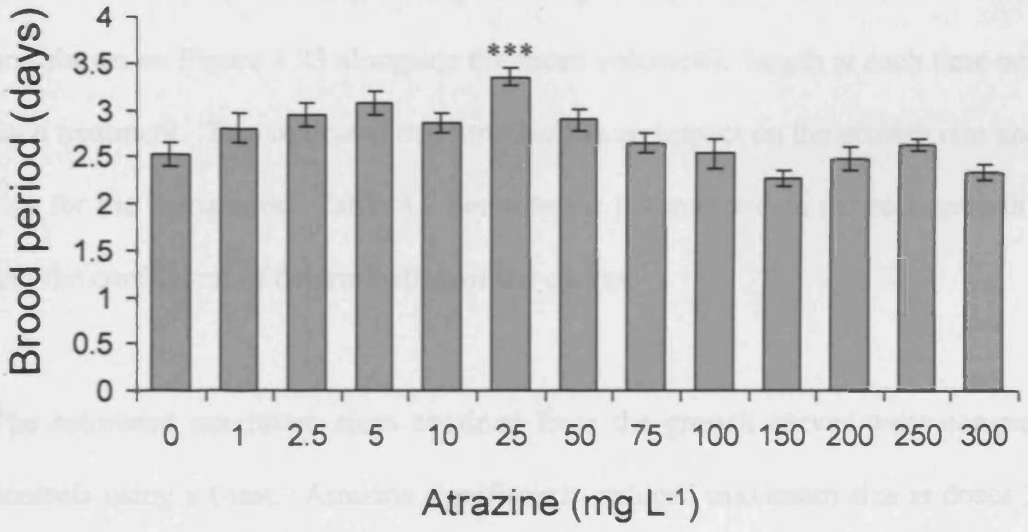


Figure 4.22: Mean reproductive period of control and atrazine exposed *C. elegans* strain GE31. Error bars represent the standard error of the mean. Values are means of between 32-42 replicates. *** $p < 0.001$ compared to controls.

4.3.5.5 Growth

Growth was measured from L1 stage larvae through to reproducing adults for 7 days. Body length and area was measured, and volume calculated as described in section 2.3.3, and volumetric length (Figure 4.23) is the cube root of the volume.

Growth was modelled using the logistic adapted equation described in section 4.2.3 and shown on Figure 4.23 alongside the mean volumetric length at each time point for each treatment. This indicated that atrazine has an impact on the growth rate and final size for the nematodes. Table 4.7 contains the parameter data for each growth curve and the coefficient of determination of the curves.

The estimated maximum sizes obtained from the growth curves were compared to controls using a t-test. Atrazine significantly reduced maximum size at doses 25 mg L^{-1} and higher ($p < 0.05$) (Figure 4.24). Growth shows a dose dependent response, with control worms growing the fastest and to the largest size and the highest atrazine dose being the slowest growing and smallest. Worms at 300 mg L^{-1} were approximately 20% smaller as adults than control worms.

4.3.5.6 Size at first reproduction

Size at first reproduction was estimated from the fitted growth curve for each treatment as described for cadmium, shown in Figure 4.25. Atrazine significantly impacted size at first reproduction (ANOVA: $F = 33.11$, $df = 12.\ 544$, $p < 0.001$). Tukey multiple comparison tests showed that dose 1 mg L^{-1} and 75 mg L^{-1} and higher

were all significantly smaller than controls. Nematode size at the highest dose of atrazine, 300 mg L^{-1}, was 10% smaller than that of controls.

Exposure to atrazine caused significant alterations to many of the life-history parameters measured. It reduced adult body size, size at maturity, and the number of eggs laid by the nematodes. Time to begin reproduction was increased, and effects were seen on the length of the reproductive period, there was an apparent increase in the length of the reproductive period at lower doses followed by a reduction at the higher end of the dose range, however this was only statistically significant at one dose, 25 mg L^{-1}, which resulted in an increased reproductive period. Lifespan remained unaltered by exposure to atrazine.

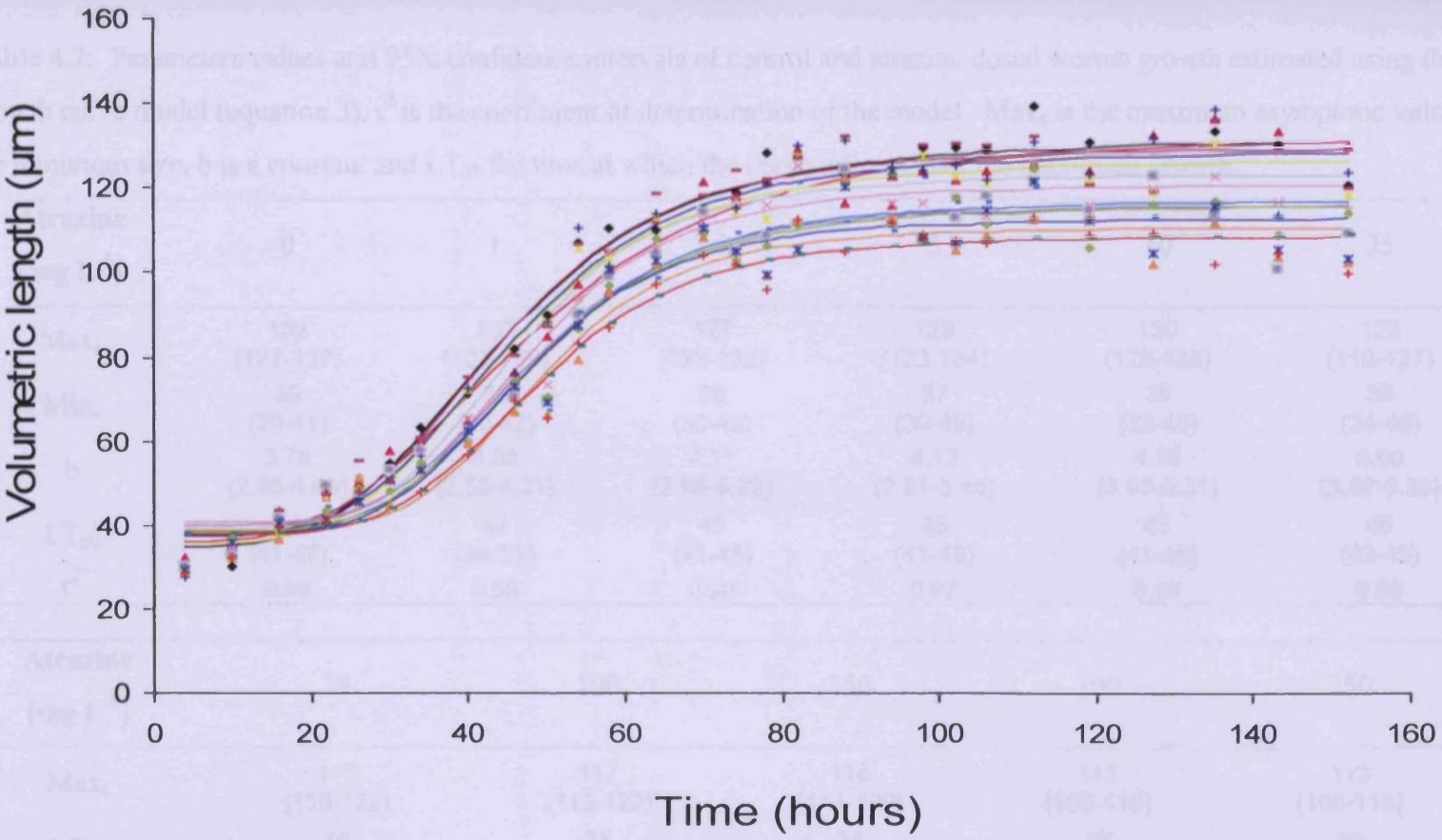


Figure 4.23: Volumetric growth of nematodes under control and atrazine exposure (— 0, — 1, — 2.5, — 5, — 10, — 25, — 50, — 75, — 100, — 150, — 200, — 250, — 300 mg L^{-1}). Unbroken lines represent the logistic growth curve, mean size at each time point is shown in the corresponding colour for each treatment.

Table 4.7: Parameters values and 95% confidence intervals of control and atrazine dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, min_s is the minimum size, b is a constant and LT_{50} the time at which the curve inflects, half the maximum growth.

Atrazine ($mg\ L^{-1}$)	0	1	2.5	5	10	25	50
Max_s	132 (127-137)	133 (127-139)	127 (122-132)	129 (123-134)	130 (125-135)	123 (119-127)	121 (117-125)
Min_s	35 (29-41)	36 (30-42)	36 (30-42)	37 (30-45)	38 (32-45)	39 (34-45)	41 (35-46)
b	3.76 (2.88-4.64)	3.38 (2.55-4.21)	4.11 (2.99-5.22)	4.13 (2.81-5.45)	4.16 (3.00-5.31)	5.00 (3.62-6.39)	5.11 (3.490-6.72)
LT_{50}	44 (41-47)	47 (44-51)	45 (41-48)	45 (41-49)	45 (41-48)	46 (43-49)	48 (45-51)
r^2	0.98	0.98	0.98	0.97	0.98	0.98	0.97

Atrazine ($mg\ L^{-1}$)	75	100	150	200	250	300
Max_s	116 (110-122)	117 (113-122)	116 (111-120)	111 (106-115)	113 (108-118)	108 (105-113)
Min_s	40 (32-47)	38 (32-44)	38 (32-44)	38 (32-45)	38 (31-45)	38 (32-43)
b	4.74 (2.59-6.90)	4.67 (3.19-6.15)	4.72 (3.18-6.26)	5.56 (3.30-7.82)	4.98 (2.97-6.99)	4.97 (3.29-6.65)
LT_{50}	48 (43-52)	48 (44-51)	48 (44-51)	49 (45-53)	47 (43-51)	49 (45-52)
r^2	0.95	0.97	0.97	0.96	0.96	0.97

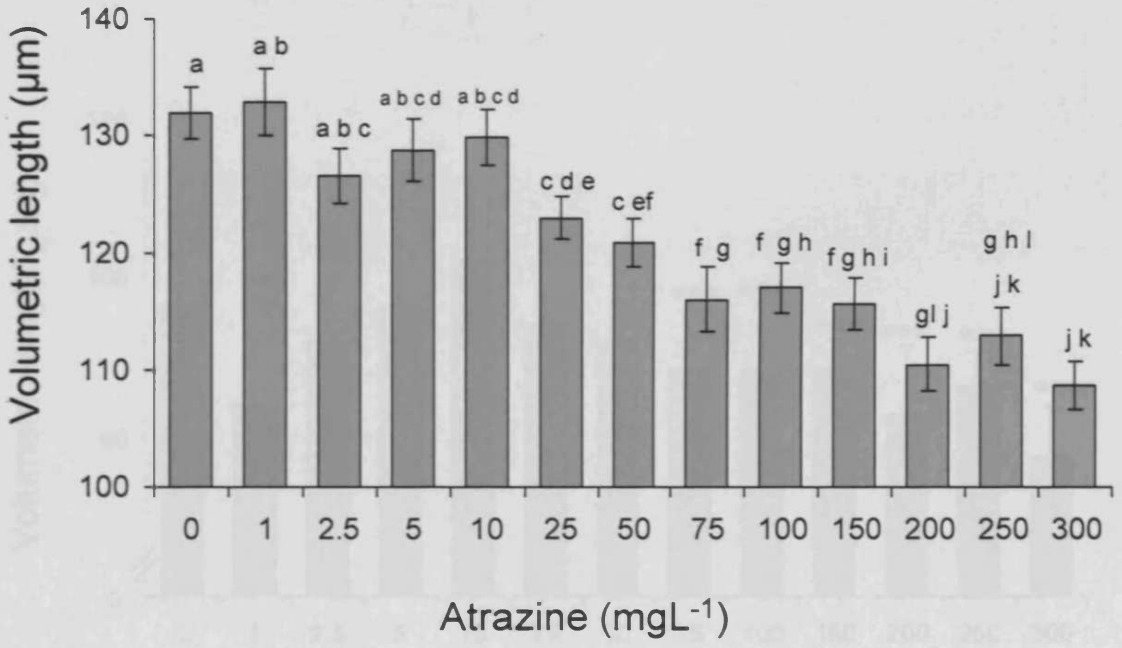


Figure 4.24: Maximum body size (estimated from the logistic growth model) in control and atrazine exposed nematodes. Error bars represent standard error of the mean. Columns which share the same letter (a-k) indicate where no statistically significant difference was seen between groups (t-test $p > 0.05$).

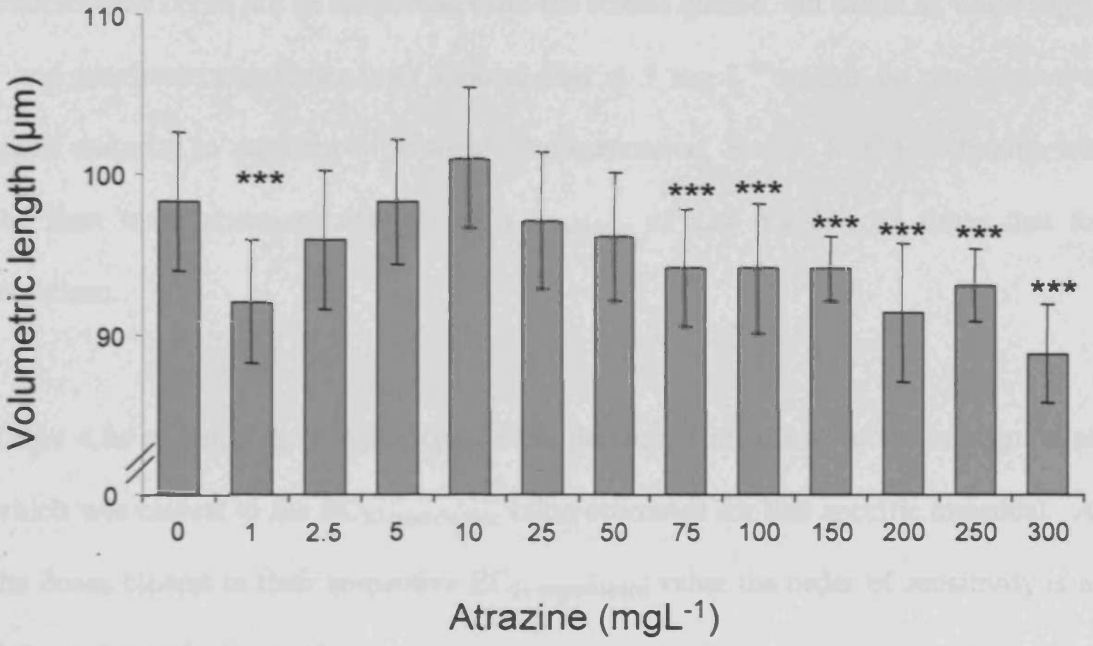


Figure 4.25: Mean estimated size at first reproduction. For control and atrazine exposed *C. elegans* strain GE31. Error bars represent the standard deviations. Values are means of between 36-46 replicates. *** $p < 0.001$ compared to control.

4.3.6 Sensitivity of life history parameters

Life history parameters responded differently to each chemical, and also to the exposure concentration. Relative sensitivity of traits is shown in Table 4.8 presented as either a % change compared to controls or as no observed effect concentration (NOECs) values. Cadmium was the most toxic chemical to *C. elegans* with an $EC_{50\text{-}reproduction}$ value of 3.6 mg L^{-1}, followed by fluoranthene. The EC_{50} value for fluoranthene could not be calculated from the results gained, but the EC_{40} was 4 mg L^{-1} and previous rangefinder tests showed that at 5 mg L^{-1} worms do not survive to reach maturity to reproduce (personal communication, Swain, S. C.). Atrazine was the least toxic chemical with an $EC_{50\text{-}reproduction}$ of 224 mg L^{-1}, 62 times that for cadmium.

Table 4.8a presents the sensitivity of each parameter measured at the concentration which was closest to the $EC_{50\text{-}reproduction}$ value estimated for that specific chemical. At the doses closest to their respective $EC_{50\text{-}reproduction}$ value the order of sensitivity is as follows for each chemical.

Cadmium: Reproduction > Reproductive period > Maximum size > Size at maturity > lifespan > time to maturity.

Fluoranthene: Reproduction > Time to maturity > Maximum size > Size at maturity > lifespan > Reproductive period.

Atrazine: Reproduction > Maximum size > Size at maturity > Time to maturity > Lifespan > Reproductive period.

Table 4.8b presents the sensitivity of each parameter measured at the highest dose tested. Differences were also seen in the sensitivity of some parameters at the highest dose tested. The order of sensitivity is as follows for each chemical.

Cadmium: Reproduction > Time to maturity > Reproductive period > Maximum size > Size at maturity > Lifespan.

Fluoranthene: Reproduction > Time to maturity > Maximum size > Size at maturity > Lifespan > Reproductive period.

Atrazine: Reproduction > Maximum size > Time to maturity > Size at maturity > Reproductive period > Lifespan.

Table 4.8c presents the NOEC values for each parameter with each chemical. NOEC values were assessed by Tukey multiple comparison test of doses against control means ($p > 0.05$). When NOEC values are used to assess the sensitivity of life history parameters to toxicants the order of sensitivity is again different to either scenario above. In each case body size, for cadmium size at maturity, for fluoranthene and atrazine maximum size, had the lowest NOEC.

Table 4.8: Life history parameter sensitivity of *C. elegans* strain GE31 in terms of % change compared to controls, exposed to cadmium, fluoranthene and atrazine. a) Sensitivity of parameters at the dose closest to the estimated $EC_{50\text{-reproduction}}$ value, b) sensitivity of parameters at the highest dose tested in each experiment and c) NOEC comparisons.

a)

Parameter	Cadmium	Fluoranthene	Atrazine
$EC_{50\text{-reproduction}}$	3.6 mg L^{-1}	> 4 mg L^{-1}	224 mg L^{-1}
Closest dose tested	4 mg L^{-1}	4 mg L^{-1}	200 mg L^{-1}
Brood size	47% decrease	42 % decrease	47% decrease
Time to reproduction	9% increase	33% increase	13% increase
Lifespan	15% decrease	8% decrease	8% decrease
Maximum size	29% decrease	10% decrease	18% decrease
Size at maturity	26% decrease	10% decrease	16% decrease
Reproductive period	40% increase	7% decrease	2% decrease

b)

Parameter	Cadmium	Fluoranthene	Atrazine
Highest dose	16 mg L^{-1}	4 mg L^{-1}	200 mg L^{-1}
Brood size	97% decrease	42% decrease	55% decrease
Time to reproduction	85% increase	33% increase	13% increase
Lifespan	20% decrease	8% decrease	6% decrease
Maximum size	47% decrease	10% decrease	20% decrease
Size at maturity	40% decrease	10% decrease	10% decrease
Reproductive period	73% decrease	7% decrease	8% decrease

c)

Parameter	Cadmium NOEC (mg L^{-1})	Fluoranthene NOEC (µg L^{-1})	Atrazine NOEC (mg L^{-1})
Brood size	1	75	50
Time to reproduction	0.5	375	25
Lifespan	16	4000	300
Maximum size	0.5	0	10
Size at maturity	0	1000	50
Reproductive period	12	4000	300

4.4 Discussion

Toxicants can have varying impacts on life history traits in individuals exposed to them. This section of work covered the effects that toxicants may have at sublethal levels on the life cycle of the nematode *C. elegans* and the sensitivity of key life cycle traits to compounds representative of chemicals taken from three major chemical classes, metal, non-specific acting organic and specific acting organic that have different modes of action. Each chemical tested showed a range of sublethal responses which was chemical specific. Cadmium was the most toxic compound to *C. elegans* with an $EC_{50\text{-reproduction}}$ of 3.6 mg L^{-1}, followed by fluoranthene, with atrazine the least toxic of the three with an EC_{50} of 224 mg L^{-1}.

C. elegans strain GE31 was shown to be a good substitute for the classic wildtype strain N2 for these studies. A parallel study on global gene expression was carried out simultaneously with the life history toxicity test. The transcriptomic study was made more powerful by collecting samples at three life stages, L1, L4 and young adults. The use of the embryonic lethal temperature sensitive strain was used to enable single stage young adult populations to be collected as the eggs that these worms laid did not hatch, and so the adults could be washed off the plates leaving the eggs behind which stick to the agar. This data is not presented in this thesis.

A comparison between the strains GE31 and N2 was performed, measuring time to reproduction, total reproduction, reproductive period, size as adults and lifespan. Slightly fewer eggs were laid by GE31 than N2 at 25°C, but this was not significantly different. The number of eggs recorded for N2 at 25°C was higher than in two other studies, who recorded 165 and 170 eggs respectively. Lifespan, brood period and

size as adults were all not significantly different between strains. GE31 had a significantly longer time to first reproduction by 7 hours (this parameter is very homogeneous in N2), however, as no other parameters were significantly different this work confirmed the suitability of GE31 as an ecotoxicological test strain.

4.4.1 Reproducibility of tests

The parameters lifespan and brood size of control nematodes were used to examine the repeatability and standardization between these experiments, which were performed at different times. Neither lifespan nor brood size was significantly different between the three experiments which indicated that test conditions which may influence life history, such a temperature, were kept constant between experiments. A previous study examining N2 wildtype lifespan has shown that the LT_{50}, the time at which there was 50% population survival, was 7.8 days (Hosono et al., 1982), which fits within the 95% confidence limits of the experiments reported in this chapter. Byerly et al. (1976) reported that the time to the first egg being laid was approximately 47 hours, the reproductive period lasted approximately 1.5 days and the brood size was 170 eggs in N2 wildtype worms at 25°C. The data collected in these experiments gave an approximate time to first egg between 52-56 hours and a brood period of 2.5-3.5 days. The brood size in these experiments was slightly larger with 203-211 eggs laid.

4.4.2 Cadmium

Cadmium is a highly toxic metal which is non-essential to organisms. It affected all life history parameters measured in this study. It has previously been shown that

specific detoxification pathways exist in *C. elegans* to deal with cadmium, such as the induction of metallothioneins (Swain et al., 2004) and phytochelatin synthase (Vatamaniuk et al., 2001), and that life history and behavioural changes also occur following cadmium exposure. Cadmium has been shown to affect feeding in terms of pharyngeal pumping rate (Alvarez et al., 2005; Jones and Candido, 1999), movement, growth and reproduction (Anderson et al., 2001), avoidance behaviour (Sambongi et al., 1999), and lifespan (Swain et al., 2004).

Previous rangefinders (data not shown) have indicated that cadmium levels above 20 mg L^{-1} result in few nematodes reaching maturity before dying. In the highest dose tested in this main study the mean lifespan was 20% shorter than controls. This indicates that at the test concentrations used although lifespan was reduced the nematodes were able to detoxify and repair cadmium induced damage meaning that worms were well able to reach maturity. The survival data shows a slight, but statistically insignificant, increase in lifespan at low cadmium doses, which may indicate a hormetic effect of cadmium. Although hormetic effects of cadmium have not been reported for *C. elegans* before, increased survival at low levels of cadmium in *Daphnia magna* have been identified (Kluttgen and Ratte, 1994).

Time to first reproduction was very sensitive to cadmium, leading to increases in the time taken to develop to maturity. Under control conditions it is a very homogeneous parameter with very low variance in age-synchronised populations. Time to first reproduction is an important parameter in life history analysis and its translation into population consequences, used in ecological risk assessment. A study by Kammenga et al. (1996) showed, using the nematode *Plectus acuminatus*, that although time to

reproduction was not the most sensitive life history parameter in response to toxicant exposure, when it was incorporated into a model for the intrinsic rate of population growth it produced the highest impact on fitness. This suggests that although changes in the time to reproduction were rather small (especially at low dose) these effects could have important implications for population growth rate.

During the final larval stage, L4, *C. elegans* first produces around 300 sperm and then switches to oocyte production, due to its self-fertilising hermaphroditic nature. Sperm is the limiting factor in *C. elegans* reproduction. Once the switch to producing oocytes is made no more sperm can be produced, although if a hermaphrodite is able to mate with a male then up to a thousand offspring may be produced (Riddle et al., 1997). Changes in time to reproduction may enable the nematode to produce as many sperm as possible to maximise fitness. If there is less energy available for development then extra time would allow not only the nematode to increase its body size, a factor commonly associated with higher fecundity, but also possibly to increase the time spent on producing sperm. A delay in maturation time can benefit an organism with a lower juvenile mortality rate of the offspring or an increased lifetime reproductive success due to a greater body size at maturation (Kozlowski and Wiegert, 1987). There are however costs associated with delayed maturation in that organisms that grow for longer periods of time have less likelihood to survive until reproductive age (Gemmill et al., 1999). These effects represent a version of the classic paradox between investment in long-term fecundity or in the desire to produce juveniles at the earliest opportunity.

Reproductive output was reduced by cadmium, as well as alterations to the reproductive period. Reproduction was the most sensitive trait to each chemical tested. The majority of nematodes at the highest dose 16 mg L^{-1} were able to produce at least 1 egg, but very few above that. The reproductive period was influenced by cadmium in two ways, at the lower doses by extending the reproductive period, and at high doses by reducing the reproductive period. At the lower doses fewer eggs were laid over a longer period of time, although the total number of eggs laid was still lower than in controls. This suggests that there was less energy, or ability to produce fertilized eggs at the lower doses. In wildtype nematodes fertilised eggs spend approximately 2.5 hours in the uterus before being laid and approximately 12 eggs remain within the uterus (Bany et al., 2003). From observations, cadmium did not appear to cause more than the normal number of eggs to be retained in the body, suggesting that the increased reproductive period was not as a result of egg-laying defects. Observations also showed no difference in the proportion of nematodes that died with eggs inside them, known as facultative vivipary or bagging (Chen and Caswell-Chen, 2004). Facultative vivipary was seen in control and cadmium conditions, in approximately 0.1% of the test organisms, these individuals were not included in the results. Previous studies, in chapter 3, have shown that cadmium does not affect hatching success of the eggs.

Growth followed a dose dependent response, as clearly shown on Figure 4.6. Cadmium severely impacted growth of the nematodes. Nematodes have an S-shaped growth curve, with an apparently exponential phase of growth during the larval stages and a gradual approach to a plateau in late adulthood (Byerly et al., 1976). It has been shown that growth during each larval stage is linear, but this linear rate increases at

each molt, producing an overall exponential larval growth curve. The cross section size of the buccal cavity (the tube that leads from the mouth to the pharynx) does not grow until the worm molts. Knight et al. (2002) have speculated that the reason for the lower growth rate in younger larvae is that the size of the buccal cavity limits feeding rate, as its size in L1 larvae is around the same size as the diameter of an *E. coli* cell (around 1μm), and thus resource acquisition which affects growth rate of the larval worm is limited.

Cadmium has been shown to reduce feeding rates in *C. elegans* (Jones and Candido, 1999). Whilst this tactic may reduce exposure to cadmium via the food, as a result the nematode also reduces its energy intake. Combined with the increased energy required for the production of a stress response and the activation of detoxification pathways this may go some way to explain the reduced growth rate and reproductive output of the nematodes, according to the dynamic energy budget theory.

Size at first reproduction decreased with increasing cadmium concentrations. This has also been observed by Kammenga et al. (2000). Body size at maturity is assumed to be correlated with other life history traits and a common finding in ectotherms is that an increased body size results in increased reproductive success (Roff, 2002). It is thought that there is a switch in energy investment from growth and development into reproduction once an individual has reached a critical size or weight which allows them to reproduce. This data clearly shows that nematodes are capable of reproducing at a much smaller size than that which control nematodes begin to reproduce at, thus indicating that a larger body size does indeed enhance total reproductive output. It could be that the nematode can sense its environment being

favourable and so invests more time for spermatogenesis, after the stage where it becomes large enough to produce an egg, thus allowing it to produce the maximal amount of sperm to fertilize its eggs.

Under stressed conditions size at maturity was modified so that worms were smaller as reproduction began. It is plausible that in sub-optimal conditions nematodes reproduce at a smaller size to reduce the pre-reproductive time, as they are more likely to reach maturity if they have less time to die as a juvenile. Life history fitness theory states that individuals who reproduce sooner give a fitness advantage to their offspring which then have the opportunity to exploit resources earlier than individuals whose eggs were laid later on. These offspring then also have the opportunity to reproduce at an earlier time. However, there is a balance of costs and benefits, as individuals with a larger body size are capable of producing more offspring, or their offspring may have a better chance of survival. A nematode in a highly toxic environment would theoretically want to produce as many offspring as possible before it dies. This could possibly mean that putting less effort into spermatogenesis or embryogenesis and more effort into reaching the specific body size to begin egg-laying is optimal. The effects seen on size and time at maturity could however simply be a result of toxicity.

4.4.2 Fluoranthene

Fluoranthene is toxic to organisms and its mode of action is via non-polar narcosis (partitioning into cell membrane). At the dose range examined in this study fluoranthene had significant impacts on time to reproduction, brood size, growth and size at maturity. It did not have any impact on lifespan or brood period however.

Fluoranthene did not reduce lifespan in *C. elegans* compared to controls at the dose range tested, although rangefinder tests showed that a high proportion of L1 larvae were unable to grow and survive at concentrations above 5 mg L^{-1} (personal communication, Swain, S. C.) which indicates a steep dose response for survival at a critical concentration.

Time to first reproduction was sensitive to fluoranthene exposure with significant increases seen at 500 μg L^{-1} and above. Developmental time was severely increased at the two highest doses, 2 and 4 mg L^{-1}. Size at first reproduction did not follow a dose response pattern; however the two highest doses were the smallest. The effect on time to maturity and size at maturity were similar to that seen with cadmium, in that there was a large jump in effect at the two highest doses. However in contrast fluoranthene increased time to maturity by 33% whereas cadmium induced an 86% increase. The same was seen with size at maturity with fluoranthene reducing size 10% whereas cadmium caused a 40% reduction in size at maturity.

Reproductive output was sensitive to very low doses of fluoranthene, showing an initial reduction in brood size of approximately 25%, however this plateaued out and only small reductions above this were seen in the higher doses. The apparent plateau of the response in reproduction may be because *C. elegans* is simply able to cope with fluoranthene in the environment, but reproduce at a lower but consistent rate, or that fluoranthene exerts its toxic impacts on a different parameter. The plateau may also be explained by a saturation effect of fluoranthene in the agar. Fluoranthene is almost completely insoluble in water and so needed to be dissolved in a solvent before

addition to the agar. There was no apparent crystallisation of the fluoranthene in the agar, but it is possible that as the amount of fluoranthene increased the solubility of it in the agar was affected. Unlike the response of cadmium, the reproductive period was unaltered by fluoranthene.

Growth was not as obviously impacted by fluoranthene as exhibited by the effect of cadmium. Whereas cadmium affected growth rate and final body size, fluoranthene had more impact on the exponential part of the growth curve but only small effects on the final body size. The growth curve of the 4 mg L^{-1} group took longer to begin the exponential growth phase, although the slope of the growth curve (rate) was higher than other treatment groups. This may suggest that L1-L2 stage nematodes were more sensitive to fluoranthene than the later stages.

The slippery pharynx phenotype was investigated further using high power microscopy. The black colouration was discovered to be packed *E. coli* cells in the procorpus area of the pharynx, although the tube was not blocked (personal communication, Redshaw, N. and Sturzenbaum, S. R.). This phenomenon was not seen with any other chemical tested. In cadmium exposed nematodes, reduced feeding rates are observed but this does not lead to the *E. coli* remaining in the procorpus, which indicates that a reduced feeding rate is not the cause for the collection of bacteria in the pharynx under fluoranthene exposure. It is possible that fluoranthene affects the passage of food into the pharynx in some way. One possible reason may be that changes in the cells of the procorpus mean the *E. coli* could not move down to the pharyngeal bulbs, where the food is ground up, easily. Very little data is available on the toxic cellular effects of fluoranthene; however, one

histopathological study has shown that fluoranthene causes death of the outer layer of the integument of bullfrog larvae (Walker et al., 1998). They showed that there was necrosis of the cells and structural disorganisation caused by fluoranthene; however this effect was seen upon UV exposure, indicating UV-induced toxicity of fluoranthene, which is not relevant to this study. Fluoranthene has also been shown to cause DNA damage in cells of the polychaete *Capitella* sp. I, including strand breaks and loss of single nucleotides (Palmqvist et al., 2003).

4.4.3 Atrazine

Atrazine is a highly toxic herbicide which exerted toxic effects on *C. elegans* also. As its mode of action is in the inhibition of photosynthesis, this is irrelevant in *C. elegans* so its toxic mode of action is unknown in nematodes. Induction of GSTs in plants can offer effective resistance to atrazine (Dautermann, 1989), increased GST activity has also been measured in zebrafish embryos (Wiegand et al., 2001). Atrazine has recently come under scrutiny due to reported endocrine disrupting effects in *Xenopus laevis* frogs (Hayes et al., 2002), who reported that at ecologically relevant concentrations atrazine caused feminization effects in larval frogs. They found that at doses as low as 0.1 $\mu g\ L^{-1}$ atrazine tadpoles developed as hermaphrodites, and at 1 $\mu g\ L^{-1}$ a significant reduction in size of the larynx was seen. The larynx is an organ that the male uses to attract potential mates. Further studies have aimed to clarify the possible endocrine disrupting effects of atrazine in other frogs and other organisms. A separate study found increased levels of intersex and discontinuous gonads in *X. laevis* under atrazine exposure (Carr et al., 2003), however at 250 times the concentration of that reported by Hayes et al. (2002). A study with the fathead

minnow did not find any significant estrogenic effects of atrazine (Bringolf et al., 2004).

Atrazine had significant impacts on reproduction, time and size at reproduction and growth. Lifespan and reproductive period were unaffected. Increasing atrazine concentrations gradually increased the time taken to develop to maturity in a dose dependent manner. This was a different response to that seen for either cadmium or fluoranthene. Although there was a gradual increase in time to maturity the maximum % change was just 13% longer at the highest dose, compared to the 33% and 85% increase in time to begin reproduction seen for fluoranthene and cadmium respectively.

Reproduction was impacted, initially with a large reduction in brood size at low dose followed by only small decreases as dose increased, showing a plateau effect. The most likely cause for this plateau was due to saturation of the agar with atrazine. In rangefinder tests adding more than 300 mg L^{-1} atrazine to the agar caused the atrazine to crystallize in the agar, due to the hydrophobic nature of this compound. Reproductive period was insensitive to atrazine.

Growth over time was dose dependent. Growth curves for atrazine showed a different response pattern than seen either upon exposure to cadmium or fluoranthene. On exposure to atrazine the timings of the exponential part of the growth curve were very similar between doses, but resulted in smaller nematodes as adults. Whereas for cadmium the growth rate and adult body size were both markedly reduced, and for

fluoranthene the growth rate and timings of the exponential part of the growth curve were dissimilar but the final body sizes were within 10% that of controls.

4.4.4 Chemical specific responses and parameter sensitivity

These three experiments demonstrate chemical class specific responses in life history parameters. The compounds tested were selected as representatives of three major chemical classes; metals, non-specific organics and specific organics. The most sensitive parameter in all cases was reproduction and lifespan was least sensitive in all cases. The growth curves of exposed worms demonstrate well the different ways in which growth can be affected by environmental chemicals, with each chemical showing a different response in growth. Cadmium drastically reduced the rate of growth and final body size. Fluoranthene reduced the rate of growth but had a far smaller effect on final body size. Atrazine had little effect on the rate and timings of growth, but resulted in a reduced final body size.

The sensitivity of parameters was different for each chemical, showing that the life history response of the nematodes was dependent on the chemical rather than being one general response to stress. Parameter sensitivity also changed according to dose in cadmium and atrazine. In cadmium the most sensitive parameter after reproduction was size and size at maturity at 4 mg L^{-1}, however at 16 mg L^{-1} time to maturity and the length of the reproductive period became more sensitive to cadmium than growth. Time to maturity also moved up the sensitivity scale in atrazine as concentration increased.

The use of NOECs to examine the relative sensitivity of life history traits to toxicants gave a different order of sensitivity to the parameters as either situation described above. Reproduction was no longer the most sensitive parameter, as was seen with the above analysis. Body size was the most sensitive parameter, having the lowest NOEC value. One problem with the use of NOEC values is that it does not account for traits which do not change monotonically. The NOEC level for the length of the reproductive period does not take into consideration the fact that there was an initial significant increase in the reproductive period, followed by a reduction in the reproductive period. The NOEC simply takes the highest dose which is not significantly different to controls.

Growth, reproduction, and maintenance are inextricably linked to each other in the competition for resources within an individual. Allocation to reproduction depends on the reserves an animal has, which depends on feeding rate, which depends on body size and thus growth. Physiological trade-offs are caused by allocation decisions between two or more processed that compete directly with one another for limited resources within a single individual (Stearns, 1989). Reduced feeding rate is one mechanism that can potentially explain reductions in survival, growth, and reproduction occurring in toxicant exposed organisms (Allen et al., 1995) another factor which must be taken into consideration is the extra energy required by the stressed organism to maintain itself, in the induction of detoxification pathways and in repairing damage caused to cells by the toxicant.

4.4.5 Summary

Whilst life-cycle experiments provide a vast quantity of information on a number of key life cycle parameters, this amount of data can be confusing to interpret. Fortunately the data from individuals can be integrated into a mathematical model which predicts potential effects on population growth rates. Toxicants or other stressors typically affect several traits of an organism simultaneously. A toxicant for instance may have adverse effects on survival as well as reproduction, and we may wish to know which of the several traits is the most important. Population growth modelling can give a framework in which to investigate this. Life history traits can be compared using sensitivity analysis, which can indicate which of the traits leads to the largest impact on population growth rate.

An underlying concept to life history changes and toxic effects of pollutants is energy flow. The dynamic energy budget theory aims to explain how individuals acquire and utilise their energy. Toxicant stress may alter how organisms acquire their energy; for example, in reducing feeding rate caused by avoidance behaviour, by direct effect of the toxicant, or via reduced ability to assimilate energy from their food. Toxic effects may also alter the way the individual allocates the energy it has between growth, maintenance, and reproduction. Toxicants may have direct effects on reproduction, but also due to the coupling between the various processes of energy uptake and use, many other effects of compounds have an indirect effect on reproduction. The DEB model describes the routes that translate these effects into an effect on reproduction (Kooijman, 1993). An extension of the DEB theory has been developed to analyse life cycle toxicity data, called DEBtox (Kooijman and Bedaux, 1996b), which describes the effects of toxicants on energy flow and can also predict the mode of action of the

toxicant.

Chapter 5

Linking Individuals to Populations

5.1 Introduction

Effects of toxicants on the stability of populations can result from effects on individual life history traits, which in turn are caused by toxicant induced effects at the cellular and molecular level. Ecotoxicological testing aims to predict toxicant effects on populations, but necessity usual means that most tests must look at single endpoints over a short period of time. Since it is not practical or particularly desirable to use populations of organisms in toxicity tests to assess population responses an alternative is needed. Life-cycle toxicity tests offer a better option than typical short-term tests as they give information on a number of life history parameters such as survival, reproduction and time to maturity, which can in combination be used to predict effects on population parameters such as population growth rate (λ). The data on the parameters measured in life-cycle tests on individuals can, thus, be integrated to predict population growth rate, and the effects of toxicants on the life history parameters can be used to predict changes to this.

Alterations in life history parameters are thought to be mediated by toxicant effects on how energy is utilised by an individual. The dynamic energy budget (DEB) theory aims to describe how individuals acquire and utilise assimilated

energy. It can be used to describe how toxicants impact energy usage in an attempt to bridge the gap between individual and ecosystem-based approaches to environmental toxicology (Kooijman and Metz, 1984). DEB, thus, represents a process based approach to describing the effects of toxicant on life-history and the ultimate integration of these parameters to describe toxicant effects on population growth rate.

5.1.1 Further introduction to demography

Demography is central to life history theory, it connects age- and stage- specific variation in mortality and fecundity to variation in fitness (Stearns, 1992). The timing of life-cycle events can be combined to gain an understanding of how changes within individuals lead to a change in population growth rate. Demographic modelling has been successfully applied in a number of methods and models including the dynamic energy budget approach (Kooijman, 2000) and matrix population models (Caswell, 2001). The intrinsic rate of population increase, r_m, (also known as the Malthusian parameter) can be calculated from survival and reproduction schedules using the *Euler-Lotka* equation, as described in Kammenga and Laskowski (2000):

$$\int_{\alpha}^{\omega} e^{-r \cdot t} l_t \cdot m_t dt = 1$$

Where α is age at maturity, ω is age at last reproduction, t is age in general, l_t age-specific survival, and m_t is age specific offspring number.

More often the measure of population growth is documented as the population multiplication rate, λ, which gives a specific growth value over a set time. It is

calculated from the *Euler-Lotka* equation, $\lambda = e^r$ (Calow et al., 1997). Whilst it is a valuable means of integrating the effects of chemicals on single parameters, analysis of population growth rate is not a simple panacea for predicting population effects in the field. For example, population growth rate modelling can only predict the *potential* performance of a population in an unlimited environment, and does not in its own right take effects due to, for example, density dependence into account. Further, population growth models in ecotoxicology do not take into account the fact that chemical concentration may change over time or that subsequent generations may not respond identically to exposure due to, for example, genetic adaptation (Stark and Banks, 2000).

There are two types of population growth models used to estimate *r*. Age-specific and stage-specific models. Each type has its own advantages and disadvantages. The stage-specific model includes time to maturity as a parameter, which has been shown to be of particular importance in changes in population growth rate as a result of toxicant exposure (Kammenga et al., 1996). Staged models do not require as much information as age-structured models; however the disadvantage of this is that stage-structured models do not take into account changes in reproduction over time. With age-specific models it is possible to determine the most important age classes affecting population growth rate however its main disadvantage is the requirement for data taken at regular time points.

The approach taken in this thesis to assess population growth rates is using a simple two stage population growth model (Caswell, 2001; Neubert and Caswell,

2000). The two stages are juveniles (pre-reproductive animals) and adults (reproducing animals).

The extent to which population growth rate responds to toxicant exposure depends on the severity of the toxicant effects on the individual life history traits as well as on the sensitivity of λ to changes in each of the individual traits contributing to it (Levin et al., 1996). If $\lambda = 1$, the population will be at a steady state, if $\lambda < 1$ the population will be declining and if $\lambda > 1$ the population will be increasing.

5.1.2 Elasticity analysis

To investigate the extent to which changes in demographic properties change under a specific condition, sensitivity analysis for each of the included life-cycle parameters can be performed. The effect on λ will vary depending on the sensitivity to changes in each of the contributing parameters when other vital rates are kept constant (Caswell, 1978). As the parameters contributing to λ are not on the same scale, sensitivity analysis of vital rates can not be directly compared. To combat this, elasticity analysis was developed, which allows comparison on a common scale between proportional changes in vital rates. Elasticity is the proportional change in λ brought about by a small proportional change in one of the vital rates that contribute to it (Caswell, 1978; De Kroon et al., 1986). So, for example, if the elasticity of a vital rate is large, it will have a greater impact on λ than other parameters when they are all changed by the same proportion.

5.1.3 DEB

The theory of Dynamic Energy Budgets (DEB) (Kooijman, 2000) describes how individuals acquire and utilize energy, based on a set of simple rules for metabolic organisation. According to the principle of allocation (Levins, 1968) and the dynamic energy budget theory (Kooijman, 2000), energy resources in an organism are partitioned between reproduction, somatic maintenance, and growth. These traits are inextricably linked to each other in the competition between one trait and another for finite energy resources.

Animals obtain their energy from oxidation and reduction reactions and get carbon from organic compounds. The food ingested by an animal is digested and transformed into faeces and egested. The energy derived from the food is taken up via the blood, which exchanges it with storage, and delivers energy to somatic and reproductive tissues as illustrated in Figure 5.1. A central assumption in the DEB model is that growth and maintenance compete more directly with each other for available energy than with reproduction. This is known as the k-rule for allocation. A fixed part of the energy, k, is used firstly to maintain the animal (this includes all the processes that are necessary to keep the organism alive) and secondly for growth. The rest, $1-k$, is used for development or reproduction. Energy requirements for maintenance always have priority over growth, so growth ceases if all energy available for maintenance plus growth is used for maintenance (Kooijman and Bedaux, 2000). The assumptions that lead to the DEB model are given in Table 5.1.

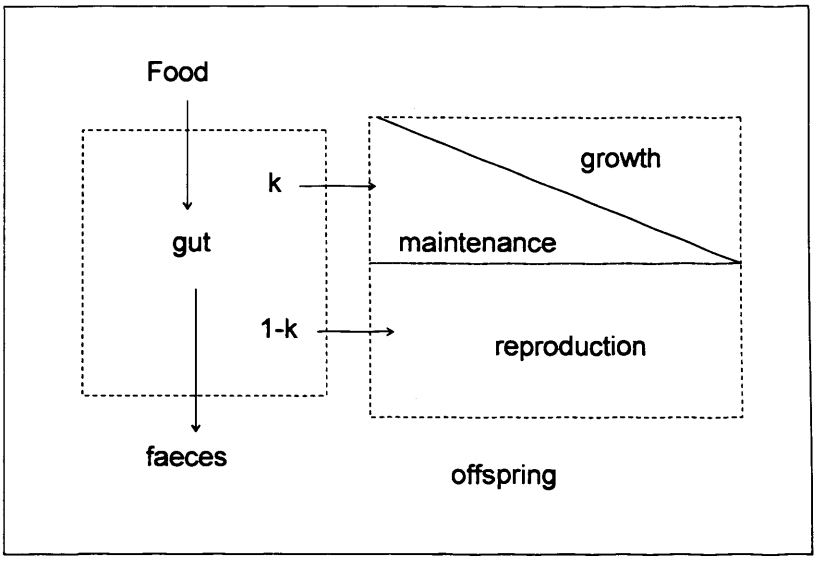


Figure 5.1: Energy channelling in the dynamic energy budget theory, taken from Kooijman and Metz (1984).

It is assumed that in a favourable environment the energy in an individual is partitioned so that the combination of these life cycle traits produces the optimal fitness for the individual. When an organism is stressed in some way then the combination of these traits may alter, caused by alterations in energetics through increased maintenance costs or decreased assimilation of energy. When an organism encounters a polluted environment, it needs to detoxify and repair damage, which it does by producing a range of stress responsive proteins. The stress responsive proteins which have been identified in invertebrates include the HSPs, MTLs, GSTs, MAPKs, CYP450s, and protein/enzymes involved in the anti-oxidant system. Since the production of these proteins, to combat and reduce stress, is energetically costly, it follows that the energy used in the production of these proteins would be diverted from growth, or reproduction. Under toxicant stress, therefore, physiological trade-offs are caused by allocation decisions between two or more processes that compete directly with one another for limited

Table 5.1: The assumptions that lead to the DEB model, as formulated for multicellular animals, taken from Kooijman (2000).

General	
1	Structural body mass and reserves are the state variables of the individuals and they do not change in composition (strong homeostasis)
2	Food is converted into faeces and assimilates derived from food are added to reserves, which fuel all other metabolic processes. These processes can be classified into three categories: synthesis of structural body mass; and of reserves (including the embryonic reserves via reproduction); and processes that are not associated with synthesis of biomass. Products that leave the organism may be formed in direct association with these three categories of processes, and with the assimilation process.
3	If the individual propagates via reproduction it starts in the embryonic stage, which initially has a negligibly small structural body mass (but a substantial amount of reserves)
Specific	
4	The reserve density of the hatchling equals that of the mother at egg formation. Foetuses develop in the same way as embryos in eggs, but at a rate unrestricted by energy reserves.
5	The transition from embryo to juvenile initiates feeding, and from juvenile to adult initiates reproduction, which is coupled to ceasing maturation. The transitions occur when the accumulated energy invested in maturation exceeds a threshold value.
6	Somatic and maturity maintenance are proportional to body volume, but maturity maintenance does not increase after a given accumulated investment in maturation.
7	The feeding rate is proportional to the surface area and depends hyperbolically on food density
8	The reserves must be partitionable, such that the dynamics is not affected; the use of reserves does not depend on food density; the reserve density at steady state does not depend on structural body mass (weak homeostasis).
9	A fixed fraction of energy, utilised from the reserves, is spent on somatic maintenance plus growth, the rest on maturity maintenance plus maturation or reproduction (the k-rule).
10	Under starvation conditions, individuals always give priority to somatic maintenance and follow one of two possible strategies • They do not change the reserve dynamics (and so continue to reproduce) • They cease energy investment in reproduction and maturity maintenance (thus changing the reserve dynamics)
11	Ageing-related damage accumulates in proportion to the concentration of damage-inducing compounds, which accumulate in proportion to the volume-specific metabolic rate.

resources within a single individual (Stearns, 1989). Evidence for the reduction of energy reserves in toxicant exposed organisms comes from De Coen and Janssen (2003) who exposed *Daphnia magna* to cadmium chloride and found that lipid reserves were significantly reduced compared to controls. Similar effects have been described by Ribeiro et al. (2001) who found that parathion exposed *Porcellio dilatatus* (woodlice) had significantly decreased protein, glycogen and lipid contents, all of which are energy reserves or used in energy generation.

Since its inception by Kooijman and Metz (1984), DEB has been used to describe how toxicants impact energy usage in an attempt to bridge the gap between individual and ecosystem-based approaches to environmental toxicology (Kooijman and Metz, 1984). They examined responses of individuals under chemical stress, in terms of individual energy balance. The general model identified that any chemical that hampers growth will necessarily affect reproductive rate. This model framework has been developed further to produce the DEBtox modelling software, both in a theoretical and practical way, to explain the allocation of energy under toxic stress (Kooijman and Bedaux, 1996a).

5.1.4 DEBtox

DEBtox was developed to provide a process-based description of results from toxicity tests (Bedaux and Kooijman, 1994; Kooijman and Bedaux, 1996a). The DEBtox software attempts to link individual responses to population effects and to produce, for risk assessment purposes, a summary of life-cycle toxicity data (Jager et al., 2004). DEBtox uses a number of parameters including the no effect concentration (NEC), defined as the highest concentration having no effect on the

test organism, for survival, and for growth/reproduction, to specify an energetic mode of action for a toxicant over an infinite exposure period. The internal concentration of a toxicant increases the probability of dying when the concentration exceeds a threshold; the NEC (Kooijman et al., 1996b). Figure 5.2 illustrates where in the energy allocation pathway toxicants can exert their effects.

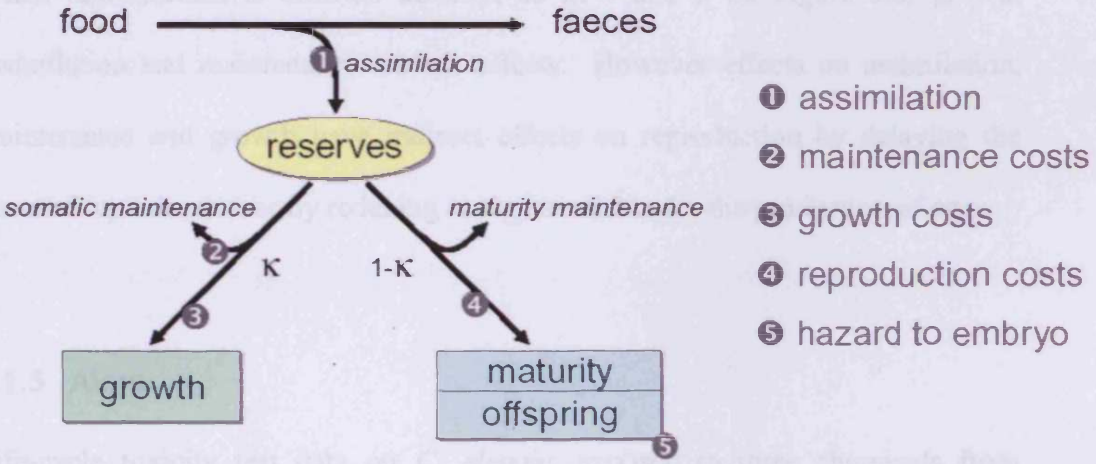


Figure 5.2: The possible places where toxicants can impacts on energy that are described by the DEBtox program.

In DEBtox there are five models which represent different toxic modes of action of a chemical. Life-cycle toxicity test data is fitted to each model and the best fit is selected. The modes of action are described below:

1 – Assimilation. The organism is unable to acquire as much energy, either from inefficient energy assimilation from the food, or the toxicant may directly affect the feeding rate.

2 – Maintenance costs. The energy costs required to keep the individual functioning properly are increased.

3 – Growth costs. The toxicant may exert its toxic effects on growth, in that it can either increase or reduce growth. As growth is inextricably linked to reproduction effects will be seen in this parameter also.

4 – Reproduction costs. There may be direct effects of the toxicant on reproduction, for example, in the increase of energy costs of producing each egg

5 – Hazard to embryo. The toxicant may directly impact the survival of embryos.

When reproduction is affected directly, as in 4 and 5 on Figure 5.3, growth assimilation and maintenance are not effects. However effects on assimilation, maintenance and growth have indirect effects on reproduction by delaying the onset of reproduction or by reducing energy available for the production of eggs.

5.1.5 Aims

Life-cycle toxicity test data on *C. elegans* exposed to three chemicals from different chemical classes (fully described in Chapter 4) were processed using a population growth model and DEBtox model to predict population level effects and specify the mechanistic basis of toxicity from data on the individual. The three chemicals used were cadmium, fluoranthene and atrazine, which were selected as representative of three classes of pollutants; metals, non-polar organic compounds and specifically acting compounds,

This study was designed to

1 – Model the effects on λ of toxicant exposed individuals.

2 – Investigate the sensitivity of λ to changes in the life history traits

3 – Investigate the toxicant mode of action using DEBtox

5.2 Approach

The data which is used in the two models described below are fully described in Chapter 4. See Section 4.2.2 for details of the life-cycle toxicity tests.

5.2.1 Population modelling framework

Life-history data from the previous chapter was integrated into a 2-stage matrix population model (Figure 5.3) as described by Neubert and Caswell (Caswell, 2001; Neubert and Caswell, 2000); the two stages are juveniles and reproductive adults. Every 12 hours the juveniles can either stay as juveniles or become reproductive adults, reproductive adults reproduce and can either stay reproductive or become post-reproductive. Juveniles and adults also have a probability of surviving during the 12 hour period.

The model has 5 parameters:

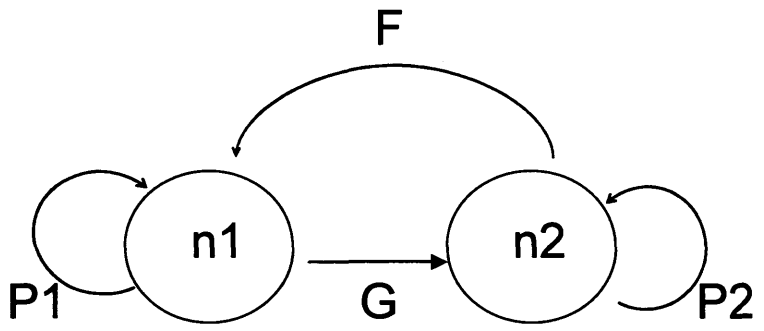
1) Maternity: the number of eggs per worm per 12 hours (F)

2) Juvenile survival rate (Sj)

3) Adult survival rate (Sa)

4) Time as a juvenile (Tj)

5) Time as a reproductive adult (Ta)



$$n_1(t+1) = P_1 n_1(t) + F n_2(t)$$

$$n_2(t+1) = G n_1(t) + P_2 n_2(t)$$

Figure 5.3: Diagrammatic representation of a two stage matrix model for population growth rate and equations. Where n_1 is the number of juveniles, n_2 is the number of adults, P_1 and P_2 are the probabilities of juvenile and adult survival respectively, G is the probability of a juvenile becoming adult and beginning to reproduce, F is the reproductive output of the adults.

For estimating the model parameters the following information was used:

- The number of eggs that the worms produce until reproduction ceases (**n**).
- The time taken for all of the eggs to be laid, the reproductive period, recorded in 12 hour time intervals (**Ta**).
- Time to maturity, defined as the time to the moment that the first egg was laid. This was measured by monitoring the worms at frequent time intervals and recording the time at which the first egg was observed (**Tj**).
- Mortality of the juveniles up to 45 hours on large NGM agar plates.
- The number of worms that die before Tj, which contributes to juvenile mortality.
- Mortality of the adults, recorded at 12 hour time intervals.

Estimating the model parameters:

1) The expected total reproduction per worm, n, was divided by Ta to calculate the reproductive rate, M.

2) Juvenile survival rate was calculated as the mean survival during the pre-reproductive time period.

3) Adult survival rate was calculated as the mean survival during the reproductive period.

4) Tj was obtained from the life-cycle data.

5) Ta was obtained from the life-cycle data.

The probability of juveniles growing into reproductive adults was calculated as the reciprocal of Tj, 1/Tj.

For each experiment mean parameter values were calculated for the control and each dose.

5.2.2 DEBtox

Life-cycle data was also used in the DEBtox model, for this analysis reproduction over time, survival and body growth over time data were required. The basis of the model is described fully by Kooijman and Bedaux (1996b). Adaptations made to this model for use with the nematode *C. elegans* and algorithmic manipulations of the life history parameters were performed by Dr. Tjalling Jager and are described below; full descriptions of the model changes can be found in the respective articles:

- Simultaneous modelling of all traits (Jager et al., 2004).

- Different shape of growth curve due to feeding limitations in juveniles, and a maximum total number of eggs for hermaphroditic *C. elegans* due to sperm limitation (Jager et al., 2005).
- Allowing the body size at the start of reproduction to decrease with stress (Alvarez et al., 2005), due to the fact that *C. elegans*, when stressed, is apparently able to decrease sperm production meaning that reproduction starts at smaller body size, with less fertilised eggs being produced.

Specific to the data sets from Chapter 4, two additional corrections to the model were required. The first correction being a general one, and the second specific to *C. elegans* (and perhaps other self-fertilising species where the number of sperm cells is limited).

1. The model described in Kooijman and Bedaux (1996b) makes use of the scaled length in the equation for reproduction (actual length is divided by the maximum length in the control). However, two modes of action affect the final body size (effects on assimilation and maintenance), and this effect has to be included in the scaling of the length. This is done in the model applied, although the quantitative effects are small.
2. The length at puberty occurs as a parameter in the reproduction equation, where it represents the maturity maintenance (the energetic costs to maintain a certain state of maturation). Both the length at puberty and the total number of eggs that can be fertilised are a function of the chemical concentration that is present in the tissues of *C. elegans*, and can thus change in time as accumulation takes place, (theoretically, this accumulation can occur up to and

even past the onset of reproduction). However, due to the small size of *C. elegans* and its intimate contact with the exposure medium, it is very likely that accumulation reaches equilibrium well before maturation onset and, thus, the costs for maturity maintenance and the total number of eggs are fixed when the transition from juvenile to adult is complete (see Kooijman (2000)).

Given the above, therefore, a correction was made to the DEBtox model to fix these two parameters as soon as reproduction starts.

5.3 Results

5.3.1 Population growth rate of controls

The population growth rates (λ) (per 12 hour period) of controls from each of the cadmium, fluoranthene and atrazine experiments were 3.52, 3.33 and 3.45 respectively confirming the reproducibility of the experiments.

5.3.2 Population growth rates

Cadmium has large impacts on population growth rate as can be seen on Figure 5.4. The λ for controls was 3.52 per 12 hour period. At the highest dose, λ was 0.95, which would indicate that under exposure to 16 mg L^{-1} cadmium, the population would decrease and tend towards extinction. Dose 14 mg L^{-1} has a value just over 1, which indicates that this population can exist but at a near steady state and would not increase in size at this concentration of cadmium.

Fluoranthene also had an effect on population growth rate as can be seen on Figure 5.5. There is approximately a 28% reduction in λ between controls and the highest dose tested 4 mg L^{-1}, reducing λ to 2.41. This is equivalent to the reduction caused by cadmium a concentration of between 2-3 mg L^{-1}, showing that cadmium is far more toxic to populations than fluoranthene. Changes in life history traits in response to increasing concentrations of fluoranthene cause small reductions in population growth rate that follow a dose response pattern.

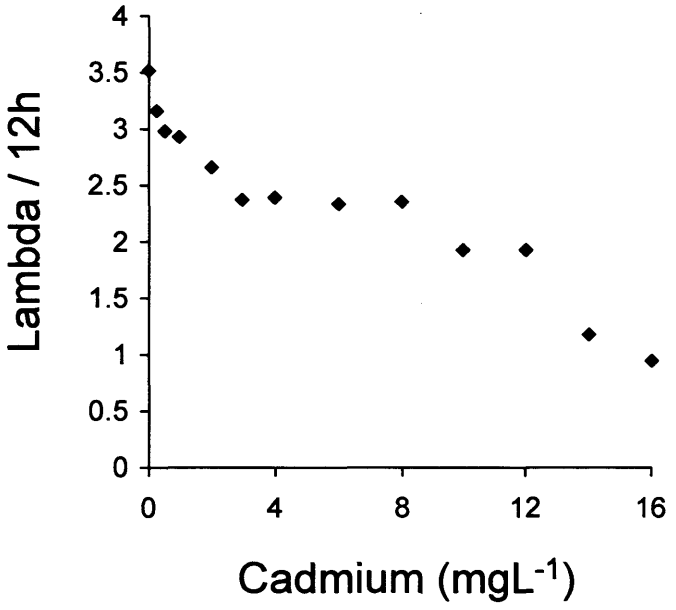


Figure 5.4: The population growth rate (λ) per 12 hours of *C. elegans* strain GE31 in control conditions and after being subjected to a range of cadmium doses.

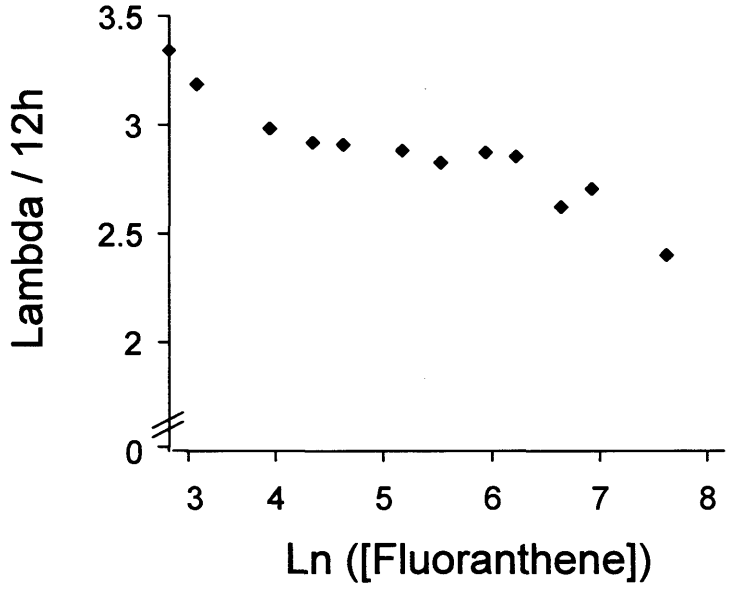


Figure 5.5: The population growth rate of *C. elegans* strain GE31 in control conditions and after being subjected to a range of fluoranthene concentrations. Fluoranthene concentrations are in $\mu g\ L^{-1}$, data point highlighted in red is for controls.

The effect of atrazine on λ was characterized by a step function response (Figure 5.6). Up to 50 mg L^{-1}, λ did not differ greatly from the controls. Doses above 50 mg L^{-1} did, however, have lower population growth rates than controls, but do not appear to follow a dose response relationship. There is a difference of approximately 34% between λ values for controls and the highest dosed worms, 300 mg L^{-1} atrazine. In terms of toxicant impacts on population growth rate atrazine was slight more toxic than fluoranthene but still has far less toxic impact on population growth rate than cadmium.

5.3.3 Elasticity analysis

Elasticity analysis was carried out to determine the relative sensitivity of λ to changes in each of the life history traits. The proportional change in λ as a result of a change in each vital rate is illustrated in Figures 5.7 – 5.9. From these figures it is easy to visualise the parameters which when changed by toxicant exposure have the greatest influence over population growth rate. Under control conditions, changes to juvenile survival would have the most impact on λ, closely followed by changes to reproductive output. Time to first reproduction was the third most important vital rate, closely followed by changes to reproductive period and finally number adult survival were least sensitive (Fig 5.7a). Large proportional changes in adult survival only produced a very small proportional change to λ.

The sensitivity of changes to vital rates on λ can change upon exposure to toxicants. In response to cadmium (Figure 5.7 b) it is possible to see that there is a change in the order of sensitivity to λ, with reproductive period having more effect on λ than time to

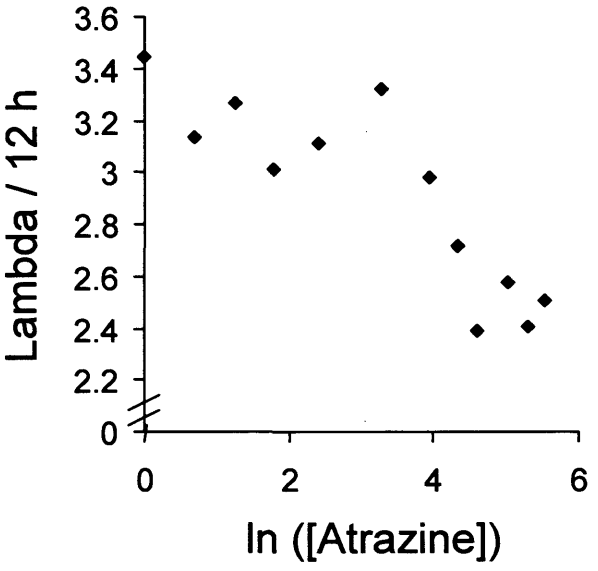


Figure 5.6: The population growth rate (λ) per 12 hours of *C. elegans* strain GE31 in control conditions and after being subjected to a range of atrazine concentrations. Atrazine concentrations are in mg L^{-1}.

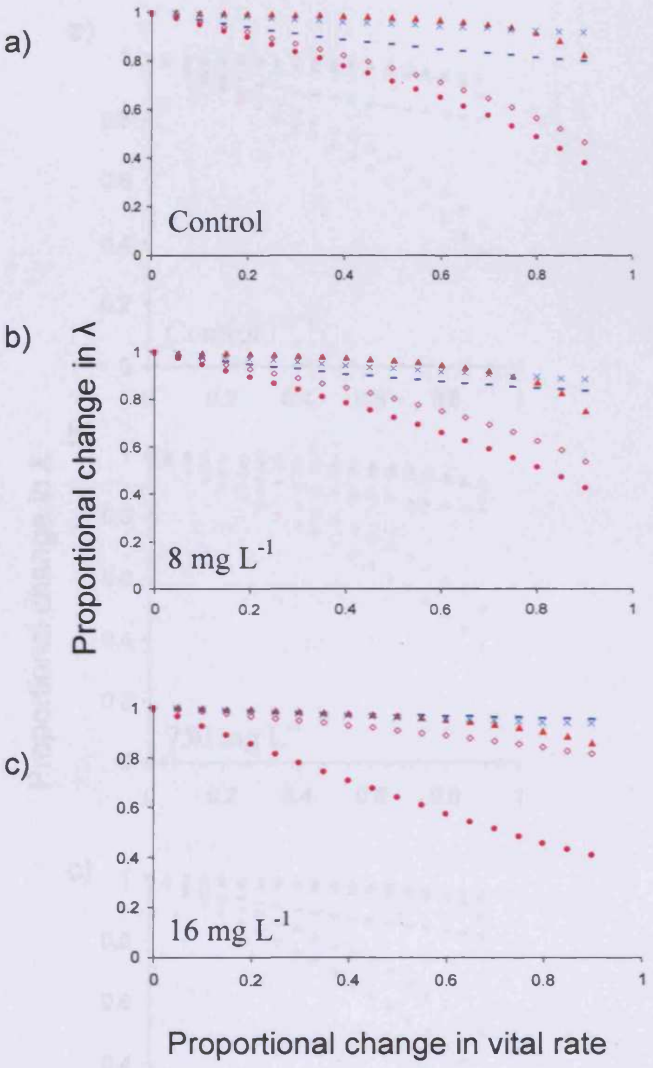


Figure 5.7: Elasticity analysis on the relative sensitivity of each life history parameter in the two-stage population growth model in response to cadmium exposure: a) control, b) 8 mg L^{-1} and c) 16 mg L^{-1} cadmium.

• Decrease in juvenile survival (Sa), ◇ decrease in reproduction (n), × decrease in adult survival (Sa), – increase in juvenile period (Tj), and ▲ decrease in reproductive period (Ta).

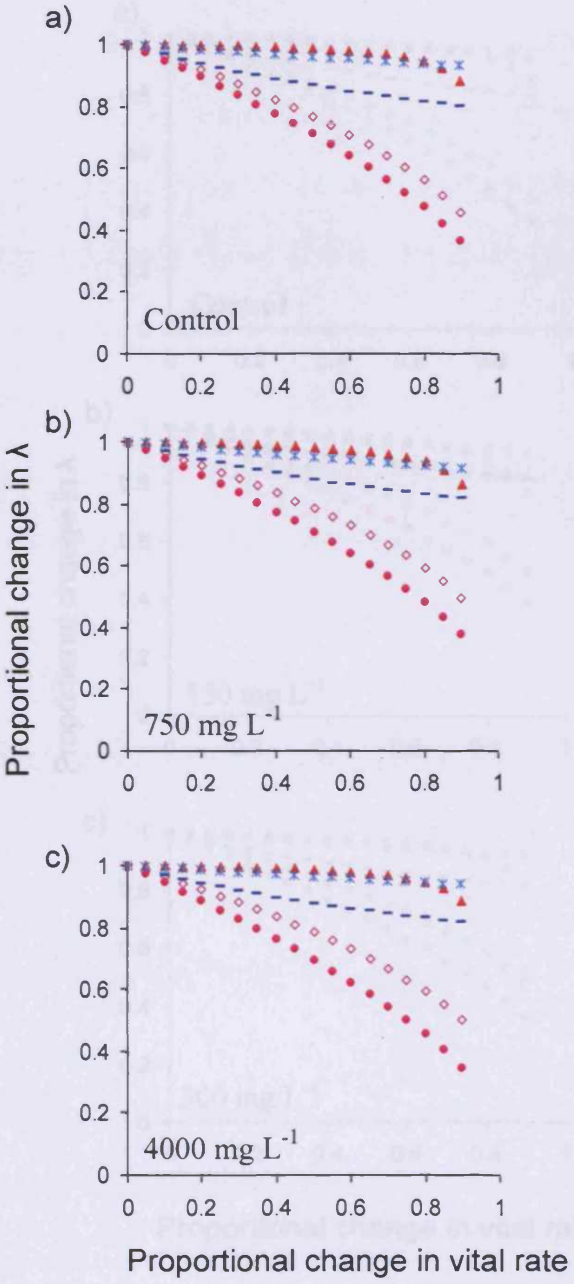


Figure 5.8: Elasticity analysis on the relative sensitivity of each life history parameter in the two-stage population growth model in response to fluoranthene exposure: a) control, b) 750 µg L^{-1},and c) 4000 µg L^{-1} fluoranthene.

• Decrease in juvenile survival (Sa), ◇ decrease in reproduction (n), * decrease in adult survival (Sa), – increase in juvenile period (Tj), and ▲ decrease in reproductive period (Ta).

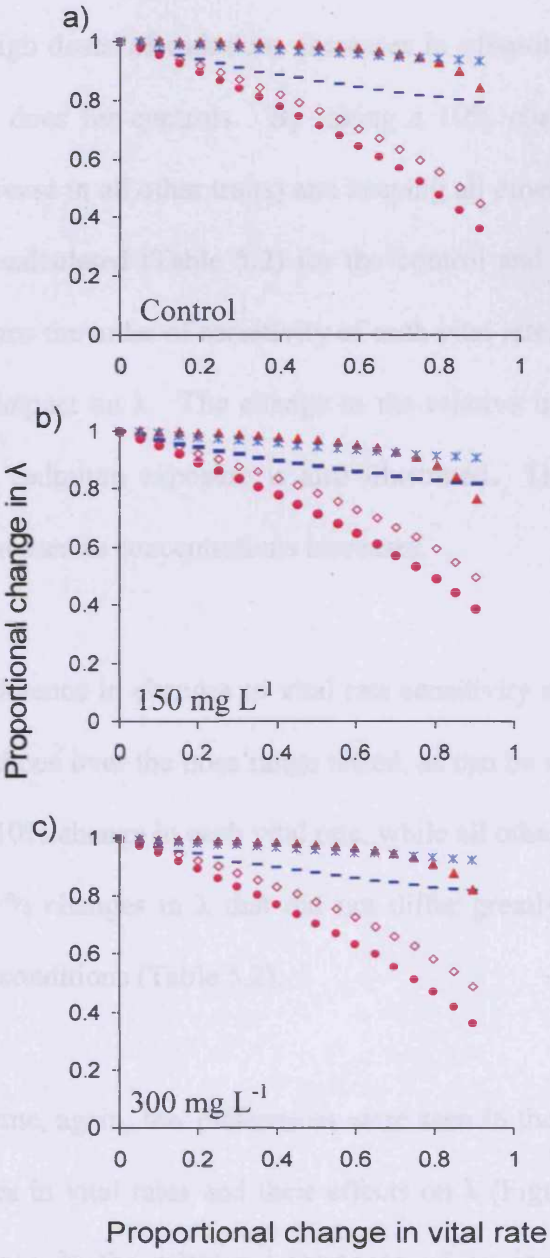


Figure 5.9: Elasticity analysis on the relative sensitivity of each life history parameter in the two-stage population growth model in response to atrazine exposure: a) control, b) 150 mg L^{-1} and c) 300 mg L^{-1} atrazine.

• Decrease in juvenile survival (Sa), ◇ decrease in reproduction (n), * decrease in adult survival (Sa), - increase in juvenile period (Tj), and ▲ decrease in reproductive period (Ta).

first reproduction. In Figure 5.7 c the change in importance of reproductive output is illustrated, so for high doses of cadmium decreases in offspring number has far less effect on λ than it does for controls. By taking a 10% change in each vital rate (increase in Tj, decrease in all other traits) and keeping all other traits the same, the % changes to λ were calculated (Table 5.2) for the control and 16 mg L^{-1} treatments. This analysis confirms the order of sensitivity of each vital rate, with juvenile survival having the largest impact on λ. The change in the relative importance of offspring number to λ under cadmium exposure is also illustrated. The contribution that Tj makes to λ also decreases as concentrations increases.

There was little difference in changes to vital rate sensitivity and their effect on λ in response to fluoranthene over the dose range tested, as can be seen in Figure 5.8 a, b, c. The results of a 10% change in each vital rate, while all other parameters were kept constant, produced % changes in λ that did not differ greatly between control and fluoranthene dosed conditions (Table 5.2).

In response to atrazine, again, few differences were seen in the comparisons between proportional changes in vital rates and their effects on λ (Figure 5.9 a, b, c). Small differences were seen in the relative importance of an increase to time to first reproduction and reductions in reproductive period. The results of a 10% change in each vital rate, while all other parameters were kept constant, produced % changes in λ that altered slightly between control and atrazine dosed conditions (Table 5.2).

Table 5.2: The % change in population growth rate, λ, when each vital rate is changed by 10%, whilst all other parameters are kept constant for cadmium, fluoranthene and atrazine under control and the highest dose tested for each chemical.

Vital rate		% change in λ	
	Cadmium	**Controls**	**16 mg L^{-1}**
N		4.0	1.7
Tj		3.4	0.8
Sj		5.1	7.4
Ta		0.2	0.4
Sa		1.0	0.9
	Fluoranthene	**Controls**	**4000 µg L^{-1}**
N		3.9	3.8
Tj		3.1	3.1
Sj		5.4	5.6
Ta		0.2	0.2
Sa		1.0	0.7
	Atrazine	**Controls**	**300 mg L^{-1}**
N		4.2	3.9
Tj		3.5	3.1
Sj		5.2	5.4
Ta		0.2	0.3
Sa		0.8	0.9

In general this analysis shows that reproductive output becomes less important in exposed animals. Tj became less important for atrazine, stayed the same for fluoranthene and much less important for cadmium. In all cases the effect of toxicant exposure caused an increase in the % change in λ caused by a 10% decrease in juvenile survival. Very small changes in Ta and Sa were shown with the importance of Ta increasing or having the same importance and Sa increasing slightly for atrazine but decreasing for cadmium and fluoranthene.

The % effects on vital rates between controls at the highest dose tested in the toxicity tests were examined for their relative effects on λ. The % change in each vital rate was calculated as the difference between controls and the highest dose tested for each compound. Table 5.3 details the % change in λ when a single vital rate was changed while all other parameters were kept constant for cadmium, fluoranthene and atrazine. It indicates that for all three compounds the adverse effects on reproductive rate contribute most to the decrease in population growth rate. For cadmium and fluoranthene time to first reproduction also had large contributions to changes in λ, in comparison with the other vital rates.

A comparison of the sensitivity of each vital rate and the sensitivity of responses to the changes in vital rates on population growth rate is also given in Table 5.3. The vital rates are ranked in order of the magnitude of the effect each toxicant has on the life history parameters, and on λ. This demonstrates that the most sensitive life history trait does not have always cause the largest impact on population growth rate. For example, the most sensitive life history trait to 4000 μgL^{-1} fluoranthene was the length of the reproductive period, however the largest influence on population growth

Table 5.3: The % change in population growth rate (λ) caused by the % change in vital rate (between control and the highest dose tested) obtained from life-cycle toxicity test data for cadmium, fluoranthene and atrazine. Each change in vital rate was assessed for its effect on λ individually, while all other parameters were kept constant.

Vital rate		% change in vital rate compared to controls	Ranking in sensitivity of changes to vital rate	% change in λ caused by % change in vital rate	Ranking in sensitivity of changes to λ
	Cadmium				
N	**(16 mg L^{-1})**	97	1	65	1
Tj		85	2	19	2
Sj		14	5	7	3
Ta		73	4	6	4
Sa		74	3	7	3
	Fluoranthene				
N	**(4000 µg L^{-1})**	42	2	19	1
Tj		33	3	10	2
Sj		3	5	2	4
Ta		7	4	0.1	5
Sa		44	1	4	3
	Atrazine				
N	**(300 mg L^{-1})**	55	1	27	1
Tj		13	3	4	2
Sj		0	5	0	5
Ta		8	4	0.2	4
Sa		29	2	2	3

rate was reproductive rate. For 16 mg L^{-1} cadmium, the most sensitive life history parameter, reproductive rate, also had the largest impacts on λ. However a large, 74%, decrease in adult survival caused only a 7% decrease in λ, compared to the same effect on λ caused by a 14% reduction in juvenile survival.

Under atrazine exposure reproductive rate was the most sensitive life history parameter and also had the largest effect on λ. The relative importance of time to maturity and length of the reproductive period was reversed between sensitivity of the vital rates and the effects on λ.

5.3.4 Modelling mode of action of chemical effect using DEBtox model suite

Life history data from Chapter 4 was fitted using the DEBtox suite of models. The parameter estimates from DEBtox are given in Table 5.4. Modelling using DEBtox can be used to evaluate the most probable energetic based "mode of action" for each toxicant. For cadmium and atrazine the mode of action that fitted the data the best was due to an effect of the compound on the amount of energy assimilated from food. For fluoranthene the mode of action that fitted the data the best was and effect of the compound on the energetic costs of growth and reproduction. The fits of the reproduction, survival and growth data to the best fitting "mode of action" model only are shown in shown in Figures 5.10, 5.11 and 5.12 for cadmium, fluoranthene and atrazine respectively.

Table 5.4: Parameters used in the DEBtox model

DEBtox parameters	Cadmium	Fluoranthene	Atrazine
Physiological			
Von Bertalanffy growth rate (day^{-1})	0.873	1.06	1.28
Initial length (μm)	17.7	24.1	32.3
Length at which ingestion is half of maximum (μm)	30.7	37.4	45.7
Length at start of reproduction (μm)	67.2	88.6	87.8
Maximum length (μm)	132	134	136
Maximum reproduction rate (eggs day^{-1})	122	208	210
Ageing			
Maximum total number of eggs	153	159	186
Damage killing rate (day^{-1})	0.00696	0.00494	0.00596
Damage amplification (day^{-1})	0.198	0.167	0.142
Toxicological			
Mode of action	Assimilation	Costs for growth and reproduction	Assimilation
Elimination rate (day^{-1})	0.0812	9.52	0.275
Half-saturation conc. for uptake (mg L^{-1})	27.7	n.e.	95.6
NEC for survival (mg L^{-1})	6.52	n.e.	85.6
Killing rate (mg L^{-1} day^{-1})	0.120	n.e.	0.0184
NEC for effects on growth/repro (mg L^{-1})	0.000131	0.000698	0.0956
Tolerance concentration (mg L^{-1})	7.54	4460	228
Decrease length at puberty due to chemical stress [-]	0.482	0.219	0.289
Decrease total eggs due to chemical stress [-]	3.69	1.69	8.13

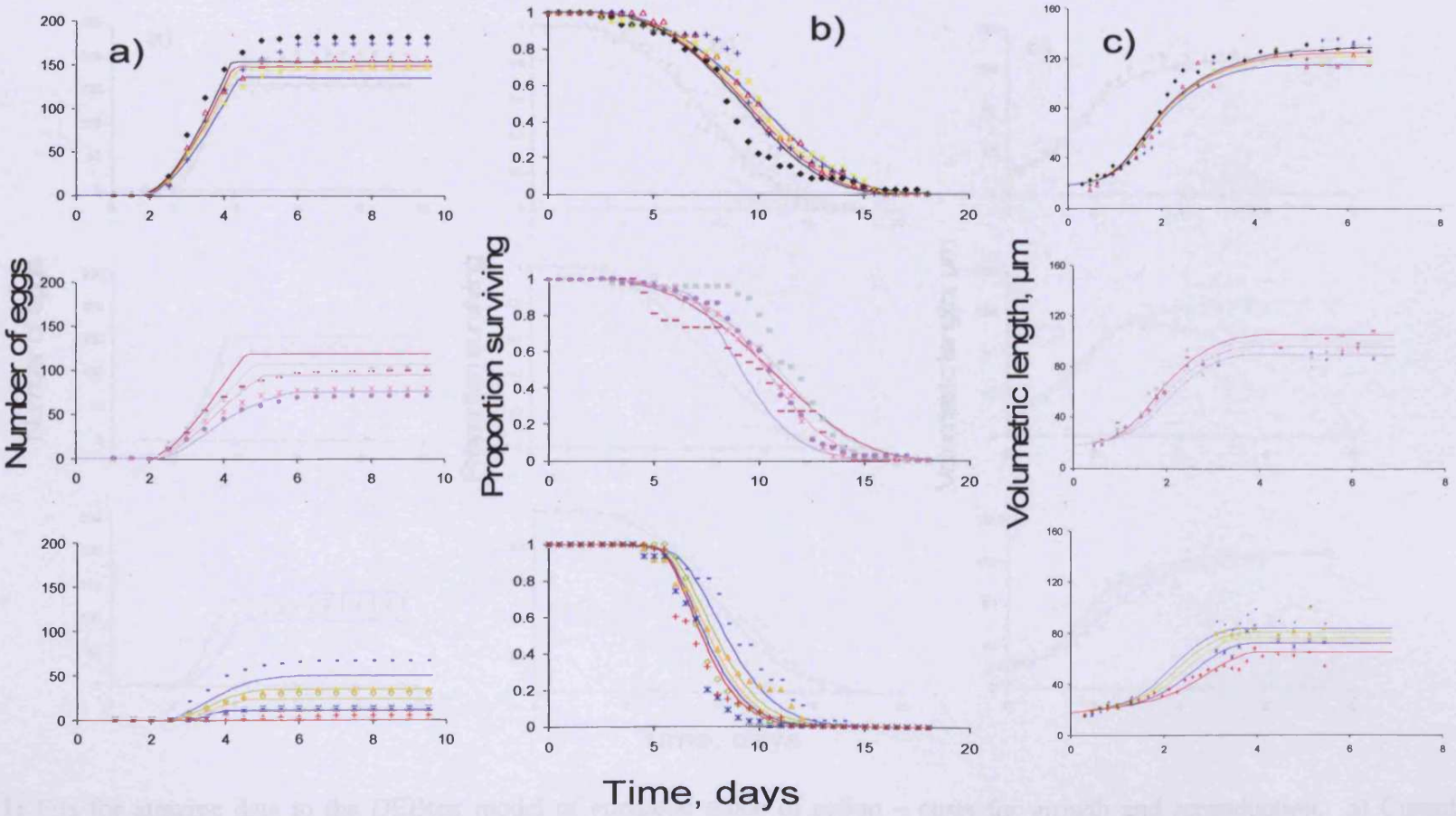


Figure 5.10: Fits for cadmium data to the DEBtox model of energetic mode of action - assimilation. a) Cumulative reproduction, b) Survival, and c) Volumetric growth. Concentrations are split over three graphs from low to high dose to enable visualisation.

— 0 — 0.25 — 0.5 — 1 — 2 — 3 — 4 — 6 — 8 — 10 — 12 — 14 — 16 mg L^{-1}.

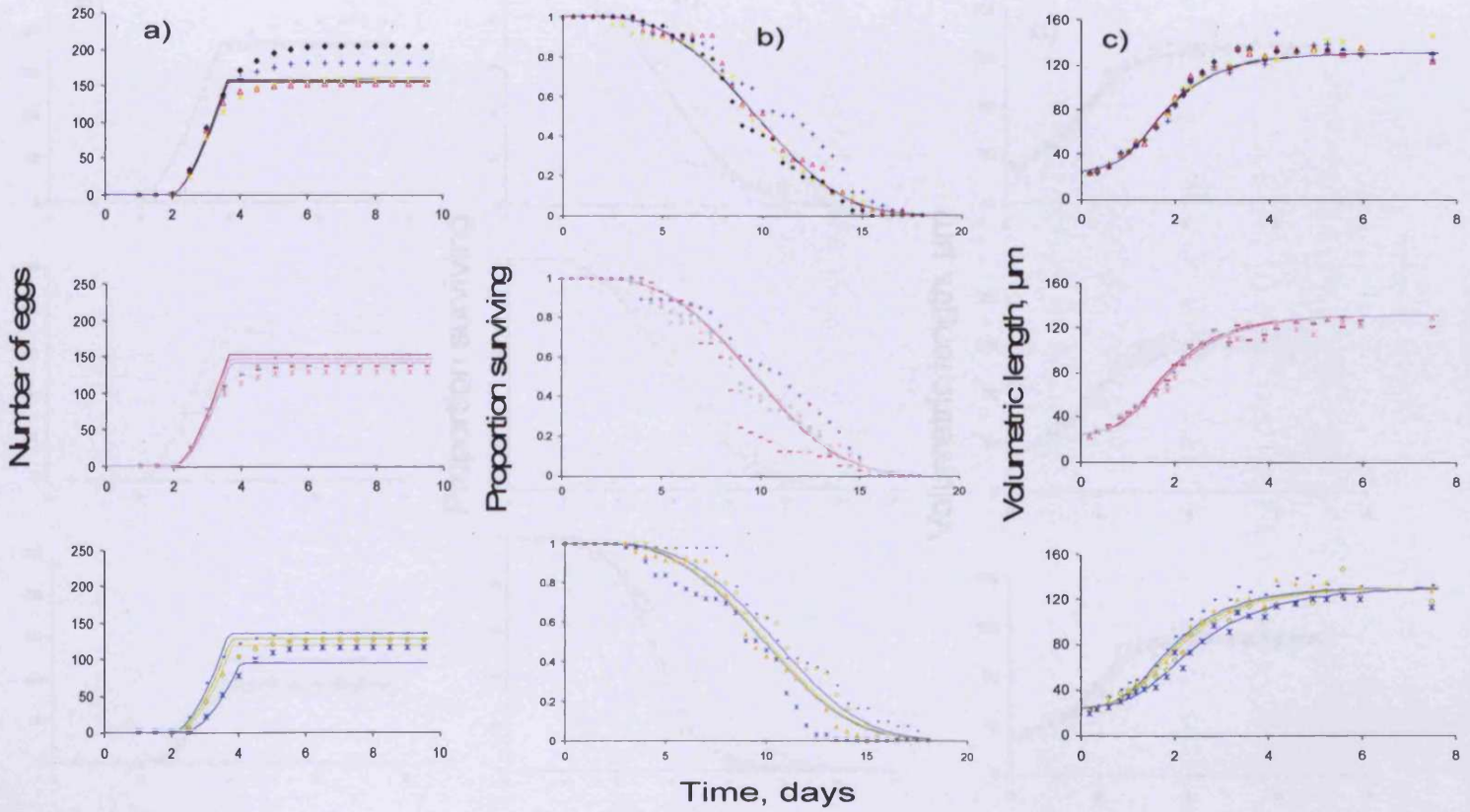


Figure 5.11: Fits for atrazine data to the DEBtox model of energetic mode of action – costs for growth and reproduction. a) Cumulative reproduction, b) Survival, and c) Volumetric growth. Concentrations are split over three graphs from low to high dose to enable visualisation. — 0 — 25 — 50 — 75 — 100 — 175 — 250 — 375 — 500 — 750 — 1000 — 2000 µg L^{-1}.

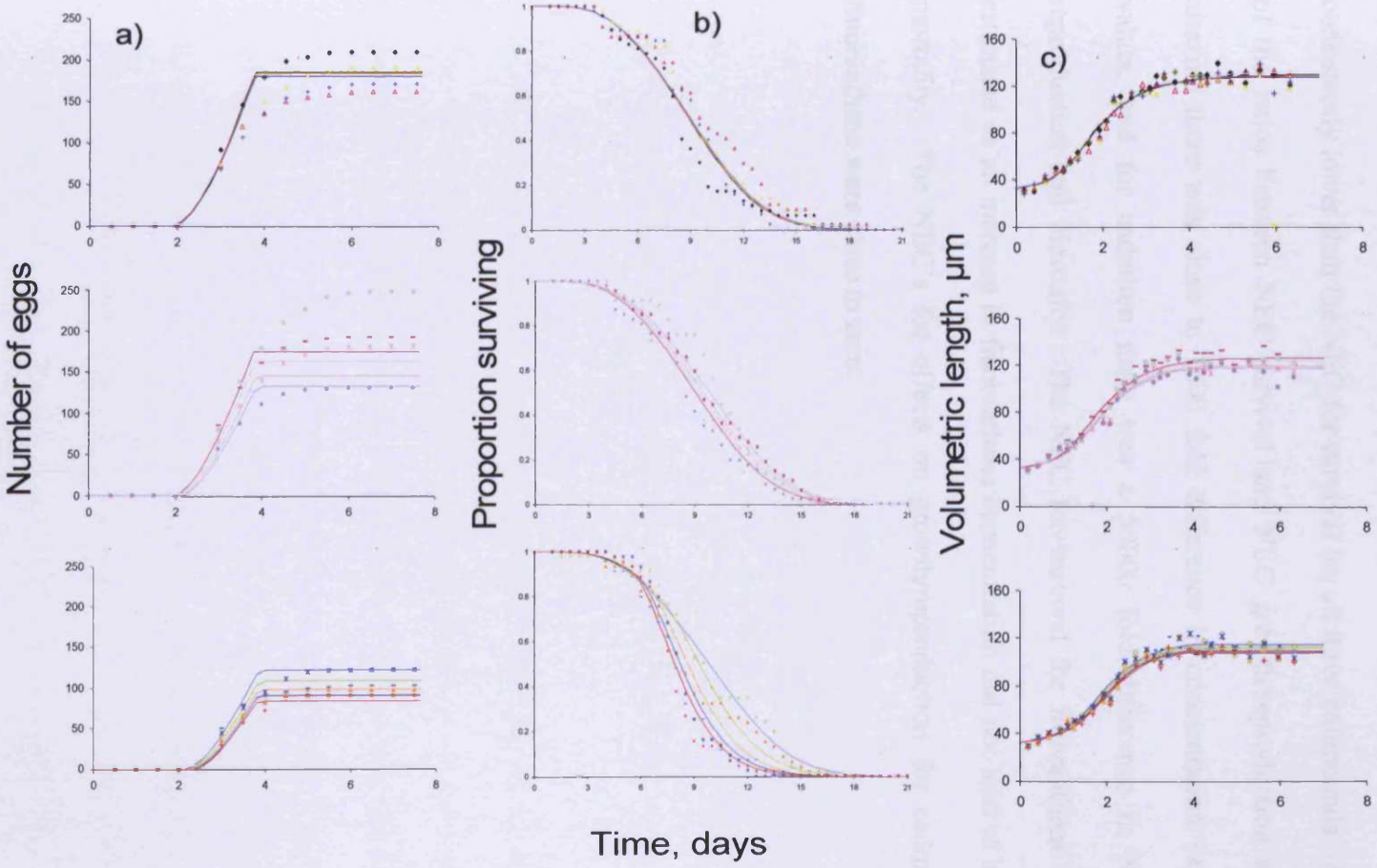


Figure 5.12: Fits for atrazine data to the DEBtox model of energetic mode of action - assimilation. a) Cumulative reproduction, b) Survival, and c) Volumetric growth. Concentrations are split over three graphs from low to high dose to enable visualisation.

— 0 — 1 — 2.5 — 5 — 10 — 25 — 50 — 75 — 100 — 150 — 200 — 250 — 300 mg L^{-1}.

DEBtox also provides a single ecotoxicological endpoint, which combined the various life-cycle endpoints described in Chapter 4, producing a No Effect Concentration (NEC) for survival and for effects on growth/reproduction assuming infinite exposure time and, thus, at internal equilibrium. The NEC for growth/reproduction is consistently lower than the NEC for survival for all three compounds. A comparison of the ratios between NEC survival and NEC growth/reproduction show that for atrazine there was close to 1000 fold difference in concentration values between values, and for cadmium there was a 50000 fold difference in NEC between reproduction and mortality. The NEC for survival for fluoranthene could not be estimated as an increase in fluoranthene concentration did not lead to an increase in mortality. The NEC's for effects on growth/reproduction for cadmium and for fluoranthene were close to zero.

5.4 Discussion

5.4.1 Population growth rate

The population growth rate and elasticity analysis presented in this chapter illustrates the potential of life table response experiments in ecotoxicology. The model used simplifies the rather complex multi stage life-history of *C. elegans*, which in fact includes four larval stages; a reproducing adult, a post-reproductive, and an alternative stress tolerant dauer stage, to a two-stage model concerned only with a pre-reproductive juvenile stage and a reproducing adult stage. Beyond reproductive age, *C. elegans* is unusual in that it has an extended post-reproductive period, which often continues for the same amount of time as both the juvenile and reproductive periods. For the purpose of this analysis post reproductive adults were not included as they do not contribute to population growth rate, however it may be that senescing worms may contribute to density dependence or have impacts, for example, on food availability.

Using the demographic approach presented, the effects of a toxicant on populations can be evaluated by integrating toxic effects on survival and fertility parameters of individual worms. In this way, it can become possible to predict long term consequences of chemical exposure (Van Straalen and Kammenga, 1998). The use of life-cycle assessment in the risk assessment of chemicals has been advocated in many studies as it provides more ecological relevance than observed effects on individual traits (Calow et al., 1997; Forbes and Calow, 2002; Kammenga et al., 1996; Maltby, 1999). However life tables do not accurately estimate the rate of growth or population size of real populations living in

contaminated ecosystems. In these wild populations, other process such as density dependence, predation and competition can all have simultaneous effects on individual and ultimately populations. There have been efforts to incorporate density dependent effects on population growth rate (Forbes et al., 2003; Linke-Gamenick et al., 1999). The demographic approach used here, however, does not take this and other complex processes into account., instead it predicts population growth in an "optimal" unlimited (e.g. space, food supply) environment assuming that the exposure concentration remains constant over time and that subsequent generations respond identically (Stark and Banks, 2000).

Each of the toxicants tested reduced population growth rate at the higher exposure concentrations. Cadmium had the largest impacts on λ reducing population growth rate in a dose dependent manner. At the two highest doses (14 and 16 mg L^{-1}) the populations were predicted to only be stable rather than increasing, due to the large effects of cadmium on the different life-history traits. At 16 mg L^{-1} the value of λ was less than 1, indicating that this population would be unsustainable and would ultimately go to extinction. Elasticity analysis showed that population growth rate is most sensitive to changes in parameters relating to the larval stage than the adult stage. This supports the finding of Stark and Banken (1999) who found that larval stages of the spider mite *Tetranychus urticae* were more sensitive to toxicants than adult stages. Changes to λ following cadmium exposure can, however, mostly be attributed to changes in reproductive rate upon cadmium exposure. This is due to the greater sensitivity to cadmium of this trait.

Fluoranthene exhibited a dose dependent reduction in population growth rate. The reduction in λ was not a large as that seen with cadmium, but this would be expected as the effects of exposure to high concentrations of fluoranthene were less than for the highest cadmium concentrations (see Chapter 4). Although there was a reduction in population growth rate for atrazine it did not follow a clear dose dependent pattern, a step pattern instead being observed. For concentrations up to 50 mg L^{-1} effects on λ was similar to that found in the controls. For doses above 50 mg L^{-1} a reduced λ value was calculated, but there was no clear dose dependent pattern.

Elasticity analysis showed that the trait which was most important in determining the population growth rate was juvenile survival in all conditions. The elasticity patterns varied in response to exposure for each of toxicants. For cadmium, the relative impact of change of selected traits on λ was reduced following exposure. For example increasing time to reproduction in control has a greater effect on λ than for worms exposed to for example 16 mg/L cadmium. This effect of exposure was also found for reproductive rate and adult survival. For other traits, however, such as reproductive period and juvenile survival, cadmium exposure decreased the effect of a proportional change on λ. For fluoranthene the same analysis showed that apart from a decrease in juvenile survival, the same proportional effect on each vital rate had either a decreasing impact or did not alter the impact on λ over the dose range. Atrazine showed that a proportional effect on reproductive rate and time to reproduction had a decreasing impact on λ following exposure, while a proportional effect on juvenile survival, reproductive period and adult survival had a greater impact.

It has been shown previously by Kammenga et al. (1996) that the most sensitive parameter to life history may have negligible effects on fitness (population growth rate). They found, using the nematode *Plectus acuminatus*, after sensitivity analysis that the most sensitive trait, the length of the reproductive period, which was reduced by 45% by cadmium, had a negligible effect on fitness; however, the least sensitive trait, time to first reproduction resulted in the largest change in λ. Kammenga et al. (1996) concluded that impacts on single life history traits need to be evaluated in a life-cycle context to ensure the proper assessment of toxicants, as simply taking the most or least sensitive parameter in risk assessment may over- or under-estimate risks to populations. A study by Forbes and Calow (1999) analysed data from a large number of life-cycle experiments, and found no general pattern with respect to the sensitivity of life history traits of individuals. This lead them to propose population growth rate was a good integrating endpoint for ecotoxicology, as it was equally sensitive to toxicant exposure as one or more individual level traits, and encompassed a number of traits together. It is not possible to determine statistically whether changes in λ caused by the changes in life history traits are more, less or equally as sensitive to the respective toxicant in this study, due to the lack of measure of variance of λ.

5.4.2 DEBtox

The DEBtox model for energetic mode of action assessment was fitted to the available life-history data for 5 different possible modes of action (costs for growth, costs for reproduction, assimilation, hazard to embryo, maintenance costs). The best fitting model was found to be toxicity through reduced

assimilation of energy from food for both cadmium and atrazine. For fluoranthene a combination of both increased costs for reproduction and increased costs for growth was found to give the best description of the observed responses.

The reduction in assimilation suggests that either of two processes could be occurring. First, the ability to efficiently acquire energy from the food may be reduced due to lower feeding activity (Kooijman and Bedaux, 1996b). Reduced feeding rate has been observed previously upon exposure of *C. elegans* to cadmium (Alvarez et al., 2005; Jones and Candido, 1999). This was done through measurement of the rate of pumping of the pharynx, which is the means by which *C. elegans* engulfs it bacterial food (Wood, 1988). In this study, pharyngeal pumping was not examined either in the cadmium or the atrazine experiment. The fit of the effects on assimilation of energy model agreed does, however, agree with a previous study by Alvarez et al. (2005) who examined the effects of cadmium on life history in *C. elegans* where an effect on pharyngeal pumping was also observed. Since assimilation has also been ascertained to be the toxic mode of action for cadmium exposed springtails *Folsomia candida* (Jager et al., 2004), this supports the fact that a change in rate of pumping of the pharynx may be a major reason for the change in energy assimilation suggested by the data.

The second possible effect of cadmium on the acquisition of energy would be through the toxicity of the metal on enzymes involved in electron transport during aerobic respiration. It has been shown that cadmium inhibits the activity of complexes II and III of mitochondrial electron transport chain (complex II – succinate: ubiquinone oxidoreductase, complex III – ubiquinol: cytochrome c

oxidoreductase) (Wang et al., 2004). As electron transport is at the basis of ATP (energy) production, any toxicant effects on components of this system could potentially affect the efficiency of energy assimilation.

The overall reduction in assimilated energy following cadmium exposure caused large reductions in body size and reproduction. The reduction in growth is likely to be caused largely by the reduction in energy available for this process. The *k*-rule of the DEB theory states that energy required for maintenance always takes precedence over energy for growth, so when available energy is reduced and extra energy is required to repair and detoxify cadmium, less energy is available for growth. It is assumed that cadmium reduces the ability to produce eggs due to the reduction in energy and resources required to produce eggs. The effects on growth will also contribute to the reduction in reproduction. Parameter values used in the cadmium model for assimilation can be compared with those used in Alvarez et al. (2005) as both studies are on *C. elegans*. One noticeable difference was in the parameter NEC for survival, which for Alvarez et al. (2005) was 0.559 mg L^{-1}, and in this study was 6.52 mg L^{-1}. This could be due to a different experimental temperatures (15 and 25°C respectively), or the use of different nematode strains GE31 and N2 respectively.

In the atrazine study effects on reproduction appear to be larger than effects on growth, indicating that the energy allocated to reproduction was reduced at a greater rate than for growth. One study on toxic effects of atrazine examined feeding rates of salamander in response to increasing atrazine concentrations but found that feeding rate was unaltered compared to controls (Rohr et al., 2004).

Feeding rate was not measured in the experiment presented in this chapter, which may have elucidated whether effects on assimilation were brought about by a reduction in feeding rate. However examining pharyngeal pumping would only highlight feeding rates, and could make no inference about the efficiency of energy assimilation from the food. The known mode of action of atrazine on plants is to decouple electron transport during photosynthesis. If atrazine has similar effects on electron transport in invertebrates, then, as with cadmium, effects on the components of the electron transport system in *C. elegans* could explain alterations in the efficiency of energy acquisition.

For fluoranthene, assimilation of energy did not appear to be the major toxicant effect on energy, instead direct costs on reproduction and costs on growth were suggest by the DEBtox analysis. The fact that there was no reduction in final body size as fluoranthene dose increased, suggests that this chemical does not have a substantive effect on the assimilation of energy from food. That greater energy is required for growth in fluoranthene exposed worms, however, is shown by their slower rate of growth (see Chapter 4, Figure 4.14) but to ultimately the same body size. The reproduction costs indicate that fluoranthene directly affects the energy required to produce offspring.

The examination of the ratios between NEC for effects on survival and NEC for effects on growth/reproduction are in agreement that sublethal effects are far more sensitive to toxicants than mortality and show support towards the use of sublethal parameters to assess toxicity of chemicals. In comparison with the NOEC values presented in Chapter 4 (Table 4.8) the NEC values were much lower. The NEC

values for effects on growth/reproduction were between 3-5 orders of magnitude smaller than NOEC values for reproduction and final body size. The NOEC and NEC values for survival were closer to each other, within one order of magnitude of each other for cadmium and atrazine. The NEC for fluoranthene could not be estimated as an increase in fluoranthene concentration did not lead to an increase in mortality, which was also indicated by no significant difference in lifespan seen at any of the doses tested, hence in this case NOEC is taken as the highest dose tested. There has been much debate over the use of NOEC in regulatory testing (Kooijman, 1996; Van Der Hoeven et al., 1997). A number of drawbacks have been pointed out by van der Hoeven (1997) for the use of NOEC as a summary statistic. These include that the value is an experimental concentration and as such is dependent on experimental design, and based on the accuracy of the experimental test. It is calculated as the largest concentration for which there is no significant statistical difference from that of controls for a specific parameter. In this way the NOEC is dependent on sample size, variation in the parameter and on the chosen significance level. Just because there is no statistical significant effect between controls and a dose does not mean to say that there is no effect at all. Thus the NEC has been proposed as an improvement (Kooijman, 1996) to the use of NOEC, as it deals with concept of a concentration where no effect is seen, and is obtained from process based models. However it comes with its own drawbacks in that NEC estimates are model dependent (Van Der Hoeven, 1997). The data from standard ecotoxicology tests may not be sufficient for model verification, and would prove to be too costly to produce the amount of data required on a standard basis.

5.4.3 Summary

Analysis of the effects of toxicants on life history parameters through the use of demographic models can provide a powerful approach to determining the mechanistic basis of toxicant effects on population dynamics. In this study, a single value for population growth rate was obtained for each dose. This approach can be strengthened using bootstrap estimation methods (Sibly et al., 2000) which would then allow for statistical analysis of parameters such as NOEC which would allow for the comparison between NOEC, NEC, and EC_{50} values.

DEBtox proved a particularly useful tool for evaluating the most likely energetic mechanism for the toxicity of each chemical through the energy budgets. The model provided insight into how a toxicant may affect the energy use of an organism, and explains the changes in life-history traits on the basis that the changes are mediated by alterations to the amount of energy available, or required for each process. The value of this approach is two fold. First, it provides a useful starting point for the identification of possible biomarkers of the toxicity of chemicals for particular responses. For example, chemicals effecting maintenance costs may be expected to increase expression of protective systems (e.g. metallothioneins, HSPs), while those affecting assimilation may down-regulate mitochondrial associated process. Second, it can illustrate which compounds work through the same mechanism and so have the potential for joint effects to be described through the concentration addition model rather than the independent action model (Jonker et al., 2005). Finally, the NEC values could provide a better parameter for risk assessment of chemicals than NOEC values, which are calculated from toxicity tests and require a large range of concentrations to be

evaluated for the accurate prediction of NOEC (Jager et al., 2004; Kooijman et al., 1996a).

Chapter 6

Individuals, populations and molecular: the effect of the pesticide aldicarb on *C. elegans*

6.1 Introduction

Ecotoxicogenomics, a term proposed by Snape et al. (2004) to describe the integration of genomic based science into ecotoxicology, is a recent and fast growing discipline. The combination of toxicology, ecology and molecular biology will lead to a fuller understanding of the molecular mechanisms of toxic response to chemicals, the impact this has on the life history of an individual, and what this could potentially mean for populations.

6.1.1 Individuals and Populations

Ecotoxicology to date has been mainly based on the responses of individuals to toxicants at the life history level either on mortality, (Donkin and Williams, 1995; Ura et al., 2002), sublethal endpoints such as growth and reproduction (Alvarez et al., 2005; Barata et al., 2000; Kammenga et al., 1997; Linke-Gamenick et al., 1999), or cellular level effects (Popham and Webster, 1979; Svendsen et al., 2004). The few studies conducted to date at the molecular level have focussed on the responses of single or a few genes at a time to chemical exposure in an attempt to identify biomarkers (Kammenga et al., 1998; Ricketts et al., 2004; Swain et al., 2004).

Data from studies on the life history of individuals can be used to predict possible population outcomes. Population growth rates can be determined by the combination of a number of life history parameters, this being known as demography. Demography is the study of births and deaths per time interval, to determine the population growth rate, lambda (λ). The population growth model used in this chapter projects the potential performance of a population, and is therefore called the intrinsic population growth rate. Good quality data are needed to parameterise such models to predict population growth rate, including details on time taken from birth to first reproduction, reproduction over time and survival of juveniles and adults over time.

Toxicants or other stressors typically affect several life-history traits of an organism simultaneously. Use of population models can provide a framework with which to identify effects of which traits are most important for population growth, by the use of sensitivity or elasticity analysis (see Chapter 5). This is because the most sensitive trait at the life history level may not have the largest impact on population growth rate (Kammenga et al., 1996). Elasticity analysis enables the examination of the impacts of changes in one life history parameter (vital rate) on population growth rate. This can be performed for each parameter in the model to see which trait has the most impact on population growth rate (lambda, λ). Elasticity analysis can also be used to assess the relative importance of each parameter on population growth rate across the range of toxicant concentrations; this is because it may be that effects on different traits may become more important in influencing population growth rate as the level of toxicant concentration increases.

One further method of evaluating the toxic effects on individuals and populations is in the use of the energy budget model DEBtox (Alvarez et al., 2005; Kooijman and Bedaux, 1996). This model is based on the dynamic energy budget theory developed and described by Kooijman (1993). DEBtox integrates the multiple endpoints of toxicity in individuals to give No Effect Concentration (NEC) estimates and also predicts the toxicant mode of action on the individual's energy budget (see Chapter 5 for details).

6.1.2 Genomics

Genomics, and the sub-disciplines of transcriptomics, proteomics and metabolomics, is the study of an organism's genetic makeup and the use of the genes. It offers the opportunity to examine how an organism uses the genes it has under different conditions. Snape et al. (2004) identified 5 key questions that ecotoxicogenomics should address. These included asking which genes are turned on and what they do, whether there is variation in gene expression in response to environmental change and if this variation is adaptive and what the ecosystem-, community-, and population-level consequences of the molecular transformations performed by these genes is. Ecotoxicogenomics also offers the possibility to identify the mechanism of action of a single chemical or a mixture of chemicals, which is more environmentally realistic, and may be able to identify conserved responses to toxicants between species.

One of the major advances in molecular biology recently has been the advent of microarray technology. DNA microarrays were first described by Schena et al. (1995). DNA microarrays allow the expression of thousands of genes to be

monitored simultaneously, which potentially allows the activity of all genes in an organism to be studied. Currently there are only a few organisms whose genome has been fully sequenced of which the model nematode *C. elegans* is one, however, this number is rapidly increasing with many environmental sentinel species being partially sequenced in EST (expression sequence tag) studies including the earthworm *Lumbricus rubellus*, the springtail *Folsomia candida* and the water flea *Daphnia magna*. All of which are currently used in ecotoxicology testing.

The aim of transcription profiling using microarray technology is to develop a complete overview of all genes in a genome that are up- or down-regulated in response to some factor of interest in comparison with a designated reference expression (Van Straalen and Roelofs, 2006). In ecotoxicogenomics the factor of interest is toxicant exposure, with the aim of identifying toxicant responsive genes and pathways affected. Further, since chemicals interact directly with cellular components, which in turn affect the life history traits there is also the possibility to look for patterns of expression that match patterns in the changes in life history traits.

The starting point for microarrays is mRNA, which is the transcript of the genes. This mRNA is isolated from organisms taken from control and treatment conditions. The mRNA is reverse transcribed and labelled with fluorescent dyes, to produce labelled cDNA which is then hybridised onto the printed microarray slide. The microarray slide has immobilized on it 'target' nucleic acid sequences, either oligonucleotides or cDNAs. The labelled samples hybridized to the

microarray are known as the 'probe'. Competitive binding of the cDNAs to the immobilized nucleotides then gives an indication of how much of the gene is being expressed between two fluorescent dyes in a semi-quantitative way.

The use of transcriptomics in ecotoxicology is in its infancy. However, there are examples of where gene expression studies have been used to examine response to chemicals of environmental concern (Bartosiewicz et al., 2001). Williams et al. (2003) produced a 160 gene custom microarray which contained 110 stress-related and other genes to analyse environmentally sampled European flounder. The fathead minnow is also being used to discern toxicant induced gene expression alterations (Miracle et al., 2003). Terrestrial organisms have also been used in ecotoxicogenomic studies, beginning with the partial sequencing of the organism which then enabled microarrays to be developed. The earthworm *L. rubellus* is one such species whose genome was begun to be sequenced in an EST project in 2000 (Sturzenbaum et al., 2003) and since then over 15000 EST sequences have been produced and microarray studies on toxicant stressed worms performed (see www.earthworms.org).

6.1.3 *C. elegans* as an ecotoxicogenomic test species

C. elegans lends itself to terrestrial ecotoxicogenomics, in terms of its genomic relevance, and the fact that it is also a species that has been used previously in ecotoxicology (Dhawan et al., 1999; Donkin and Dusenbery, 1993; Jonker et al., 2004; Kammenga et al., 1994). As its entire genome is sequenced microarrays can cover all known genes. There are chips commercially available from Affymetrix covering the entire span of genes (22,500 transcripts). Alternative sets

of oligonucleotides (50 mers, 70 mers) can be purchased commercially and these probes printed onto a suitable slide platform. The latter approach is taken for the microarray slides used in this study, with the arrays representing over 19,000 70 mer oligos printed onto glass slides.

A number of successful microarray studies have been performed using *C. elegans* including examining differential gene expression between developmental stages and sexes (Jiang et al., 2001; Reinke et al., 2004; Wang and Kim, 2003), between ages (Golden and Melov, 2004; Lund et al., 2002) and in response to ethanol (Kwon et al., 2004). There have been just a few cases where microarrays have been used to examine the responses to environmental compounds (Custodia et al., 2001; Menzel et al., 2005; Reichert and Menzel, 2005). These studies which focused on the effects of only a single dose, in all cases did not record the effects of exposure on life-history meaning that they have been unable to complete the integration of mechanistic toxicology with the adverse life-history of contaminant exposure across a concentration range.

6.1.4 Toxicant

Pesticides are one of the most common pollutants found in the environment. Pesticides generally have specific modes of action, although these may not be species specific. Aldicarb, trade name Temik®, (2-methyl-2-(methylthio)propionaldehyde-O-methylcarbamoyloxime) is a carbamate pesticide which is used in the control of nematodes, insects and mites and is one of the most toxic pesticides produced. It is spread in the field in granules and is either tilled

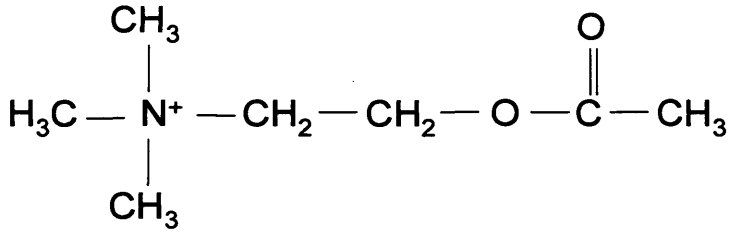
into the land or scattered on the soil surface. This 'spiking' of the soil means that the pesticide dissolves in the groundwater and is then taken up by the root system of the plants, meaning that this chemical comes into direct contact with non-target soil dwelling arthropods such as free-living nematodes.

The World Health Organisation has classed aldicarb as a class I – extremely hazardous chemical. Although it is highly toxic to target and non-target organisms, it is not thought to have carcinogenic or teratogenic effects, at least in mammals (Risher et al., 1987). Aldicarb is metabolised to the active metabolites aldicarb sulfoxide and aldicarb sulfone in the presence of soil bacteria and oxygen both of which can have a long persistence in soils.

The toxic mode of action of aldicarb and other carbamates is in the inhibition of the enzyme acetylcholinesterase (AChE), which is an essential part of the nervous system. The structure of aldicarb resembles acetylcholine (ACh) (see Figure 6.1) and thus competitive inhibition in binding to AChE occurs. Inhibition of AChE prevents the proper regulation of ACh levels in the nervous system. ACh is a neurotransmitter which is synthesized by choline acetyltransferase and is stored in synaptic vesicles until its release is stimulated by an action potential. ACh is released into the synaptic cleft when an action potential opens voltage gated ion channels it then combines with receptors on the post-synaptic membrane, which passes the action potential along. The ACh is removed from the receptors and hydrolysed to choline and acetic acid by the enzyme AChE. When AChE is inhibited the enzyme can not break down the ACh so the concentration of

neurotransmitter builds up in the synapse resulting in over stimulation of cells (Albert et al., 2002).

a)



b)

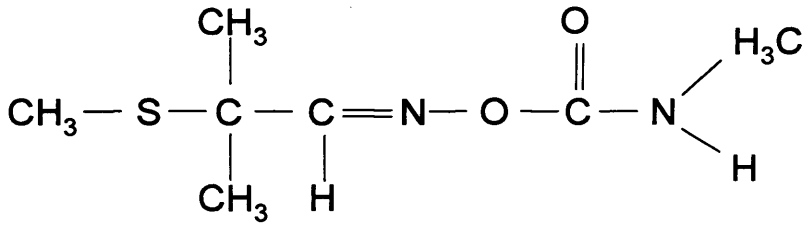


Figure 6.1: The chemical structures of a) acetylcholine and b) The pesticide aldicarb.

6.1.5 Acetylcholine and *C. elegans*

ACh is the primary excitatory neurotransmitter in *C. elegans* motor function (Rand and Nonet, 1997) and is essential for viability. AChE is an enzyme that hydrolyzes the excitatory neurotransmitter ACh at the neuromuscular synapse and thus is responsible for the termination of transmission at the cholinergic synapse. There are four genes in *C. elegans* which code for four separate isoforms of AChE, *ace-1* and *ace-2* yield about 95% of the total enzyme activity, and *ace-3* and *ace-4* yield the remaining 5% (Combes et al., 2001). Aldicarb is often used in experiments on neurobiology and behavioural studies of *C. elegans* (Bany et al.,

2003; Nguyen et al., 1995). When AChE is inhibited in nematodes (from all species) they exhibit classic twitching and convulsive behaviour (Opperman and Chang, 1992).

6.1.6 Aims

The aim of the experiments presented in this chapter was to gain an in depth understanding of the individual, population and molecular response to the toxicant aldicarb, an acetylcholinesterase inhibiting pesticide. The rate of development, total reproduction, reproductive period, lifespan and growth of nematodes under different toxicant concentrations was measured. The effects of this neurotoxin on the behavioural endpoint movement were also studied. Individual level effects were integrated to predict population level impacts of exposure and to gain an insight into the toxicant impact on energy budgets. Microarrays were then used to gain an insight into the transcriptomic changes occurring as a result of aldicarb exposure. Bioinformatic analysis of the pathways affected by aldicarb was used to link the observed toxicological effects with the molecular mechanisms of aldicarb toxicity.

6.2 Approach

The *C. elegans* strain used in this experiment was the laboratory wild type Bristol, referred to as N2. Nematodes were cultured as described in Section 2.2.4. at 15°C.

6.2.1 Chemicals

All chemicals were of the highest analytical grade available. A stock solution of 5 mg ml^{-1} aldicarb (Sigma) was made by dissolving aldicarb in sterile bidistilled water. The stock solution was pushed through a 0.2μM filter to ensure sterility. Aldicarb was added to the NGM agar at the required concentrations after the agar had been autoclaved and cooled to 50°C. Aldicarb concentrations used were 1, 4, 8, 12 and 16 mg L^{-1} agar.

6.2.2 Toxicity tests

A full life-cycle toxicity test was performed as described in Section 2.3.2, with worms being age synchronised twice by transferring 15 gravid nematodes onto NGM plates for 4 hours, then removing the worms and letting the eggs develop into adults. The second synchronisation step was carried out on Petri plates containing cadmium dosed agar corresponding with the test doses, again allowing 15 gravid adult worms to lay eggs for 4 hours. This ensured that there would be no more than 4 hours difference in the age of all worms used in the experiment and that maternal effects were limited as the environment that the mother experiences could affect the phenotype of her offspring independently from her

genotype. The life history traits time to reproduction, survival, length of the reproductive period, reproduction over time and growth over time were measured. Growth was measured as described in Section 2.3.3.

6.2.3 Movement assays

Movement assays were performed by Miss Samantha Hughes.

Movement of the nematodes was quantified in 2 ways, firstly by the distance moved on agar and secondly by looking at tail thrashing in liquid medium. Nematodes had been kept at the desired aldicarb concentration on agar plates from hatching until their use in the movement assays when they reached young adult stage. All movement studies were performed on nematodes within 8 hours of age of each other.

(i) Movement on agar

NGM plates (60 mm diameter Petri dishes) with a thick layer of OP50 covering the entire plate were used in these tests. The OP50 was prepared as in Section 2.2.2, with the exception that prior to being spread on the agar plates it was centrifuged at 5000 x g for 5 minutes and the majority of the supernatant removed to concentrate it. 200µl OP50 was added to each plate.

Age synchronised nematodes (Section 2.2.5i) were placed onto the corresponding dosed Petri dish, allowed to move for 30 seconds and then photographed. The worm was then removed. 20 replicates were used per dose, except at 16 mg L^{-1} where the replicate number was 10. The distance the worms moved which was

visible by track lines in the OP50 lawn were photographed and measured using Image Pro Express.

(ii) Movement in liquid

Movement in liquid was quantified as the number of tail thrashes. Age synchronised nematodes (Section 2.2.5i) grown on dosed agar plates at the respective concentrations were placed individually into 1ml M9 buffer (containing aldicarb at the desired concentrations) in a well of a multi-well plate. Each worm was allowed to acclimatise for 30 seconds before measurements of the number of times the tail thrashed were taken. The number of thrashes in 30 seconds was recorded and repeated four times with the same worm and an average taken. There were 10 replicates at 0-12 mg L^{-1} and 5 reps at 16mg L^{-1}. A single thrash was counted as the tail moving from one side to the other and back again.

6.2.4 Statistics

EC50 values for reproduction were calculated using a logistic model, as below.

$$N = \frac{c}{\left(1+\left(dose/EC50\right)^{b}\right)}$$

Where c is the maximal response (of controls), b is a slope parameter and EC_{50} is the concentration at which there is a 50% reduction in reproduction compared to that of controls.

Survival was analysed using the Weibull survival model. Percentage survival values at each time interval were subjected to least squared fitting of a Weibull curve according to:

$$L_t = \exp\left[-\ln 2 \cdot \left(\frac{t_a}{LT50}\right)^c\right]$$

Where L_t is the proportion of the population alive at time t, c is the slope of the Weibull curve, and LT50 is the estimated time for 50% mortality of the population.

Growth was modelled over time using a logistic adapted equation as below:

$$V_t = \frac{(Max_s - Min_s)}{1 + \exp^{-b(\ln t - \ln LT50)}} + Min_s$$

Where V_t is the size at time t, Max_s is the maximum adult size, Min_s is the minimum size, LT50 is the time at which size is 50% that of Max_s (point of inflection of the curve) and b is a shape parameter.

GLM was used to investigate treatment effects on life-history and movement decreases. To compare survival and dose response curves between populations and treatments, F-tests were performed.

Censored individuals were either lost during the experiment as a result of the individual crawling up the side of the well and becoming desiccated or lost during transfer, these were not included in lifespan analysis, or reproduction if the nematode was lost prior to cessation of the reproductive period. Nematodes which exhibited 'bagging', where eggs hatch and the larvae grow inside the parent killing it, were only included in the assessment of time to maturity.

6.2.5 Microarrays

6.2.5.1 Microarray sample collection

Nematodes were exposed to the same conditions as used in the life cycle test at 15°C (Section 6.2.2). The microarray experiment is a comparative analysis of control and a single treatment of aldicarb (16 mg L^{-1}), with five biological replicates for each of the control and aldicarb treatments, compared to reference RNA. Worms used as the reference sample in the hybridisations were grown and frozen away simultaneously with the treatment and control worms. Samples for microarray analysis were age synchronised by treatment with sodium hypochlorite (Section 2.2.5ii). Each array replicate contained approximately 3-4000 nematodes at the egg-L1 stage; these worms were split onto 6 NGM plates (90mm diameter) to avoid density dependent effects on life-history that would uncouple observations in the toxicity test from microarray results. The treated worms were exposed to 16 mg L^{-1} aldicarb for a pre-exposure of one generation and the sample worms were exposed either to aldicarb or not and harvested at the young adult stage. This was once the gonads became clear (after L4 stage) but before any eggs were visible in the gonad.

To harvest the worms the NGM Petri dishes approximately 5 ml M9 buffer was pipetted onto the plate to wash the worms off. These nematodes were then centrifuged at 1800 x g for 1 minute at room temperature. The supernatant was removed and the nematode pellet frozen immediately in liquid nitrogen before being stored at -80°C until the total RNA was extracted (Section 2.4.2.).

6.2.5.2 Microarray experimental design

The microarrays described in this chapter follow a reference design, that being that each sample (control and aldicarb exposed) was compared to a reference sample. The reference sample was compiled from control and aldicarb exposed worm mRNA in equal proportions. The aim of this is to provide optimal coverage of the genes spotted on the array. The reference RNA was labelled with Cy3.

6.2.5.3 Microarray labelling and hybridisation

RNA from the control and aldicarb exposed populations was extracted (Section 2.4.2), purified (Section 2.4.3) and labelled with fluorescent dyes (Section 2.5), the control and aldicarb samples were labelled with Cy5 and the reference RNA sample labelled with Cy3. The samples were hybridised to the microarray slides overnight at 42°C and washed (Section 2.5).

6.2.5.4 Image capture

Following hybridisation the microarrays were washed and scanned using a ScanArray™ Express HT microarray scanner (Perkin Elmer) at the appropriate wavelengths as described in Section 2.5.9. The images generates by the scanner were saved as TIFF (tagged image file format) files, one file for Cy3 channel and one file for the Cy5 channel.

6.2.5.5 Image analysis

The TIFF image files were imported directly into ImaGene™ microarray analysis software as described in Section 2.5.10, which located spots and quantified the

amount of fluorescence at each spot, by taking the number of pixels at each spot as the intensity. To quantify the intensity of each spot accurately the program was given information about the precise location and boundary of each spot. During the printing process of the microarray slides a file was made which provided information allowing the spot position and oligo identification to be traced. The template file for ImaGene™ provided a meta-grid and sub-array layout of the spots on the slide. The meta-grid was firstly aligned with the corner spots of the microarray, and the sub-arrays were then placed directly over the spots. Once this was done ImaGene™ automatically located each individual spot and was able to modify the spot size to each individual spot.

The quantification process is based on segmentation, where the spot fluorescence and the background fluorescence along with other contaminants (such as a fleck of dust) on the slide were separated from each other. ImaGene™ does this automatically and quantifies the fluorescence of each spot and its immediate background. Data on each spot, which includes spot position, diameter, background fluorescence and spot fluorescence, was saved in .txt files, again one file for the Cy3 channel and one for the Cy5 channel. To quantify accurately the amount of probe hybridised to each oligo it is necessary to subtract the background signal from the probe signal (Yang et al., 2001). Background fluorescence may come from non-specific hybridisation, auto-fluorescence of the glass slide or from chemicals in the buffers used during the hybridisation process.

6.2.5.6 Data transformation

The raw data in the .txt files was imported into the software package GeneSpring GX 7.3 (Agilent Technologies, USA). Prior to analysis of differentially expressed genes the raw data needed to be transformed and filtered to remove dubious data points and to eliminate systematic errors which occur during the microarray process.

Data was firstly $\log_2$ transformed so that up and down regulated genes, and expression ratios could be treated similarly (Quakenbush, 2002). Logged intensities are used rather than absolute intensities because the variation of logged intensities and ratios of intensities is less dependent on absolute magnitude, and gives a more realistic sense of variation (Dudoit et al., 2002).

Lowess (locally weighted linear regression) normalisation was then applied to the entire dataset, which is used to eliminate intensity-dependent effects in the $\log_2$ (ratio) values (Yang et al., 2002a; Yang et al., 2002b), which can cause the graph of raw versus control signal to be curved.

6.2.5.7 Data filtering

Normalised data was filtered so that only spots which were present in both the Cy3 and Cy5 channels remained in the dataset. This data was then filtered so that only those genes that were present in a subset of the experimental samples were included. The next filtering step specified that the reference (in this case Cy3) signal intensity was more than 100 pixels, as relative error increases at lower intensities. The final step was to filter on fold change, in the experiment presented

in this chapter only those genes whose expression changed more than 1.4 fold up or down were used in the further analysis.

6.2.5.8 Data analysis

The normalised, filtered data set was then statistically analysed with a t-test, and multiple testing correction using the Benjamini and Hochberg false discovery rate procedure (1995) set to $p = 0.05$. This provided a list of genes that were differentially expressed between the control and aldicarb treatments. Use of the Benjamini and Hochberg false discovery rate test corrects for the occurrence of false positives. In microarray analyses false positives are genes that are found to be statistically significant between conditions, but are not in reality. During a t-test on microarray data each gene is treated independently from one another. The incidence on false positives is proportional to the number of tests performed and the critical significance level. Multiple testing correction adjusts the individual p-value for each gene to keep the overall error rate to less than a user-specified p-value cut-off, or error rate. Benjamini and Hochberg false discovery rate is the least stringent of the tests in GeneSpring GX and provides a good balance between discovery of statistically significant differences in gene expression and protection against false positives.

6.2.5.9 Functional analysis

The statistically significant up and down-regulated gene lists were functionally analysed in GeneSpring GX using the Gene Ontology (GO) annotation tool, and using the online software DAVID 2.1 beta. DAVID provides a way of annotating

gene lists imported from GeneSpring GX, and EASE (Expression Analysis Systematic Explorer) provides statistical methods (one-tailed Fisher exact probability) for discovering the significance of the functional grouping (Dennis et al., 2003; Hosack et al., 2003). DAVID and EASE can be accessed online at: http://david.niaid.nih.gov/david/ease.htm

6.3 Results

6.3.1 Life-history effects of aldicarb

6.3.1.1 Lifespan

Mean lifespan for control nematodes was 16.0 days. Aldicarb had a significant impact on lifespan of the nematodes (ANOVA: $F = 9.37$, $df = 5, 131$, $p < 0.001$) (Figure 6.2). Tukey multiple comparison tests showed that the only dose whose lifespan was significantly altered compared to controls, in this case increased, was dose 4 mg L^{-1}. Lifespan at 4 mg L^{-1} was 37% longer than control lifespan, going from 16 to 22 days. The lifespan of dose 4 mg L^{-1} was also significantly increased compared to the mean lifespan measured in treatment groups 1, 12 and 16 mg L^{-1}. There was also a significant decrease in lifespan between doses 1 and 12 mg L^{-1}.

Survival was modelled using the Weibull survival curve, shown on Figure 6.3, alongside the actual data. The Weibull model gives an estimated LT50, which is the time at which there is 50% population mortality, and a slope parameter c, these values are given in Table 6.1 along with the correlation coefficient for each curve. F-tests were performed to assess whether the survival curves were significantly different to each other. All exposure survival curves were significantly different to the control survival curve ($p < 0.05$).

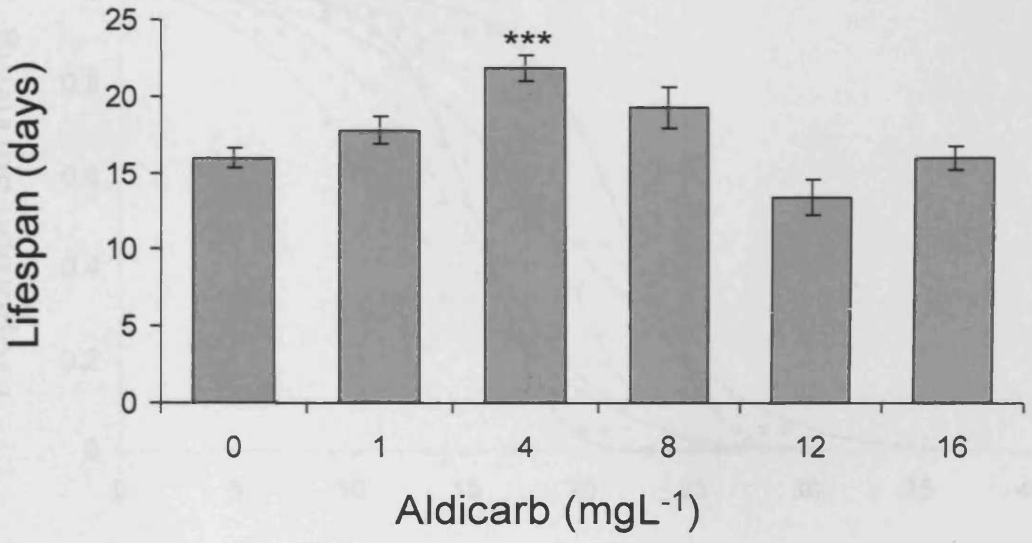


Figure 6.2: Mean lifespan of *C. elegans* strain N2 at increasing concentrations of aldicarb (15°C). Error bars represent standard error of the mean. Values are means of 24 replicates, excluding individuals that were censored (range 22-24). *** $p < 0.001$ compared to controls.

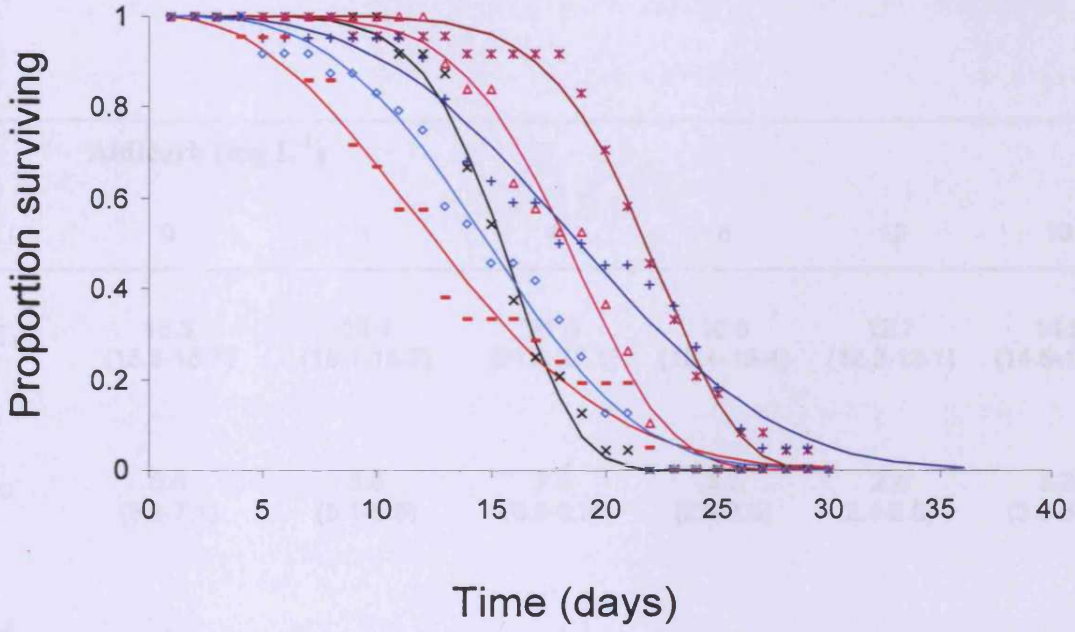


Figure 6.3: Survival curves (unbroken lines) and data points (in the corresponding colour) for nematodes exposed to aldicarb (— 0, — 1, — 4, — 8, — 12, — 16 mg L^{-1}).

Table 6.1: Parameter values and 95% confidence intervals of control and aldicarb exposed nematode survival, modelled using the Weibull survival model. LT_{50} is the time at which there is 50% mortality, and c is the slope parameter, r^2 is the coefficient of determination for each curve.

	Aldicarb (mg L^{-1})					
	0	1	4	8	12	16
LT_{50}	15.5 (15.3-15.7)	18.4 (18.1-18.7)	21.8 (21.6-22.1)	18.9 (18.4-19.4)	12.7 (12.2-13.1)	14.9 (14.6-15.3)
c	6.5 (5.9-7.1)	5.8 (5.1-6.5)	7.6 (6.8-8.3)	3.2 (2.8-3.5)	2.4 2.1-2.6)	3.3 (3.0-3.6)
r^2	0.9965	0.9920	0.9934	0.9804	0.9874	0.9912

6.3.1.2 Time to reproduction

Exposure to aldicarb had a significant effect on the time taken to begin reproduction (ANOVA: $F = 15.83$, $df = 5, 190$, $p < 0.05$) (Figure 6.4). Tukey multiple comparison test showed significant differences between controls and aldicarb doses of 1 and 16 mg L^{-1}. Significant differences were seen in all doses compared to 16 mg L^{-1} ($p<0.05$). Time to reproduction at 16 mg L^{-1} was just over 10% longer than that of controls.

6.3.1.3 Reproduction

Mean brood size for control nematodes was 245. Aldicarb significantly reduced reproductive output (ANOVA: $F= 26.99$, $df = 5, 123$, $p<0.05$) as shown on Figure 6.6. Tukey multiple comparison test showed significant reductions in brood size compared to controls at 12 mg L^{-1} and 16 mg L^{-1} aldicarb. Doses 1, 4 and 8 mg L^{-1} all produced significantly more eggs than nematodes at doses 12 and 16 mg L^{-1}. There was no significant difference between brood sizes at 12 mg$L^{-1}$and 16 mgL^{-1}. Total reproduction in the highest dose tested was just over 50% reduced compared to controls. The dose response was modelled using a logistic dose response equation to give an estimated $EC_{50\text{-reproduction}}$ value and a slope parameter b. EC_{50} (95% confidence limits) was 14.9 (13.6-16.2) mg L^{-1} aldicarb. Slope parameter b value was 3.5 (2.0-5.0).

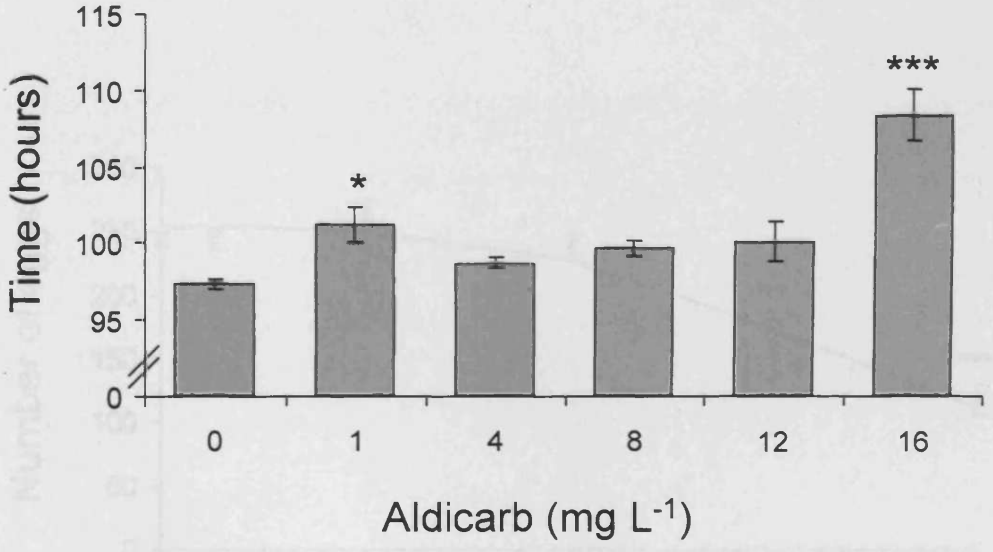


Figure 6.4: Mean time to first reproduction of control and aldicarb exposed nematodes. Error bars represent standard error of the mean. Values represent 38 replicates, excluding any individuals that are censored (range 28-38). * $p < 0.05$, *** $p < 0.001$ compared to controls

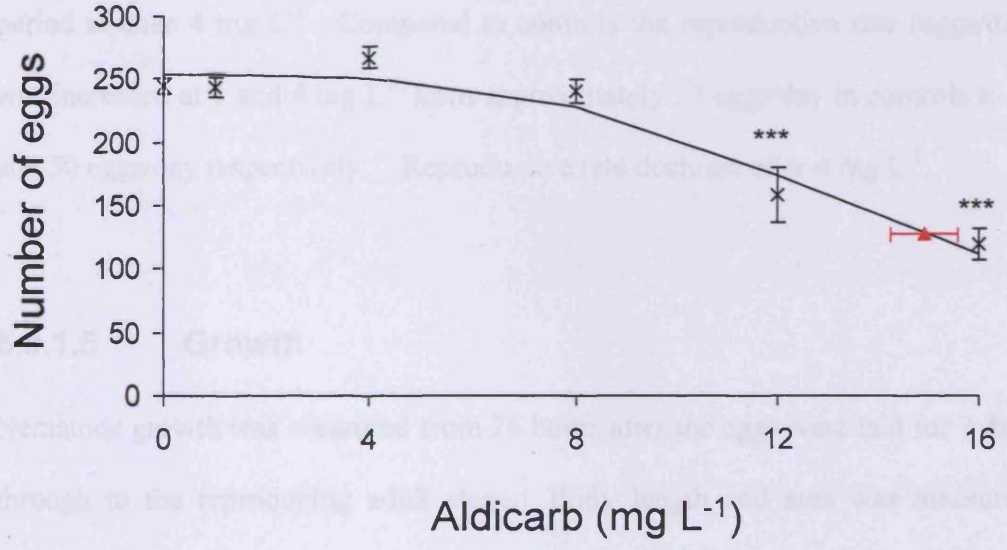


Figure 6.5: Mean total reproduction measured as total number of eggs laid, of *C. elegans* under control and aldicarb exposure. Individual data points represent the mean values (of between 18-24 replicates); the unbroken line is the modelled logistic dose response. The EC50-reproduction is shown in red. Error bars represent the standard error of the mean. *** $p < 0.001$ compared to controls.

6.3.1.4 Reproductive period

Reproductive period was significantly affected by aldicarb (ANOVA: F= 18.26, df = 5, 144, $p < 0.05$) (Figure 6.6). Tukey test showed a significant decrease in reproductive period compared controls at 1 mg L^{-1}, and significant increase in the length of the reproductive period at 16 mg L^{-1} ($p < 0.05$). Length of the reproductive period in dose 16 mg L^{-1} was significantly longer than in all other doses. There was also a statistically insignificant decrease in the reproductive period at dose 4 mg L^{-1}. Compared to controls the reproductive rate (eggs/day) was increased at 1 and 4 mg L^{-1} from approximately 37 eggs/day in controls to 55 and 50 eggs/day respectively. Reproductive rate declined after 4 mg L^{-1}.

6.3.1.5 Growth

Nematode growth was measured from 26 hours after the eggs were laid for 7 days through to the reproducing adult stage. Body length and area was measured, volume calculated as described in Section 2.3.3, and volumetric length shown in Figure 6.7. is the cube root of the volume.

Growth was modelled using the logistic adapted equation described in Section 6.2.4 and shown on Figure 6.7 alongside the mean volumetric length at each time point for each treatment. This clearly shows the impact that aldicarb had on growth rate and final size of the nematodes. Table 6.2 contains the parameter data for each growth curve. The growth model estimates four parameters, maximum size, minimum size, LT_{50} and a slope parameter *c*. T-test comparisons between estimated maximum body sizes showed that aldicarb significantly reduced final

body size in all doses compared to controls ($p < 0.05$). There was an initial 25-30% reduction in maximum body size, (Max_s) from 1-8 mg L^{-1} aldicarb and at 16 mg L^{-1} maximum size was reduced by more than 60%.

6.3.1.6 Size at T_j

Size at first reproduction was estimated from the growth curve models, shown in Figure 6.8. It was not possible to photograph and measure each worm as it began reproducing, so the size was estimated in the following way. The time to reproduction of each individual was used to obtain a value for body size using the growth curve parameters which had been estimated in Section 6.3.1.5. Aldicarb significantly altered mean size at first reproduction (ANOVA: $F = 821.59$, $df = 5, 190$, $p < 0.001$). Tukey multiple comparison tests showed that a significant increase in size at first reproduction compared to controls was seen at 1 mg L^{-1} ($p < 0.05$). Significant decreases in size compared to controls were seen in all other doses, with the exception of 8 mg L^{-1}. All other doses were significantly different to each other. Nematodes at the highest dose tested were over 30% smaller at maturity than controls.

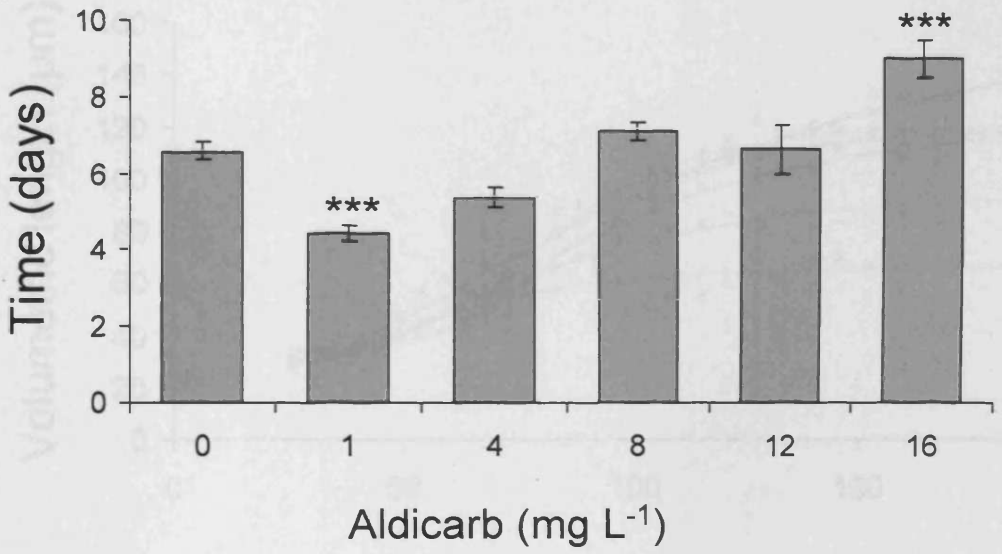


Figure 6.6: Mean reproductive period of control and aldicarb exposed *C. elegans*. Error bars represent the standard error of the mean. Values are the means of 24 replicates excluding any individuals that were censored (range 18-24). *** $p < 0.001$ compared to controls.

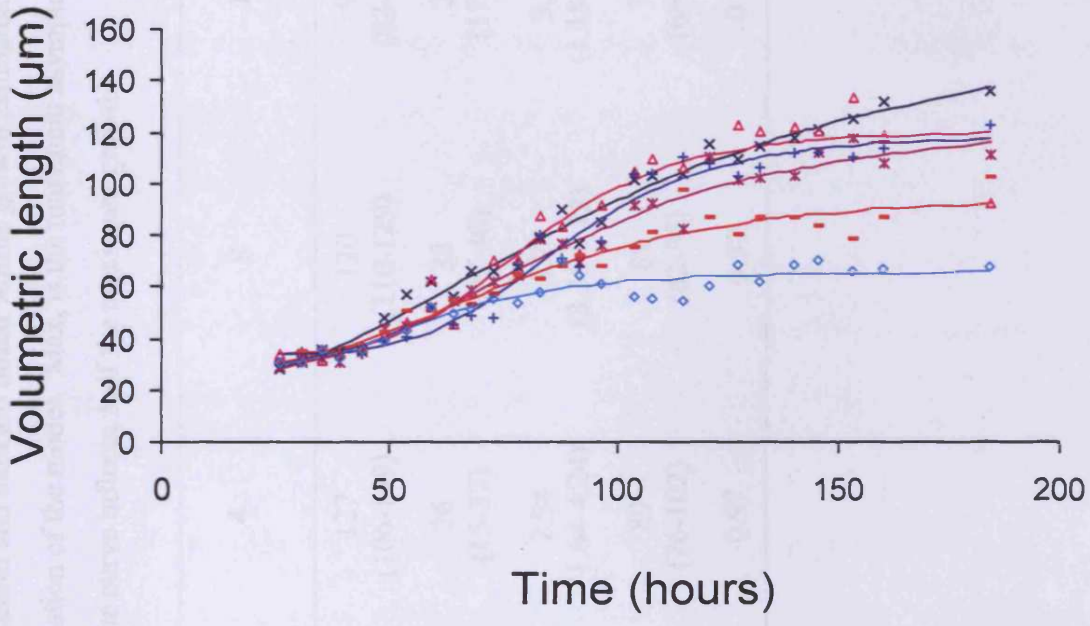


Figure 6.7: Volumetric growth of nematodes under control and aldicarb exposure (— 0, — 1, — 4, — 8, — 12, — 16 mg L^{-1}). Unbroken lines represent the logistic growth curve. Mean size at each time point is shown in the corresponding colour for each treatment.

Table 6.2: Parameters values and 95% confidence intervals of control and aldicarb dosed worms growth estimated using the adapted logistic growth curve model (equation 3), r^2 is the coefficient of determination of the model. Max_s is the maximum asymptotic volumetric length, Min_s is the minimum size, b is a constant and LT_{50} the time at which the curve inflects, half the maximum growth.

Aldicarb (mg L^{-1})	0	1	4	8	12	16
Max_s	171 (128-214)	122 (109-135)	127 (106-149)	120 (110-129)	98 (82-114)	66 (61-71)
Min_s	21 (8-35)	34 (24-44)	26 (15-37)	33 (27-40)	29 (17-40)	28 (18-38)
B	2.18 (1.26-3.10)	5.04 (2.51-7.56)	2.94 (1.64-4.24)	5.24 (3.36-7.12)	3.00 (1.18-4.82)	3.87 (1.23-6.51)
LT_{50}	104 (79-128)	82 (73-90)	89 (76-102)	89 (82-95)	79 (66-92)	59 (48-70)
r^2	0.98	0.93	0.97	0.97	0.94	0.9

6.3.1.7 Movement

Movement was quantified in two ways, on agar and in liquid.

i) Movement on agar

Aldicarb significantly reduced movement on agar, shown on Figure 6.9a (ANOVA: $F = 44.53$, $df = 5, 98$, $p < 0.001$). Tukey multiple comparison tests showed that dose 4 mg L^{-1} and above were all significantly reduced compared to controls ($p < 0.05$). Movement decreased as concentration increased, with complete inhibition of movement on agar at 16 mg L^{-1}. Linear regression gives the following equation: Distance moved = 3895 – (257 * dose), with an $r^2 = 0.96$. $EC_{50\text{-distance}}$ was estimated to be 7.6 mg L^{-1} aldicarb, with the nematodes exposed to 16 mg L^{-1} showing no movement on agar.

ii) In liquid, the average number of thrashes the nematode makes during 30 second time periods over 3 minutes was recorded as shown on Figure 6.9b. Aldicarb significantly reduced the number of tail thrashes in a dose dependent manner (ANOVA: $F = 43.23$, $df = 5, 44$, $p < 0.001$). Tukey multiple comparison tests showed significant differences between controls and all doses above 4 mg L^{-1} ($p < 0.05$). All other doses compared to each other were significantly different with the exception of doses 4 and 8 mg L^{-1}, and 12 and 16 mg L^{-1}. Linear regression gives the following equation: Number of tail thrashes = 45.4 – (2.7 * dose), with an $r^2 = 0.98$. $EC_{50\text{-thrashing}}$ was estimated to be 7.8 mg L^{-1} aldicarb. The number of tail thrashes at the highest dose 16 mg L^{-1} was almost 70% less than that of controls.

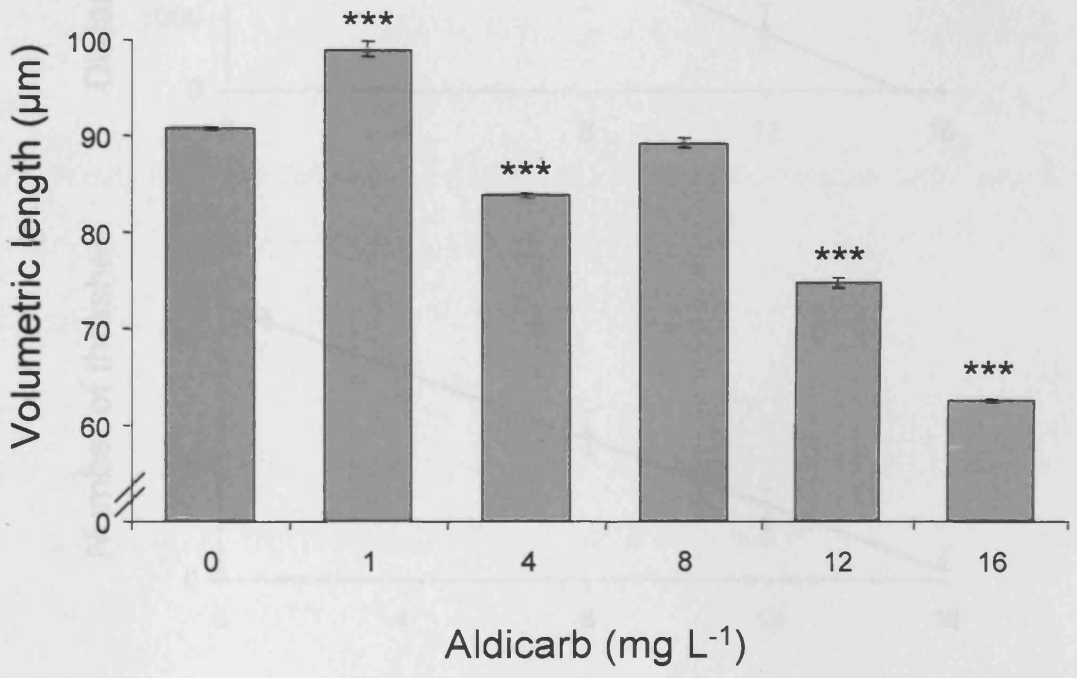


Figure 6.8: Mean estimated size at first reproduction. For control and aldicarb exposed *C. elegans*. Error bars represent the standard error of the mean (30-39 replicates). *** $p < 0.001$ compared to controls.

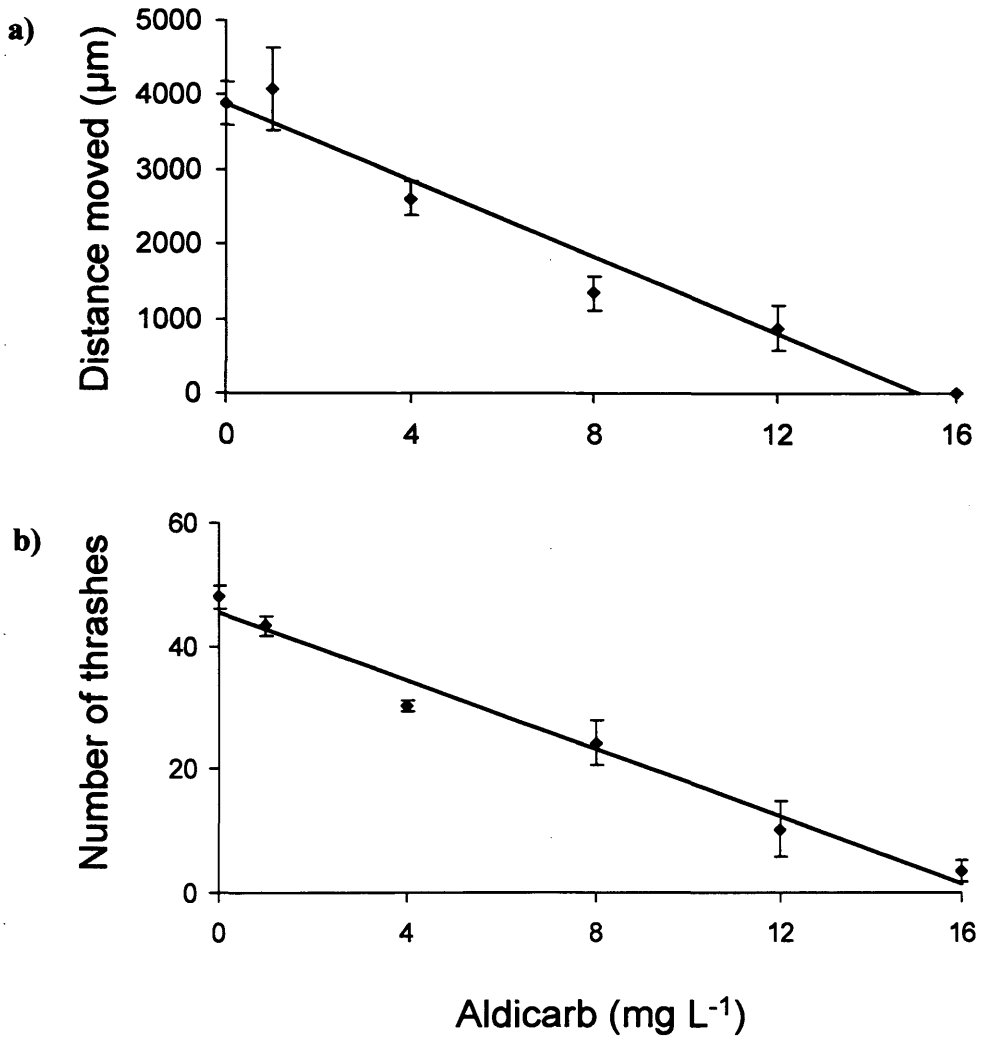


Figure 6.9: Movement of L4 stage nematodes under control and aldicarb exposure: a) movement on agar, measured as the distance moved by the nematode in 30 seconds; b) movement in liquid medium, measured as the mean number of tail thrashes in 30 seconds. Error bars represent standard error of the mean.

6.3.2 Population growth rate and elasticity analysis

Aldicarb has large impacts on population growth rate, λ, as can be seen on Figure 6.10. The population growth rate for controls was 2.4 per 12 hour period, so the control population more than doubles every 12 hours. At the highest dose, 16 mg L^{-1}, lambda was 1.5, approximately 40% lower than control population growth rate. So under exposure to aldicarb the population still increases in size but would only more than double every 24 hours. There was an increase in lambda at the two lowest doses of aldicarb tested, with an increase of 13% and 9% for 1 mg L^{-1} and 4 mg L^{-1} respectively.

The extent to which each life history trait (vital rate) used in the two-stage population model contributes and affects population growth rate was investigated by elasticity analysis (Figure 6.11). Under control growth conditions the trait which has most impact on lambda was juvenile survival, closely followed by adult survival. Decreases in reproductive rate caused large, but less significant impacts on lambda, whilst pre-reproductive period and reproductive period changes had little impact on lambda. The same order of sensitivity was seen at the highest dose 16 mg L^{-1} aldicarb, however adult survival had less impact and was closer in effect to a change in reproduction than controls.

6.3.3 DEBtox

The life-cycle data was put into the DEBtox suite of models, as described in Section 5.2.2. The model whose proposed mode of action of the toxicant which best fitted the data was maintenance (Table 6.3). DEBtox also provides a single

ecotoxicological endpoint, which combines the various life cycle endpoints described previously, producing a No Effect Concentration (NEC) for aldicarb exposure over infinite time. NEC values for survival and for effects on growth/reproduction are presented in Table 6.3. This value is the estimated concentration of aldicarb at which no adverse effects on either survival or growth and reproduction would be seen assuming infinite exposure time. The NEC for growth and reproduction is a factor of 10 smaller than the NEC for survival, confirming that survival is not the most sensitive parameter to aldicarb. Figure 6.12 shows the fits of the raw data to the DEBtox maintenance model, to the parameters, survival, growth and cumulative reproduction.

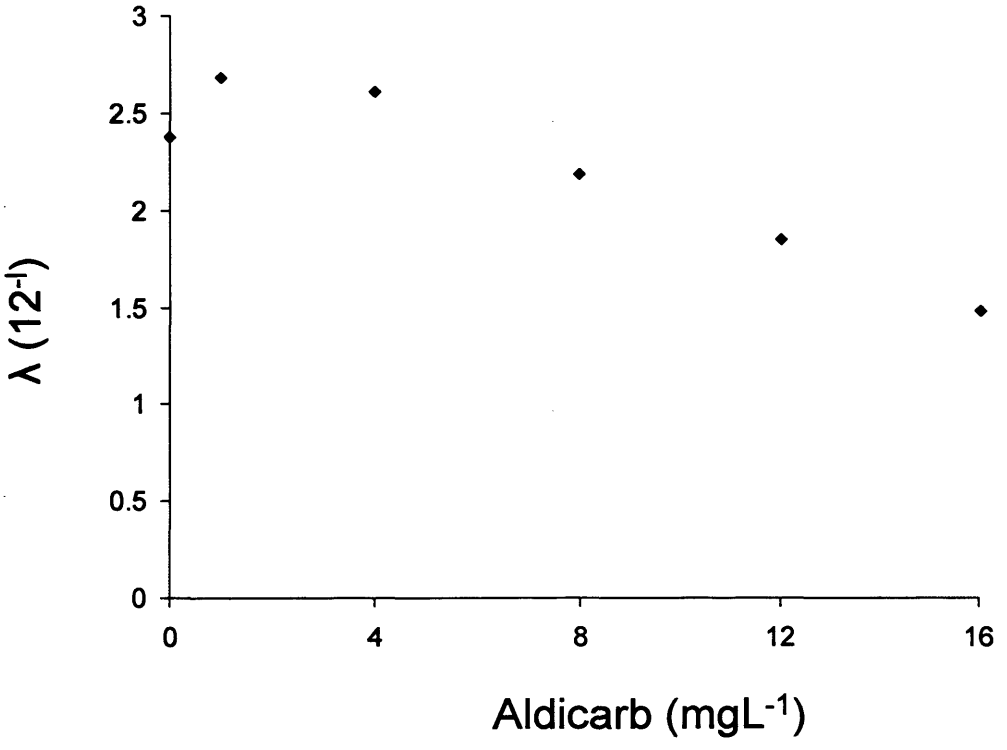


Figure 6.10: Population growth rate (λ) on control and aldicarb exposed nematodes, obtained from the integration of life cycle parameters into a two-stage matrix population model.

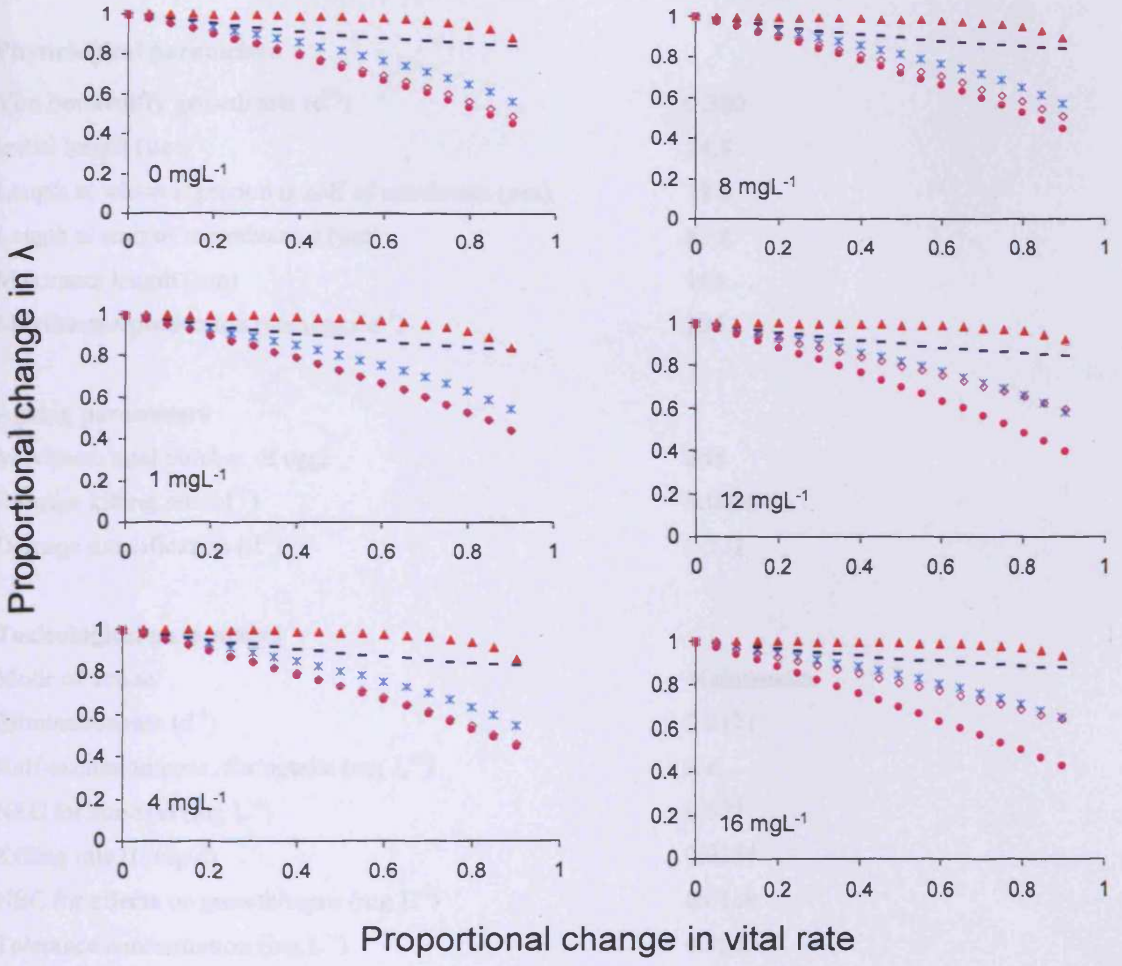


Figure 6.11: Elasticity analysis on the relative sensitivity of each life history parameter in the two-stage population growth model.

• Decrease in juvenile survival (Sa), ◇ decrease in adult survival (Sa), ✳ decrease in reproduction (n), – increase in juvenile period (Tj) and ▲ decrease in reproductive period (Ta).

Table 6.3: Parameters used in the DEBtox model.

	Aldicarb
Physiological parameters	
Von Bertalanffy growth rate (d^{-1})	0.380
Initial length (μm)	24.8
Length at which ingestion is half of maximum (μm)	38.6
Length at start of reproduction (μm)	87.8
Maximum length (μm)	156
Maximum reproduction rate (eggs d^{-1})	236
Ageing parameters	
Maximum total number of eggs	248
Damage killing rate (d^{-1})	0.00264
Damage amplification (d^{-1})	0.172
Toxicological parameters	
Mode of action	Maintenance
Elimination rate (d^{-1})	0.0121
Half-saturation conc. for uptake (mg L^{-1})	n.e.
NEC for survival (mg L^{-1})	0.127
Killing rate (L/mg/d)	0.0354
NEC for effects on growth/repro (mg L^{-1})	0.0169
Tolerance concentration (mg L^{-1})	0.993
Decrease length at puberty due to chemical stress [-]	0.816
Decrease total eggs due to chemical stress [-]	1.193

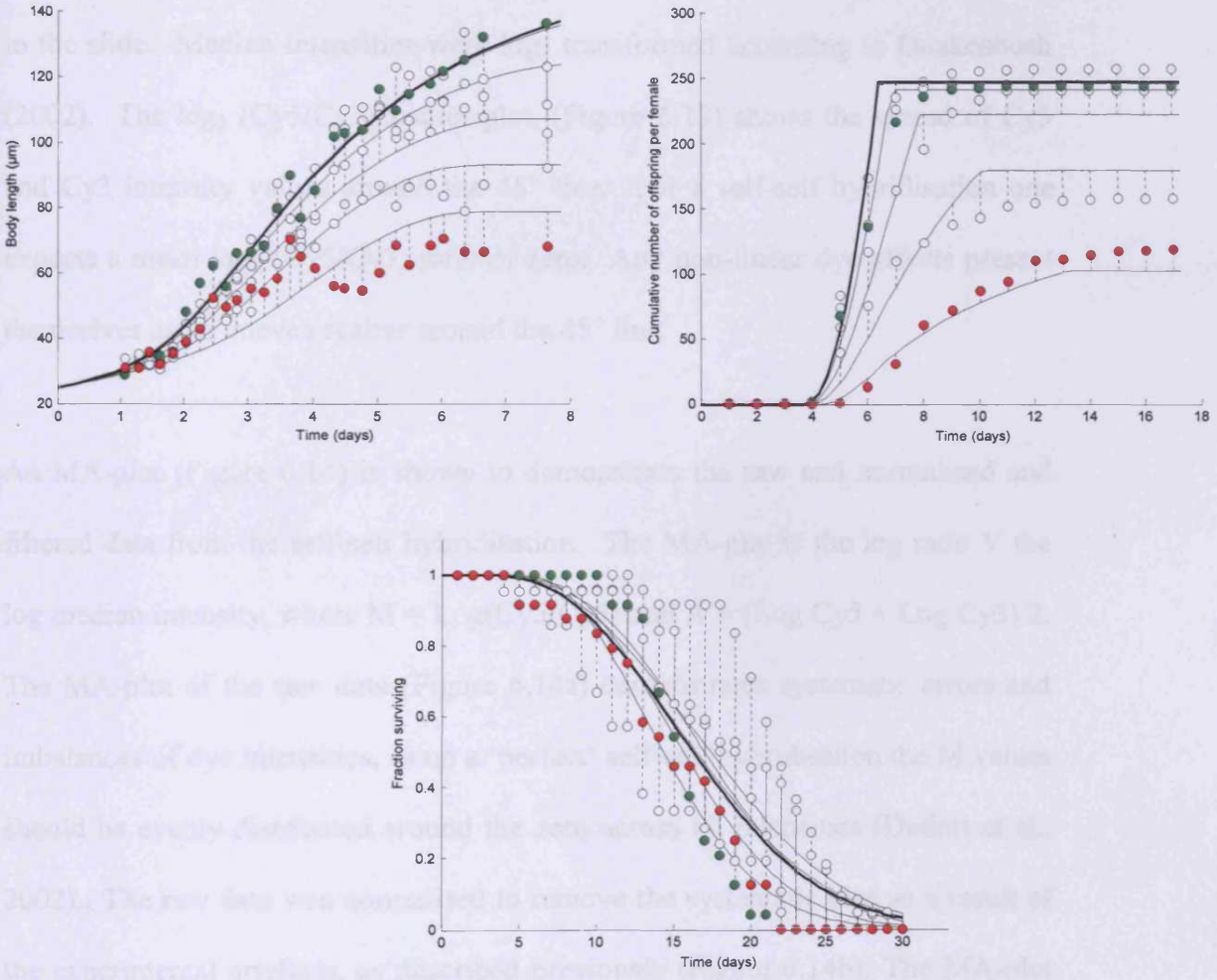


Figure 6.12: DEBtox fits to model assuming mode of action of effect is through effects on maintenance energy. Green represents control nematodes, red represent 16 mg L^{-1} aldicarb exposed nematodes.

6.3.4 Gene Expression

6.3.4.1 Self-self hybridisation

RNA from the same sample was labelled with both Cy3 and Cy5 and hybridised to the slide. Median intensities were $\log_2$ transformed according to Quakenbush (2002). The $\log_2$ (Cy5/Cy3) scatter plot, (Figure 6.13) shows the spread of Cy5 and Cy3 intensity values around the 45° line. For a self-self hybridisation one expects a mean $\log_2$ (Cy5/Cy3) ratio of zero. Any non-linear dye effects present themselves as an uneven scatter around the 45° line.

An MA-plot (Figure 6.14) is shown to demonstrate the raw and normalised and filtered data from the self-self hybridisation. The MA-plot is the log ratio V the log median intensity, where M = Log(Cy5/Cy3) and A = (Log Cy5 + Log Cy3)/2. The MA-plot of the raw data (Figure 6.14a) demonstrates systematic errors and imbalances of dye intensities, as on a 'perfect' self-self hybridisation the M values should be evenly distributed around the zero across all intensities (Dudoit et al., 2002). The raw data was normalised to remove the systematic bias as a result of the experimental artefacts, as described previously (Figure 6.14b). The MA-plot shows how this normalisation and filtering process created a data set equally spread around zero.

Synthetic mRNA was included in the Cy3 and Cy5 labelling reactions, which hybridise to specific spots on the microarray. These mRNAs are used as controls, known as the Lucidea scorecard. After hybridisation, the Cy3 and Cy5 signals obtained from the ratio controls can be compared with the known amount of

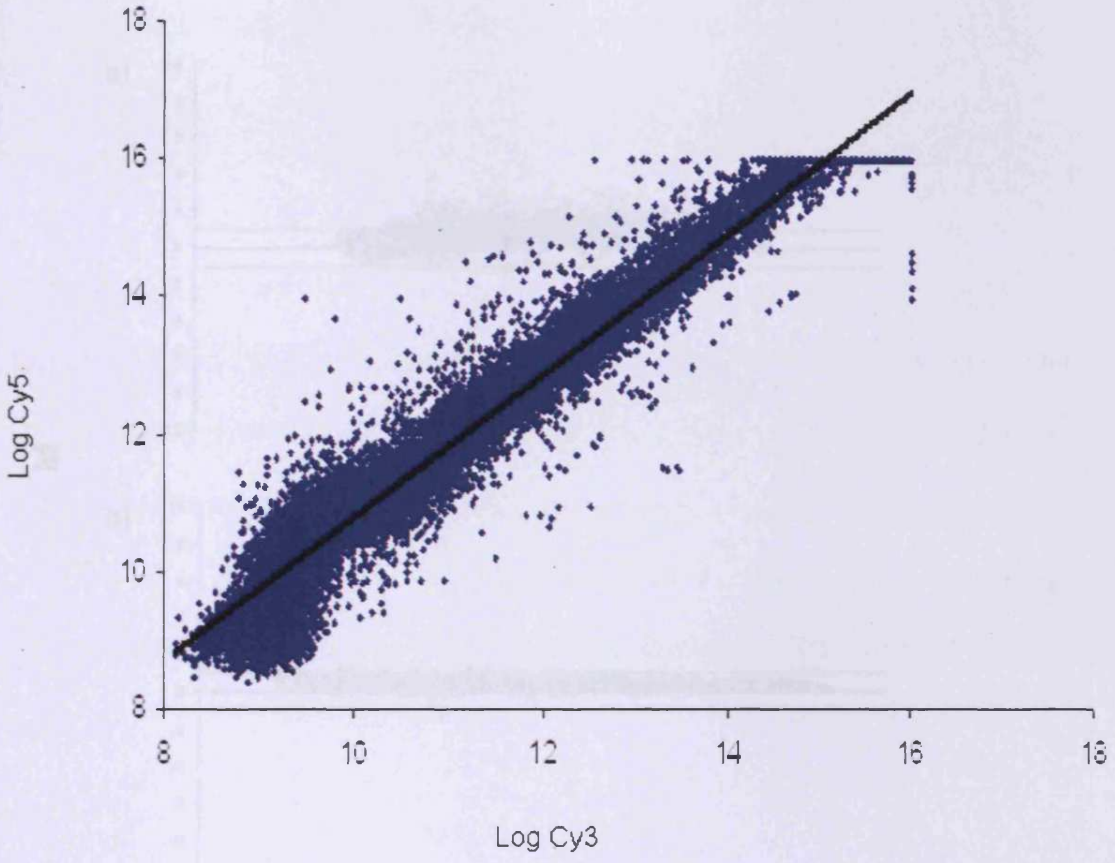


Figure 6.13: Scatter plot of the log-transformed expressions of a self-self microarray experiment in which two identical mRNA samples were labelled with different dyes and hybridised to the same slide. This shows the 1:1 ratio of target hybridisation when an identical target labelled with both Cy3 and Cy5 is hybridised overnight at 42°C. Non-linear dye effects can be seen if there is not an even scatter around the 45° line.

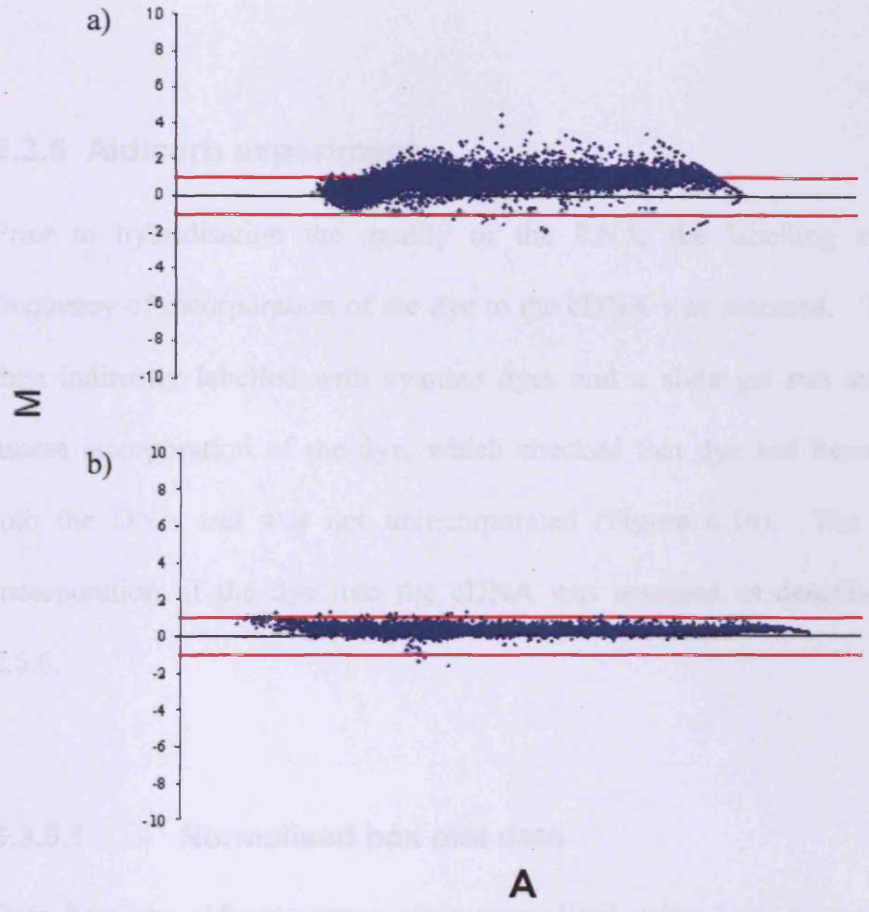


Figure 6.14: MA-plots of self-self hybridisation a) raw data b) normalised and filtered data. The MA-plot is the log ratio V the log median intensity, where M = Log2(Cy5/Cy3) and A = (Log2 Cy5 + Log2 Cy3)/2.

mRNA spiked into the labelling reactions and the theoretical known ratios (Amersham, 2002). The scorecard analysis for the self-self hybridisation is shown in Figure 6.15. This confirms that the hybridisation worked well.

6.3.5 Aldicarb experiment

Prior to hybridisation the quality of the RNA, the labelling efficiency and frequency of incorporation of the dye to the cDNA was assessed. The RNA was then indirectly labelled with cyanine dyes and a slide gel run and scanned to assess incorporation of the dye, which checked that dye had been incorporated into the DNA and was not unincorporated (Figure 6.16). The frequency of incorporation of the dye into the cDNA was assessed as described in Section 2.5.6.

6.3.5.1 Normalised box plot data

Data from the aldicarb arrays were normalised using Lowess per chip and per gene, and plotted as box plots to examine the variance between arrays. Following Lowess normalisation the distribution of the data was visualised using a box plot which indicated that sample C5 displayed an aberrant profile, therefore this sample was removed from all further analysis. A box plot of the remaining data is shown in Figure 6.17 and represents; a) each of the remaining 9 individual microarrays and b) the arrays grouped together into treatment, in this case controls and 16 mg L^{-1} aldicarb exposed nematodes. The microarray data complies with quality control requirements and was included in the further statistical analysis.

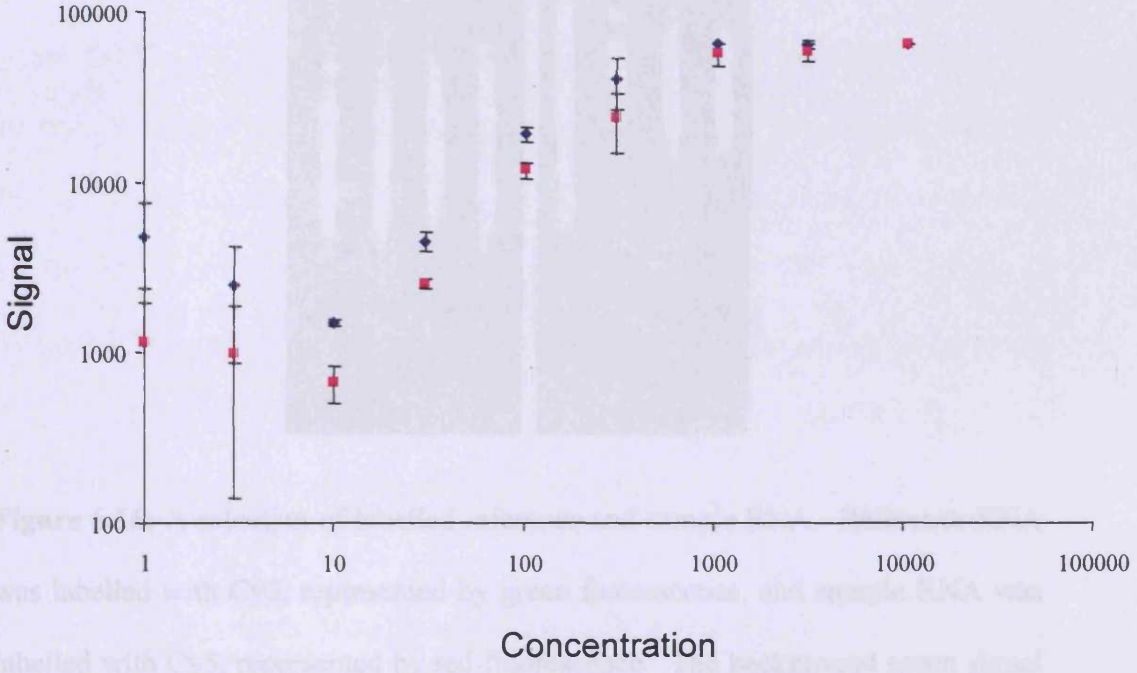


Figure 6.15: Analysis of Lucidea Scorecard calibrators from a self-self hybridisation. Each Scorecard reporters appeared 8 times on each array, and the data given represents the median and standard error of these measurements.

◆ Median test signal ■ Median reference signal

Figure 6.16: A selection of labelled reference and sample RNA. Reference RNA was labelled with Cy3, represented by green fluorescence, and sample RNA was labelled with Cy5, represented by red fluorescence. The background green signal resulted from fluorescence of residual traces of salt of the electrophoretic buffer.

6.3.5.2 Statistically significant differentially expressed genes

The raw dataset covered 19,881 genes on each microarray, 16,332 passed the filter on spots being present in 4 of the 9 datasets, filtering on the reference signal being more than 100 pixels resulted in all 16,332 genes remaining in the dataset. Filtering on expression level of more than a 1.4 fold change resulted in 8282 genes (4960 1.4 fold up-regulated, 3322 1.4 fold down-regulated). The normalised filtered dataset as described in Sections 6.2.4.6 - 6.2.4.8, was analysed for statistically significant differences in expression by performing a t-test using the Benjamini and Hochberg false discovery rate test set to 0.05. The full list of 380 genes that were significantly up-regulated in response to aldicarb is given in Appendix 3, Table 1.1. The full list of 282 genes that were significantly down-regulated in response to aldicarb is given in Appendix 3, Table 1.2.

Selected genes involved with growth, reproduction and lifespan were identified from the gene lists. Six genes were identified as being significantly induced in the gene ontology term 'determination of adult lifespan' (Figure 6.18). However, their induction was relatively small, with the highest induction being close to two-fold, these genes included two 7-transmembrane receptors and GTP-ase activating protein (which is also required for co-ordinated locomotion).

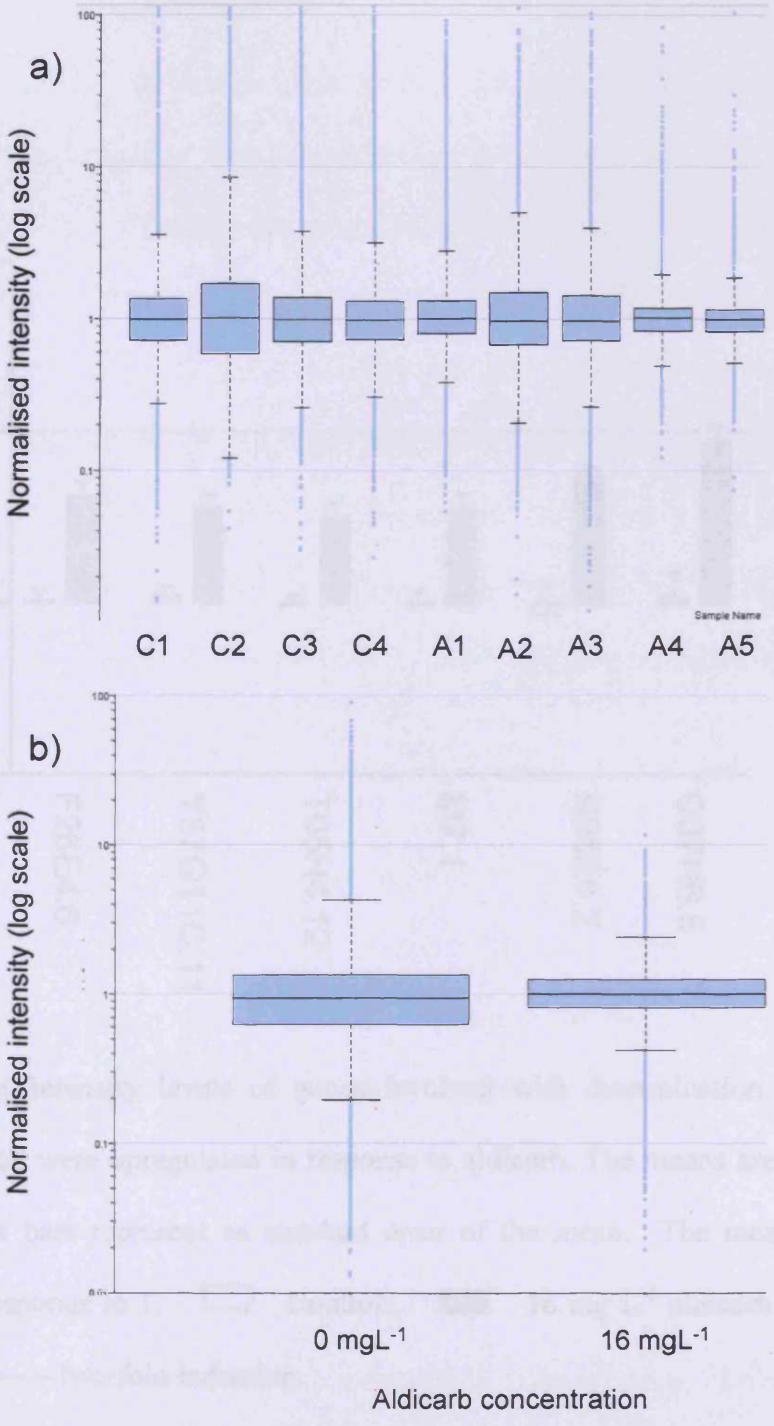


Figure 6.17: Box plots of normalised intensity microarray data. a) C1-C4 are arrays hybridised with control mRNA, A1-A5 are arrays hybridised with aldicarb exposed worm mRNA and b) arrays groups into their treatment group.

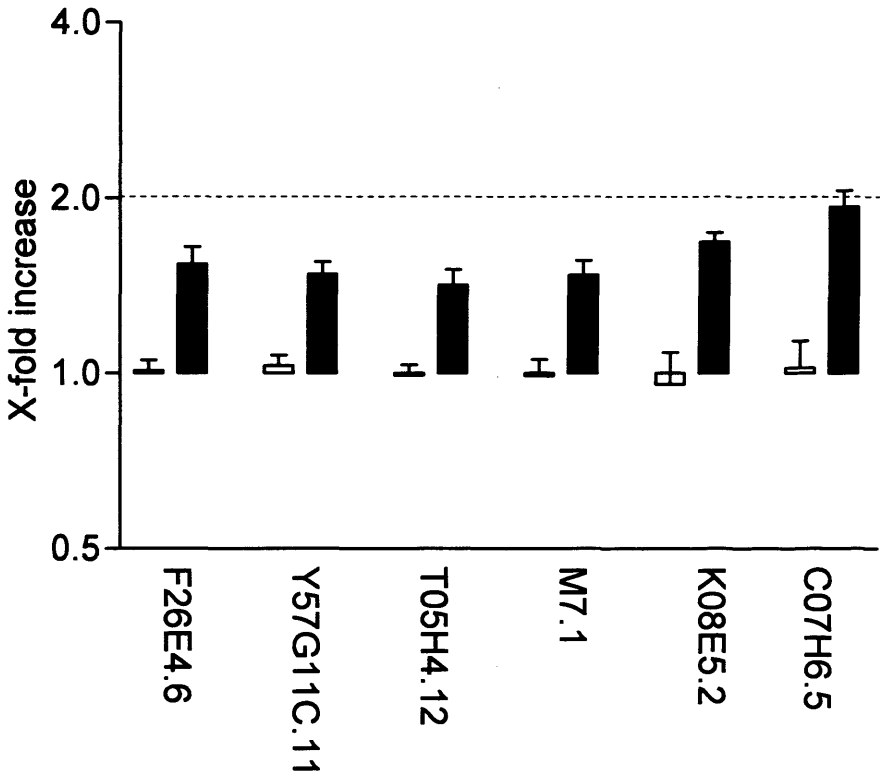


Figure 6.18: Intensity levels of genes involved with determination of adult lifespan which were upregulated in response to aldicarb. The means are semilog plotted, error bars represent as standard error of the mean. The mean of the control corresponds to 1. ☐ Controls, ■ 16 mg L^{-1} aldicarb exposed nematodes, ------ two-fold induction.

Another group of genes of interest in terms of the life history data presented was that of reproduction. There were genes both significantly up and down regulated from the gene ontology term reproduction (see Supplementary Material). Reproductive output of the nematodes was halved compared to controls, the genes that were upregulated may be compensating for direct effects for the toxic effects of chemical exposure on the processes of reproduction. The same was true of both the development and growth categories (Supplementary Material). One gene of interest involved with growth that was up-regulated by almost 9-fold was *hoe-1* (E04A4.4). *Hoe-1* is required for normal rapid growth. RNAi studies have shown *hoe-1* repression slows growth (Kamath et al., 2003; Smith and Levitan, 2004).

There were a number of genes involved in receptor activity that were down-regulated in response to aldicarb, these included a number of 7-transmembrane olfactory receptors, 7-transmembrane receptor, hormone receptors, G-protein coupled receptors and a GABA receptor. Receptor activity was of particular interest in this experiment due to the known effects of aldicarb on intracellular signalling.

One significantly overrepresented gene ontology term from the up-regulated gene list is that of chromatin assembly or disassembly. The expression of these genes is represented in Figure 6.19.

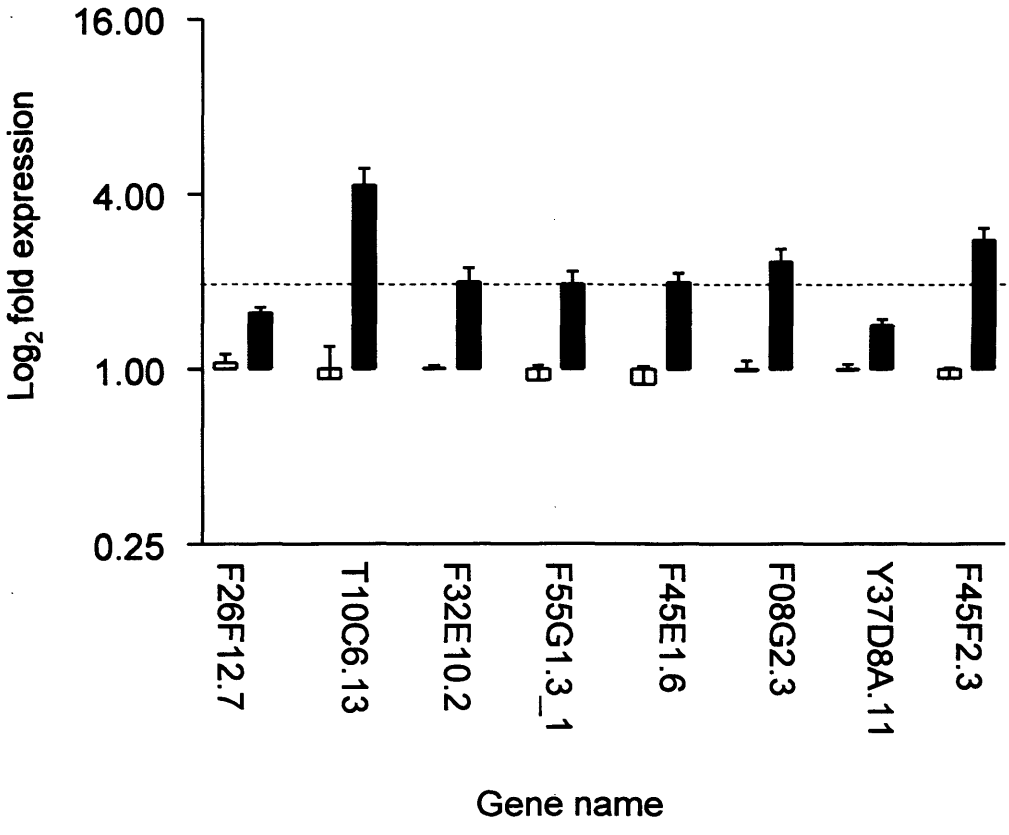


Figure 6.19: Intensity levels of genes involved with chromatin assembly and DNA packaging which were upregulated in response to aldicarb. The means are semilog plotted, error bars represent standard error of the mean. The mean of the control corresponds to 1. ☐Controls, ■ aldicarb exposed nematode (16 mg L^{-1}), ------ two-fold induction.

6.3.5.3 Functional analysis of microarray data

The statistically significant up and down-regulated gene lists were functionally analysed in GeneSpring GX using the Gene Ontology (GO) annotation tool, and using the online software DAVID 2.1 beta (Section 6.2.5.9). Gene ontologies are used to annotate genes and gene products with a list of attributes. The ontologies are organised into three principles, biological process, cellular component and molecular function. The use of the GO terms with the gene lists in Section 6.3.5.2, provided a way of examining which GO categories were enriched for the genes of interest, which may indicate common pathways being affected by aldicarb exposure.

Using the GO annotation tool in GeneSpring GX only 40% of the genes in the significantly differentially expression genes were annotated. From 380 of the up-regulated genes 167 were annotated, and of the 282 gene down-regulated only 95 were annotated. These genes were categorised into each of the Gene Ontology groups, biological process, cellular component and molecular function. GO terms were given a *p*-value by the Gene Ontology tool to indicate functional categories that had more genes from the imported lists in a category than would be expected if the genes were randomly selected. Those GO terms whose p-value < 0.05 are presented in Tables 6.4-6.6, each table deals with up and down regulated gene lists within each gene ontology theme. The GO terms represented with each gene list for each GO category is presented visually in Figures 6.20 – 6.25. These pie charts detail the terms represented by the gene lists and the number of genes in each term for up and down regulated gene list separately.

Table 6.4a: Overrepresented Biological Processes Gene Ontology categories in the significantly up-regulated gene list analysed in GeneSpring GX.

GO term	GO description	p-Value
GO:6333	chromatin assembly or disassembly	0.000593
GO:6323	DNA packaging	0.000775
GO:6325	establishment and/or maintenance of chromatin architecture	0.000775
GO:7001	chromosome organization and biogenesis (sensu Eukaryota)	0.00113
GO:51276	chromosome organization and biogenesis	0.0012
GO:43283	biopolymer metabolism	0.00137
GO:43170	macromolecule metabolism	0.00307
GO:19538	protein metabolism	0.00712
GO:7568	aging	0.0106
GO:8340	determination of adult life span	0.0106
GO:44267	cellular protein metabolism	0.0108
GO:6334	nucleosome assembly	0.0119
GO:44260	cellular macromolecule metabolism	0.0143
GO:31497	chromatin assembly	0.0148
GO:6259	DNA metabolism	0.0154
GO:6526	arginine biosynthesis	0.0193
GO:9306	protein secretion	0.0193
GO:16567	protein ubiquitination	0.0195
GO:6461	protein complex assembly	0.022
GO:30163	protein catabolism	0.0234
GO:18987	osmoregulation	0.0262
GO:16265	death	0.0267
GO:6512	ubiquitin cycle	0.027
GO:8150	biological_process	0.0278
GO:9057	macromolecule catabolism	0.0284
GO:6996	organelle organization and biogenesis	0.0319
GO:50874	organismal physiological process	0.0327
GO:42592	homeostasis	0.0371
GO:44238	primary metabolism	0.0379
GO:51	urea cycle intermediate metabolism	0.0383
GO:6525	arginine metabolism	0.0383
GO:9139	pyrimidine nucleoside diphosphate biosynthesis	0.0383
GO:9197	pyrimidine deoxyribonucleoside diphosphate biosynthesis	0.0383
GO:6233	dTDP biosynthesis	0.0383
GO:9221	pyrimidine deoxyribonucleotide biosynthesis	0.0383
GO:9133	nucleoside diphosphate biosynthesis	0.0383
GO:9189	deoxyribonucleoside diphosphate biosynthesis	0.0383
GO:9263	deoxyribonucleotide biosynthesis	0.0383
GO:9138	pyrimidine nucleoside diphosphate metabolism	0.0383
GO:9196	pyrimidine deoxyribonucleoside diphosphate metabolism	0.0383
GO:46072	dTDP metabolism	0.0383
GO:9132	nucleoside diphosphate metabolism	0.0383
GO:9186	deoxyribonucleoside diphosphate metabolism	0.0383
GO:6914	autophagy	0.0383

GO term	GO description	p-Value
GO:43285	biopolymer catabolism	0.039

Table 6.4b: Overrepresented Biological Processes Gene Ontology categories in the significantly down-regulated gene list analysed in GeneSpring GX.

GO term	GO description	p-Value
GO:7214	gamma-aminobutyric acid signaling pathway	0.0219
GO:6826	iron ion transport	0.0433

Table 6.5: Overrepresented Cellular Component Gene Ontology categories in the significantly up-regulated gene list analysed in GeneSpring GX.

GO term	GO description	p-Value
GO:785	chromatin	0.000593
GO:5694	chromosome	0.000938
GO:43232	intracellular non-membrane-bound organelle	0.00156
GO:43228	non-membrane-bound organelle	0.00156
GO:43229	intracellular organelle	0.00822
GO:43226	organelle	0.00822
GO:786	nucleosome	0.0106
GO:5622	intracellular	0.0137
GO:502	proteasome complex (sensu Eukaryota	0.0216
GO:5839	proteasome core complex (sensu Eukaryota	0.0216
GO:5623	cell	0.029
GO:5575	cellular_component	0.0431

There were no significantly over-expressed categories in Cellular Component ontological analysis in the significantly down-regulated gene list.

Table 6.6a: Overrepresented Molecular Function Gene Ontology categories in the significantly up-regulated gene list analysed in GeneSpring GX.

GO term	GO description	p-Value
GO:3682	chromatin binding	0.00946
GO:43176	amine binding	0.00964
GO:16874	ligase activity	0.0176
GO:8169:	C-methyltransferase activity	0.0193
GO:30580	quinone cofactor methyltransferase activity	0.0193
GO:8425	2-polyprenyl-6-methoxy-1,4-benzoquinone methyltransferase activity	0.0193
GO:16743	carboxyl- and carbamoyltransferase activity	0.0193
GO:4070	aspartate carbamoyltransferase activity	0.0193
GO:4086	carbamoyl-phosphate synthase activity	0.0193
GO:8158	hedgehog receptor activity	0.0197
GO:16879	ligase activity, forming carbon-nitrogen bonds	0.0254
GO:4842	ubiquitin-protein ligase activity	0.0277
GO:16881	acid-amino acid ligase activity	0.0353
GO:4693	cyclin-dependent protein kinase activity	0.0383
GO:4798	thymidylate kinase activity	0.0383
GO:15925	galactosidase activity	0.0383
GO:4565	beta-galactosidase activity	0.0383
GO:4827	proline-tRNA ligase activity	0.0383

Table 6.6b: Overrepresented Molecular Function Gene Ontology categories in the significantly down-regulated gene list analysed in GeneSpring GX.

GO term	GO description	p-Value
GO:4252	serine-type endopeptidase activity	0.00885
GO:4607	phosphatidylcholine-sterol O-acyltransferase activity	0.011
GO:15928	fucosidase activity	0.011
GO:4560	alpha-L-fucosidase activity	0.011
GO:4329	formate-tetrahydrofolate ligase activity	0.011
GO:5099	Ras GTPase activator activity	0.011
GO:16229	steroid dehydrogenase activity	0.0326
GO:3854	3-beta-hydroxy-delta5-steroid dehydrogenase activity	0.0326
GO:8374	O-acyltransferase activity	0.0326
GO:8199	ferric iron binding	0.0433
GO:5096	GTPase activator activity	0.0433
GO:16747	transferase activity, transferring groups other than amino-acyl groups	0.0453

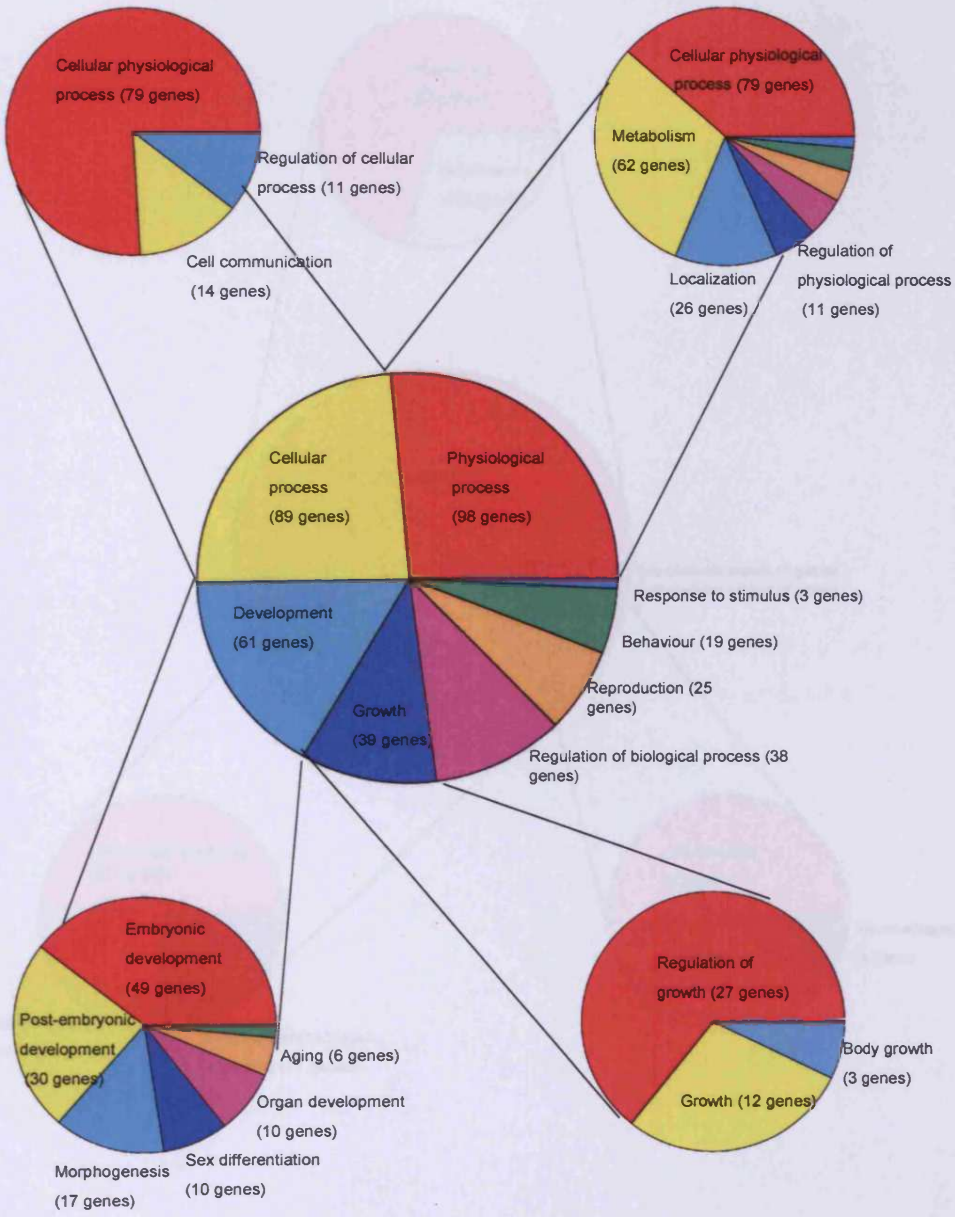


Figure 6.20: Biological Process GO for significantly up-regulated genes in response to aldicarb.

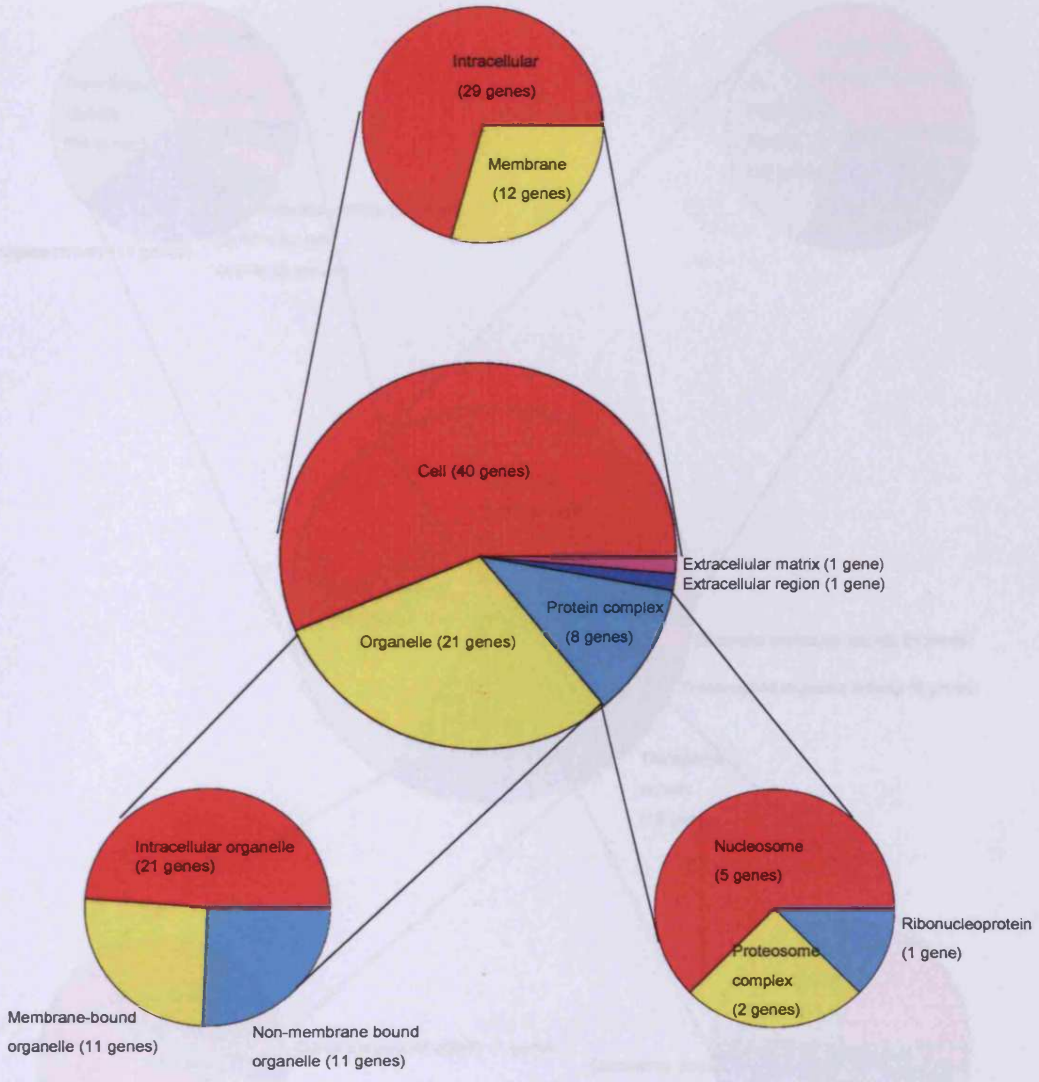


Figure 6.21: Cellular component GO for significantly up-regulated genes in response to aldicarb.

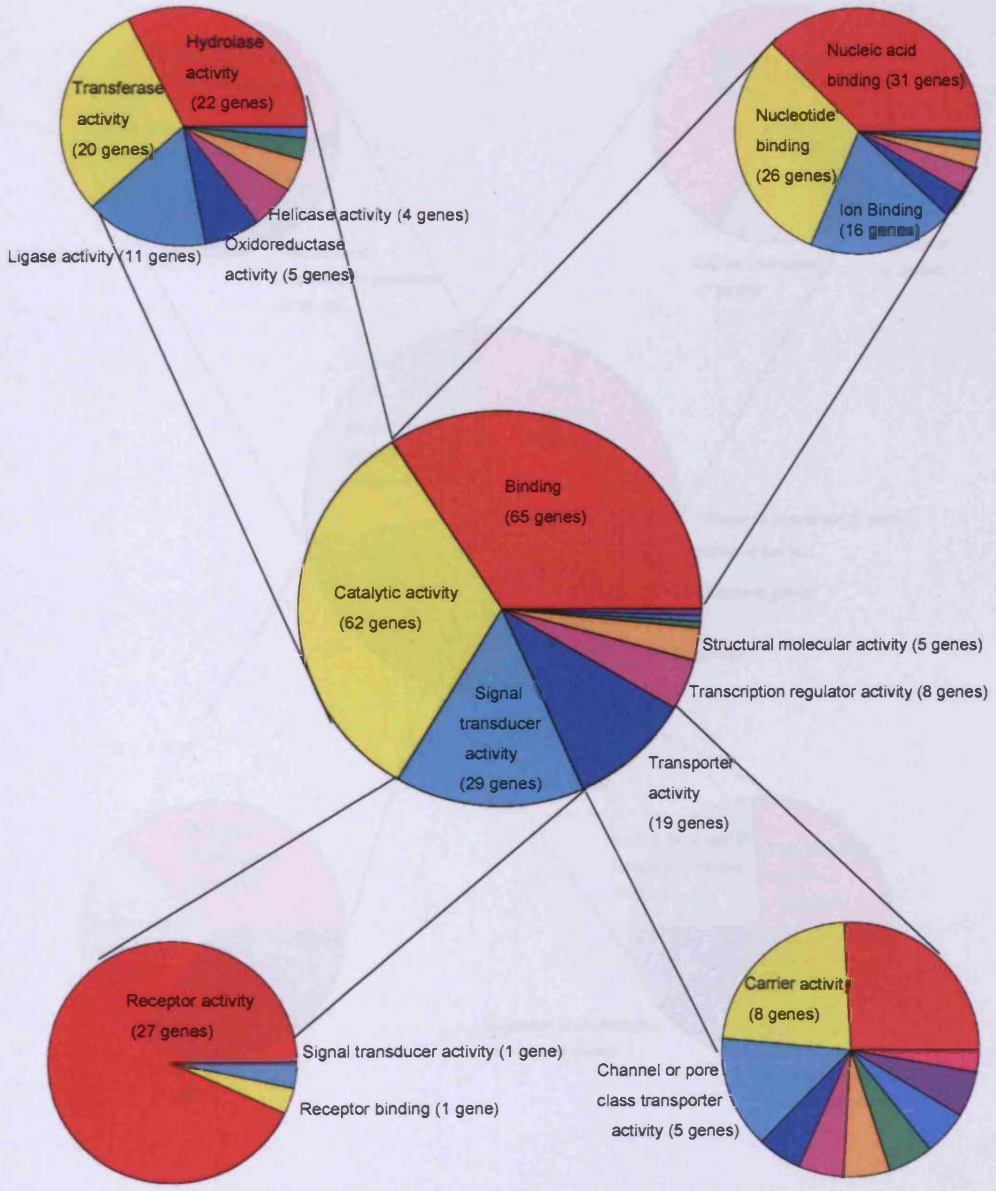


Figure 6.22: Molecular function GO for significantly up-regulated genes in response to aldicarb.

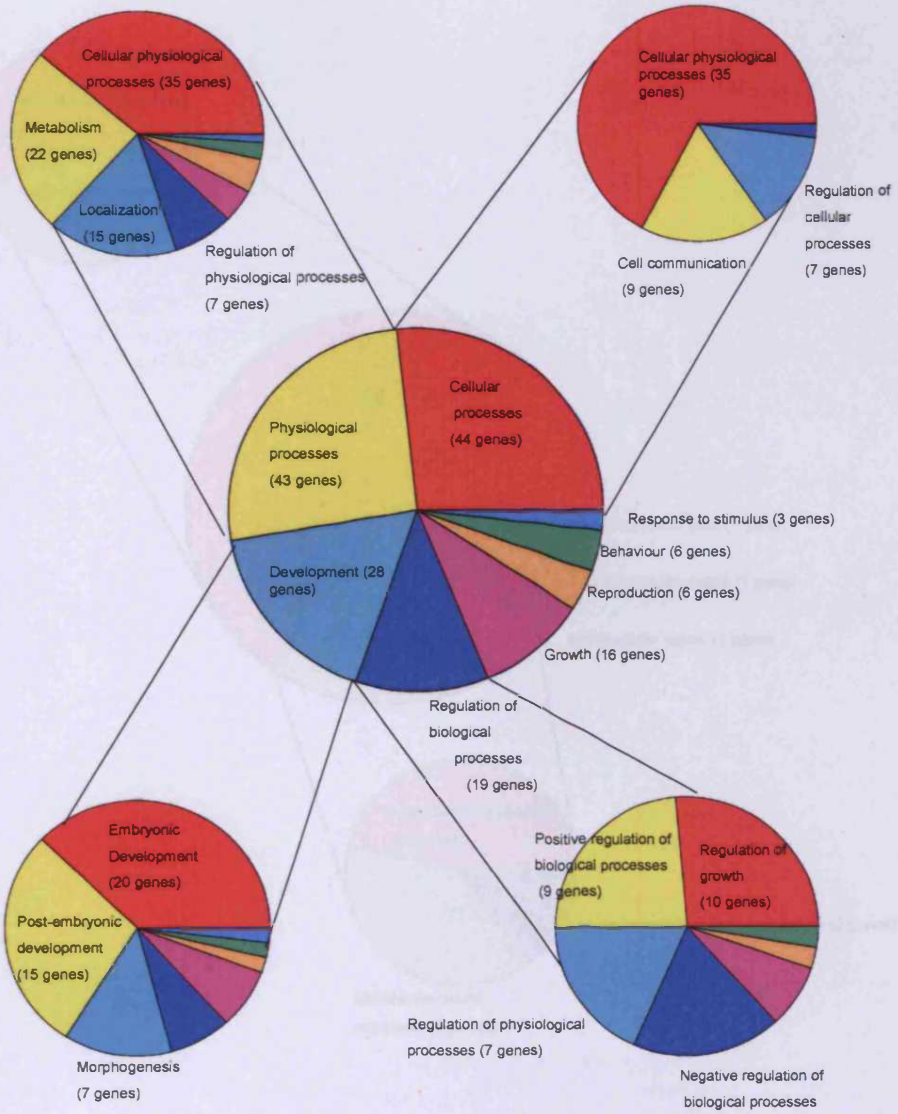


Figure 6.23: Biological Process GO for significantly down-regulated genes in response to aldicarb.

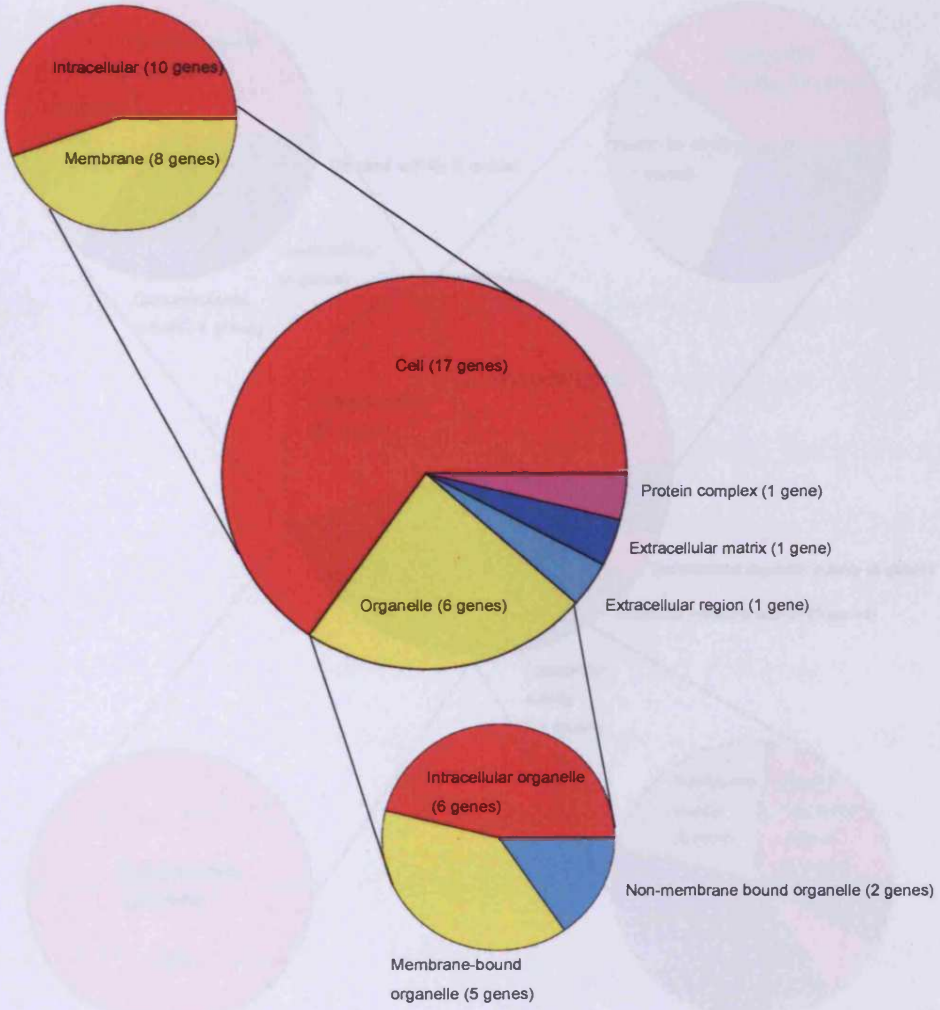


Figure 6.24: Cellular component GO for significantly down-regulated genes in response to aldicarb.

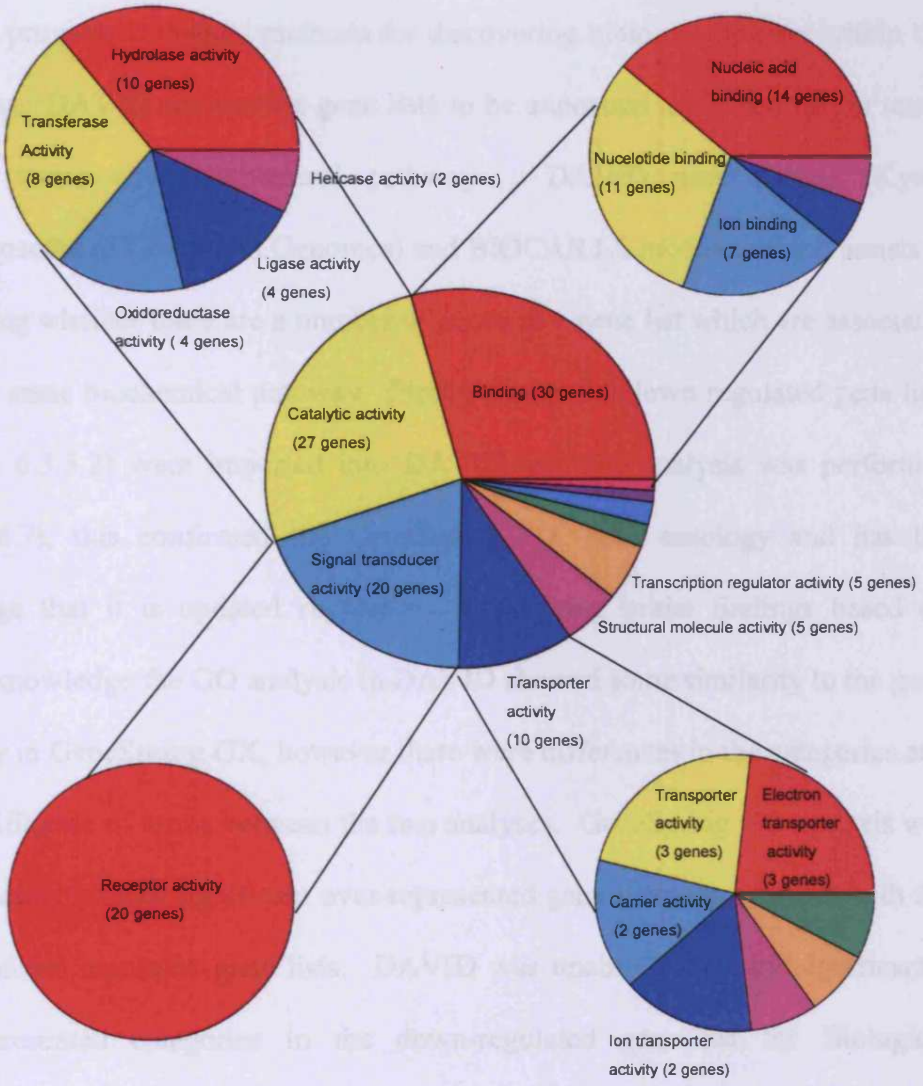


Figure 6.25: Molecular function GO for significantly down-regulated genes in response to aldicarb.

The up and down statistically significantly regulated gene lists were imported into DAVID 2.1 beta. The DAVID program annotates and ranks functional categories based on co-occurring sets of genes in a gene list. EASE, the program linked to DAVID provides statistical methods for discovering biological themes within the gene lists. DAVID enables the gene lists to be annotated and examined in terms of GO terms and biochemical pathways. DAVID uses KEGG (Kyoto Encyclopaedia of Genes and Genomes) and BIOCARTA modules which assists in examining whether there are a number of genes in a gene list which are associated with the same biochemical pathway. Firstly the up and down regulated gene lists (Section 6.3.5.2) were imported into DAVID and GO analysis was performed (Table 6.7), this confirmed the GeneSpring GX gene ontology and has the advantage that it is updated regularly. Confirming initial findings based on current knowledge the GO analysis in DAVID showed some similarity to the gene ontology in GeneSpring GX, however there were differences in the categories and the significance of terms between the two analyses. GeneSpring GO analysis was able to identify more significant over-represented gene ontology terms in both the up and down regulated gene lists. DAVID was unable to list any significantly over-represented categories in the down-regulated gene list for Biological Function and Cellular Component categories.

Pathway analysis using the combined gene lists for up and down regulated genes was performed in DAVID using firstly the *C. elegans* gene names; this list however did not detect any pathways where the genes encoding core components were significantly overrepresented (significant pathways). The same procedure was repeated using those genes which were annotated with a human homologue,

which enabled the identification of statistically significant pathways, presented in Table 6.8 with the EASE score set to 0.1. The pathway for metabolism of xenobiotics by cytochrome P450 (KEGG - HSA00980) was of particular interest in this study, which pinpointed cytochrome P450's (families 2 and 3), alcohol dehydrogenase and UDP-glucuronosyltransferase encoding genes (Figure 6.26). Another pathway identified was the fatty acid pathway (Figure 6.27).

Table 6.7: Functional annotation of statistically significant genes using DAVID/EASE tool. Table a) significantly up-regulated gene list, b) significantly down-regulated gene list.

a) Up-regulated gene list

Gene Ontology Category	GO term	p-value
Biological Process	osmoregulation	0.012125
	protein ubiquitination	0.02408
	homeostasis	0.026206
	development	0.026897
	determination of adult life span	0.027088
	aging	0.029096
	organismal physiological process	0.043336
Cellular Component	ubiquitin ligase complex	0.018372
Molecular Function	ligase activity	0.002578
	ligase activity, forming carbon-nitrogen bonds	0.007228
	ubiquitin-protein ligase activity	0.008163
	FK506-sensitive peptidyl-prolyl cis-trans isomerase	0.025447
	cyclophilin-type peptidyl-prolyl cis-trans isomerase activity	0.025447
	cyclophilin	0.025447
	obsolete molecular function	0.027954
	chromatin binding	0.040828

b) Down-regulated gene list

Gene Ontology Category	GO term	p-value
Down BP	No significant ontologies	
Down CC	No significant ontologies	
Down MF	sugar binding	0.010173
	carbohydrate binding	0.014748

Table 6.8: Pathway analysis using DAVID using KEGG and BIOCARTA pathways. Human homologues of the *C. elegans* genes from the combined up and down-regulated gene lists were analysed.

Pathway analysis	Pathway description	Genes from list identified in pathway	P-value
BIOCARTA-Pathway			
h_mta3	Downregulated of MTA-3 in ER-negative Breast Tumors	3	0.025187
h_atm	ATM Signaling Pathway	3	0.043278
h_atrbrca	Role of BRCA1	3	0.074394
KEGG-Pathway			
HSA00980	Metabolism of xenobiotics by cytochrome P450	6	0.012094
HSA00500	Starch and sucrose metabolism	6	0.01676
HSA00071	Fatty acid metabolism	5	0.018164
HSA00860	Porphyrin and chlorophyll metabolism	4	0.032535
HSA00511	N-Glycan degradation	3	0.047367
HSA05010	Alzheimer's disease	3	0.068028
HSA00040	Pentose and glucoronate interconversions	3	0.079284

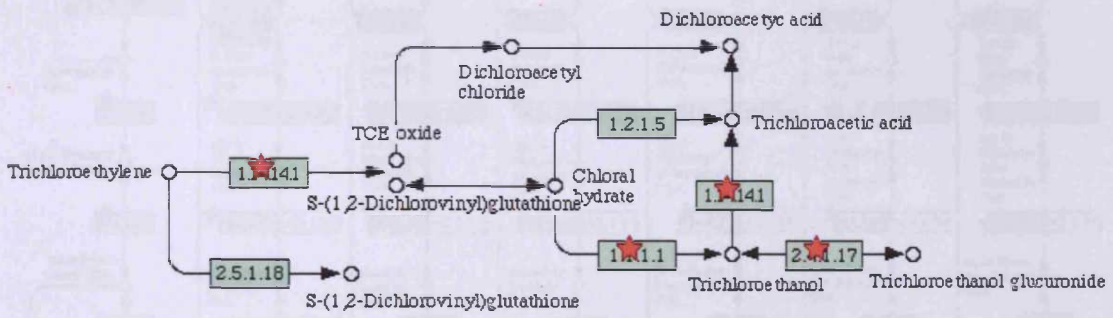


Figure 6.26: Metabolism of xenobiotics by Cytochrome P450. Pathway generated by KEGG, pathway shown is for the metabolism of trichloroethylene. Starred boxes represent genes from list. 1.14.141 are Cytochrome P450's, of families 2 and 3, 1.1.1.1 is an alcohol dehydrogenase gene and 2.4.1.17 is the gene encoding UDP-glucuronosyltransferase. Gene 1.14.141 has also been implicated in the metabolism of naphthalene, 1-nitronapthalene, bromobenzene and benzo(a)pyrene (KEGG-pathway: HSA00980).

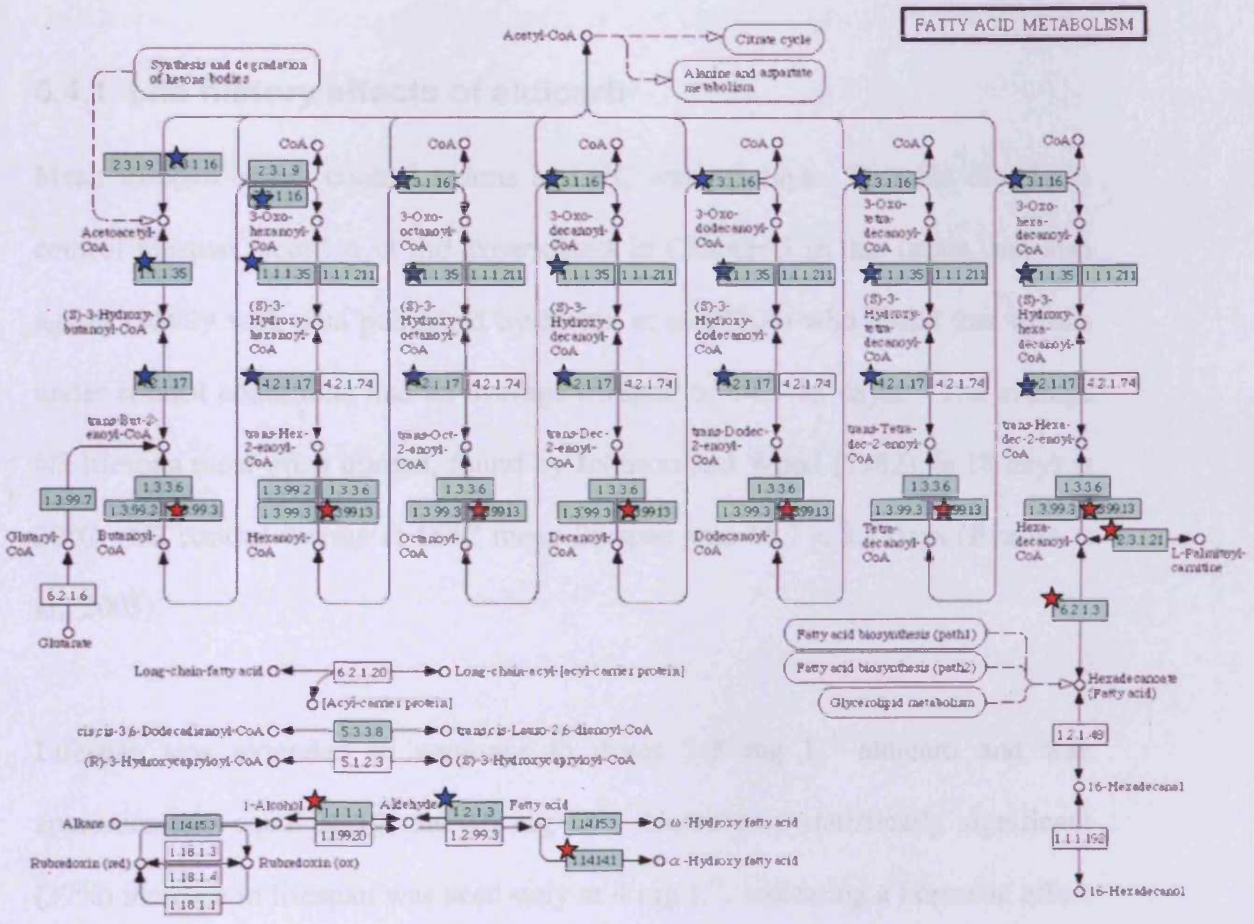


Figure 6.27: Fatty acid metabolism pathway (KEGG). Red stars indicate genes from the statistically significant gene list identified in the pathway. Blue stars represent additional genes identified in the pathway from a gene list representing all genes more than two-fold changed in response to aldicarb (up and down).

6.4 Discussion

6.4.1 Life history effects of aldicarb

Mean lifespan of N2 control worms at 15°C was 16 days. This fits closely to control lifespan recorded in the experiments in Chapter 3 in this thesis, and also agrees closely with data published by Swain et al. (2004) who found that worms under control conditions had an average lifespan of 14.5-15 days. The average N2 lifespan most often quoted, found by Johnson and Wood (1982), is 18 days at 20°C. N2 control worms at 15°C mean lifespan was 16.7 ± 3.7 days (Evason et al., 2005).

Lifespan was extended in response to doses 1-8 mg L^{-1} aldicarb and was approximately equal at 12 and 16 mg L^{-1}. However a statistically significant (37%) increase in lifespan was seen only at 4 mg L^{-1}, indicating a hormetic effect from exposure to aldicarb. Hormesis is the phenomenon whereby a sublethal stress event can lead to improved survival or future performance. It has been suggested that hormesis may be the result of a low dose exposure resulting in an increase in stress responsive pathways that then confer protection from other stresses (including endogenous production of free radicals). One example where such cross tolerance has clearly been shown in *C. elegans* is where a short term exposure to a high temperature conferred tolerance to cadmium (Treinin et al., 2003). Thus in this case it is possible that aldicarb induces stress responsive pathways, which also confer protection against the endogenous product of metabolism that may result in age-related declines of physiological processes, thereby conferring lifespan extension.

One possible age-delaying process may be that the AChE inhibition activity of aldicarb affects the ability of the nematode to pump its pharynx and thus eat, causing a reduction in the assimilation of energy and thus a decrease in metabolism, however this does not fit with the DEB prediction of maintenance. It has been widely researched that restricted calorie intake in *C. elegans* can result in an extended lifespan (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). A compound that diminishes the feeding rate is likely to extend lifespan through caloric restriction. ROS that are generated during normal metabolism can damage a variety of macromolecules and have been proposed as a cause of aging (Masoro and Austad, 2001) so a decrease in metabolism would lead to fewer ROS and thus a slower rate of aging. *C. elegans* pharyngeal muscle contracts upon ACh release from the MC motor neurons and ACh is the critical neurotransmitter in fast pumping through nicotinic receptors and also in regulating pumping through the muscarinic receptors (McKay et al., 2004; Raizen et al., 1995; Steger and Avery, 2004). Although pumping rate has not been directly measured in response to AChE inhibition either in this current study or in previous studies, it may be that chemicals which inhibit AChE and thereby affect nerve function could have some impact on feeding rates.

Lifespan extension caused by exposure to specific chemicals has been reported previously, in response to anticonvulsant medications (Evason et al., 2005). The results from this study indicated that these drugs stimulate synaptic transmission in the neuromuscular system. Ethosuximide increased mean lifespan of wildtype nematodes by 17%, from 16.7-19.6 days at 15°C. Mutations that stimulate

synaptic transmission cause hypersensitivity to aldicarb-mediated paralysis (Miller et al., 1999). Overall, the genetic and pharmacologic studies of *C. elegans* provides substantial support that neural activity limits lifespan by accelerating the aging of non-neuronal cells, as reviewed by Kornfeld (2006). This raises the possibility that the increase in lifespan at low aldicarb can be attributed to the effect of the chemical on nerve synapses. The reduction in lifespan at higher doses being caused by direct mortality attributed to the exposure (higher "kill rate" in DEB terminology).

Developmental period was significantly lengthened in doses 1 mg L^{-1}, and 16 mg L^{-1}. At 16 mg L^{-1}, an 11% lengthening in time to reproduction was seen. Lower doses of aldicarb did not have large impacts on the time taken to begin reproducing. Although the 1 mg L^{-1} dose was statistically significantly different, the change compared to controls was just a 3% lengthening of the developmental period. The extension in time to maturity in 1 mg L^{-1} correlated with an increased estimated body size at maturity.

Mean brood size of 245 is representative of data from other experiments at similar temperatures. Byerly et al. (1976) 275 eggs laid by N2 worms at 16°C and 265 eggs recorded by Alvarez et al. (2005). The $EC_{50\text{-reproduction}}$ was 14.9 mg L^{-1}. At the doses tested aldicarb did not elicit a complete knockdown of reproduction so the steepness of the dose response can not be inferred. It appears that there is a critical concentration at which point reproduction begins to be affected, which was above 8 mg L^{-1} aldicarb.

The length of the reproductive period was shorter at the lowest doses tested 1 mg L^{-1} (statistically significant) and 4 mg L^{-1} (not statistically significant) than in controls although the brood size was similar to controls, thus a higher reproductive rate was observed in the low dose nematodes. Worms at 1 mg L^{-1} were particularly interesting as their time to reproduction was longer than controls also. Egg laying is controlled by 16 egg laying muscle cells which are stimulated by the HSN (hermaphrodite specific neurons) and the VC (ventral-type C) neurons (Desai et al., 1988). Bany et al. (2003) have proposed that acetylcholine inhibits egg laying, after finding that an increase in synaptic ACh decreases egg laying. Some mutants defective for ACh signalling show a hyperactive egg laying phenotype (egl-C, egg laying constitutive). It has however been shown that the nicotinic ACh agonist, levamisole, stimulates egg laying; however aldicarb, which acts on both the nicotinic and muscarinic receptors, inhibited egg laying. The concentrations of aldicarb used in their assay however were 10-1000μM aldicarb, which covers the range used in the study presented here (53-840μM). The increase in egg laying rate seen in the three lowest doses in this study here may be as a consequence of the control over the stage that the eggs are laid at being altered by the altered synaptic transmission caused by the AChE inhibition. Under control conditions eggs are normally laid at about the 100 cell stage, however in egg laying mutants eggs are laid earlier than normal pre-10 cell stage (Bany et al., 2003).

Time to maturity was longer and reproductive period in the highest dose, 16 mg L^{-1}, was significantly longer than all other groups, indicating that it took longer for the nematodes to mature and then to produce and lay a fertilised egg, which is a

similar response to chemical exposure to that seen in the previous toxicity studies (Chapter 4).

Nematode growth was significantly impacted by aldicarb. Final body size was significantly reduced at all doses tested compared to controls and was the most sensitive parameter measured. Some changes in body size may be accounted for because of the mode of action of the drug, in that AChE inhibition causes muscle contraction, which may make the nematode shorter than normal. Growth reduction may also have been caused if the feeding rate was reduced, as less energy would have been available to grow. This is consistent with the theory of lengthened lifespan through restriction of food intake and thus lower endogenous ROS production. AChE inhibition may have direct impacts on growth caused by the disruption of synaptic transmission and thus improper signalling.

A trade-off between reproduction, growth and lifespan is evident at the low doses of aldicarb. At these low doses lifespan was increased without any reductions in reproduction compared to controls, however growth was reduced, suggesting that there was a trade-off between these parameters. The evident trade-off between growth and lifespan at low dose can possibly be explained by a reduction in feeding rate leading to lower metabolism and, thus, ROS production. This concept however raises a question as to why adults at 1 mg L^{-1}, which were between 25-30% smaller than control adults, were able to produce as many eggs, in some cases over a shorter amount of time, as controls?

Under control conditions, optimal energy usage is expected, so the animals should grow to a size at which reproduction and survival is maximised. However if nematodes are capable of producing the same number of offspring at a 30% smaller size, why do control nematodes grow this extra %? Possibly there may be effects on the quality of the offspring, which are not measured in this experiment. It may be the case that the offspring of the exposed nematodes are less fit and unable to survive or reproduce as much as control offspring.

Another explanation may be that at low dose aldicarb only exerts toxicity directly on the nervous system, the consequences of which is to inhibit somatic tissue assembly. Since there is no increased demand for energy to produce a stress response this leaves the same amount of energy available for growth and then reproduction. Since aldicarb exposed nematodes use less energy for growth (to a smaller size) then they may have more available energy for reproduction.

6.4.2 Behavioural response to aldicarb

Nematode sinusoidal movement has been used as a phenotype in many studies of *C. elegans* development behaviour and physiology (Cronin et al., 2005; Dhawan et al., 1999; Gerhardt et al., 2002; Wei et al., 2004). Body movement declines gradually with increasing age in *C. elegans* (Duhon and Johnson, 1995), becoming progressively less continuous and coordinated until it ultimately stops altogether (Huang et al., 2004). Movement may be more sensitive to toxicants that interact with the neuromuscular system. Aldicarb has previously been shown to initiate paralysis in *C., elegans*, analysis of movement parameters by Cronin et al. (2005) who showed that mean point velocity, centroid velocity, track amplitude

and track wavelength were all equally sensitive to aldicarb and that these alterations were apparent at the lowest concentration tested (0.1mM). The $EC_{50\text{-}movement}$ value reported by Cronin et al. (2005) was 0.39mM aldicarb in agar and that paralysis was induced at 6.4 mM aldicarb in wild type N2 nematodes. This is higher than the $EC_{50\text{-}movement}$ values reported in this study of 0.04 mM (7.6 mg L^{-1}) for distance moved and 0.041 mM (7.8 mg L^{-1}) for tail thrashing. The differences in EC_{50} movement between this study and the work of Cronin et al. (2005) can be explained in terms of exposure time. In this study nematodes were exposed to aldicarb from egg to adult while Cronin et al. (2005) took adult worms that had been reared under control conditions and placed them on aldicarb containing agar plates (0 - 6.4 mM aldicarb) for 30 minutes to assess movement. Thus it would be expected that higher concentration of a toxicant would be required to elicit a response in a short-term exposure compared to a long-term exposure.

6.4.3 Population growth rate and elasticity analysis

Aldicarb enhanced population growth rate at low dose. This can be explained by the increased reproductive rate and survival of these worms compared to controls. Comparing the sensitivity of population growth rate to the most sensitive individual life-history parameter the most sensitive parameter tested was growth in terms of final body size, which showed statistically significant reductions at 1 mg L^{-1}, the lowest dose measured. Population growth rate was not reduced at this exposure concentration.

The elasticity analysis showed that the trait which was most important in determining the population growth rate was juvenile survival in all conditions.

The relative importance of adult survival increased at low dose and then decreased as concentration increased from 4 mg L^{-1} aldicarb. The decrease in reproduction was the third most important parameter influencing population growth rate. Reproductive period reductions and increase in time to maturity were the least sensitive traits influencing population growth rate. This analysis indicated that it is stage specific mortality rather than the timing of life-cycle events that is the principal driver of changes in population performance in nematodes exposed to aldicarb.

6.4.4 DEBtox

The DEBtox model for energetic mode of action assessment which best fitted the data of the five used (growth costs, maintenance costs, hazard to embryo, assimilation, and reproduction costs) was the one that described the toxicity by an increase in the amount of energy required for maintenance of somatic tissue. This proposes that the amount of energy used in maintaining the nematode, to keep it alive and functioning was much increased, more than either direct effects on energy needed for reproduction, growth or assimilation of food or hazard to embryo. Increased maintenance costs may come from an increased amount of stress protein production which is needed to repair cellular damage caused by the toxicant. The difference between no effect concentrations on survival and growth/reproduction was nearly 10 fold, which indicates that the sublethal endpoints in ecotoxicological testing such as growth and reproduction are far more sensitive than mortality. However in comparison to the NEC values presented in Chapter 5 for atrazine and cadmium this 10 fold change is relatively

small. For atrazine the difference between NEC for survival and the NEC for effects on growth/reproduction was almost 1000 fold, and for cadmium the same comparison was 50,000 fold different. Previously, Herbert et al. (2004) have proposed that the difference between sub-lethal sensitivity and mortality may be smaller for more specifically acting compounds. These results provide some support for this suggestion.

6.4.5 Gene expression

Microarrays offer huge potential for rapidly examining the responses of individuals to environmental pollution, particularly with the nematode *C. elegans*. However there are limitations, in that the results can be affected at a number of stages. The efficiency of detection is reliant on the efficiency of labelling and hybridisation of an individual array and it may also be difficult to detect low-copy number transcripts.

The amount of data produced by a microarray experiment is phenomenal. Specific analysis routes and functional analyses were employed to reduce and interpret the data. Statistical testing was used to obtain lists of significantly over or under expressed genes in response to aldicarb. Functional analysis of these gene lists was performed using gene ontologies and pathway analysis using both the *C. elegans* gene names and human homologues. There were limitations to the functional analysis using gene ontologies and pathway analysis in that it requires the availability of published information regarding the genes in a list and in this case only 40% of the genes were annotated by the gene ontology used.

Scientific papers on microarray studies regularly present genes whose expression is more than 2-fold changed rather than a statistical analysis (Momose and Iwahishi, 2001; Reichert and Menzel, 2005) which produces a much larger gene list for analysis. In the experiments presented here the number of genes whose expression was induced by a factor of 2 or more was 2734, and those that were repressed by a factor of 2 or more were 1762. This demonstrates the need for proper statistical testing given that the respective statistically significant gene lists numbered 380 and 282 genes for up- and down-regulation respectively. However, the use of much larger gene lists may produce a much wider range of results from the functional analysis, as demonstrated in the identification of the fatty acid pathway in the functional analysis (Figure 6.27). When the human homologues of genes that were more than 2-fold altered in response to aldicarb were mapped to the fatty acid pathway a much larger number of genes involved in the pathway were affected than when just the statistically significant gene list was used. However the confidence with which this gene list was used was lower. Thus there is a need to balance statistical rigour against excessive stringency when analysing and interpreting microarray data.

In the ontological analysis of up-regulated genes categories of interest were the chromatin assembly/disassembly and DNA packaging groups. The genes up-regulated in response to aldicarb from this group included genes encoding for histones 2, 3 and 4. Enrichment for histones suggests that in aldicarb exposed worms the chromatin is altered, possibly to reduced overall transcription rate or assume a structure less susceptible to damage. Histones are the major proteins of

chromatin, and form histone octamers (consisting of two of each of H2A, H2B, H3 and H4) on which DNA is wound around which is known as a nucleosome (Lewin, 1997). Nucleosomal DNA is generally repressive to transcription as the DNA of genes and their regulatory regions are unavailable for the binding of the transcriptional machinery (Wolffe, 1994). One study examining the effect of metals on the RNA/DNA ratio in *C. elegans* has shown that RNA concentrations decrease but DNA remains stable within a specific stage (Ibiam and Grant, 2005). Alternatively it may be that aldicarb directly results in the induction of histones/transcription as a step to protein replacement.

The fatty acid pathway was also identified as having several genes upregulated in response to aldicarb. This would suggest that either overall metabolism is increased possibly because more energy is required to survive and repair damage caused to cells by aldicarb, or that aldicarb impacts upon lipids again leading to increased expression as part of the process of increasing lipid biosynthesis.

In an attempt to link transcriptomic information and life history information selected genes were identified from the induced and repressed gene lists. Of particular interest in the significantly repressed list were genes *let-75*, *nhx-2* and *jph-1*, which are implicated in the processes of feeding, growth and locomotion respectively. *Let-75* is required for pharyngeal muscle contraction and thus normal feeding behaviour (Avery, 1993) and is expressed exclusively in pharyngeal muscle (Ardizzi and Epstein, 1987). The repression of this gene could indicate that pharyngeal pumping was altered compared to controls and thus feeding rate declined. *Nhx-2*, is a Na^+/H^+ exchanger which has been shown to be

required for normally high growth rate (Kamath et al., 2003; Nehrke, 2003), which would suggest that repression of *nhx-2* expression would confer the reduction in growth rate seen in aldicarb exposed nematodes. RNA interference (RNAi) of *nhx-2* produced nematodes with reduced body size, brood size and pharyngeal pumping rate and a loss of fat stores along with an increased post-reproductive lifespan of 40% (Nehrke, 2003). The repression of these two genes links back to the idea proposed earlier that nematodes exposed to aldicarb have a reduced energy intake, which may be caused by reduced pharyngeal pumping. The reduction in energy would contribute to the reduction in growth, although there are also a number of genes involved in the control of growth as well.

RNAi has shown that *jph-1* is needed for normal locomotion, and that when it is repressed by RNAi a locomotion defect is seen which has been suggested to be caused by impaired body wall muscle function.

6.4.5 Summary

Aldicarb is thought to be a specifically acting pesticide inhibiting the enzyme acetylcholinesterase at the synaptic junction. It has previously been described to produce paralysis and death in organisms. The locomotion experiments support this as with increasing aldicarb concentrations the ability to move in a co-ordinated way decreases. However at sublethal levels over the entire lifespan this study has shown that it produces alterations in life-history events and timing of events. The toxic response is somewhat different to that seen with other compounds (Chapter 4) in that for cadmium, atrazine and fluoranthene, when

significant effects were seen on reproduction, time to maturity and growth, lifespan was reduced alongside. However under aldicarb exposure lifespan was increased at low concentrations and unaffected at concentrations where reproductive and growth effects were seen. The transcriptomic data also suggests that aldicarb does not solely exert its effects on acetylcholine signalling and receptor activity. It also indicates that many non-acetylcholine signalling and receptor activity genes are affected including the GABA receptor and olfactory receptors. The array data also suggests that other response pathways are induced, such as xenobiotic metabolism via cytochrome P450's, chromatin assembly and fatty acid metabolism.

The scope of transcriptomic studies is far beyond what is discussed here. In this simple single dose experiment the amount of information gathered was immense. The advantage of using *C. elegans* was that all of its genes have been sequenced, so each microarray results in over 19,000 pieces of information on signal intensity for each gene. However these 19,000 genes have not all been described, so there is a large proportion of genes whose expression may be altered in response to chemical stress that have unknown functions. As was seen with the gene ontology analysis 60% of the genes had not been functionally described. The limited information on the function of genes limits the analysis, and thus there may be unidentified response pathways. As the information regarding genes increases the possibilities of describing the data from this array experiment will be enhanced.

Linking transcriptomic changes to the responses seen in life history of the individuals will be one of the major challenges faced in ecotoxicogenomics,

because life history traits are controlled by intricate interactions of many genes which are influenced by environmental factors. The work conducted here; however, represents a first attempt to bridge observations of life-history to underlying mechanisms. In this context application of individual based models such as DEB may have a role to play, since this provides a simple framework for interpreting chemical effects that can be linked to behavioural (movement, pharyngeal pumping) and transcriptional observations.

Chapter 7

Final Discussion

7.1 Introduction

In the past century the problem of pollution has increased enormously, due largely to increased industrialisation and the development of synthetic chemicals which when released into the environment cause deleterious effects. Of uppermost importance is the need to understand the impacts that these toxicants have on the environment, (including the animals that live in the environments), to regulate for acceptable levels and to characterise newly developed chemicals.

Chemical impacts can be monitored at any biological level; molecular, cellular, individual, population, community and ecosystem; but the effect is always seen in the individual. For effective ecotoxicological risk assessment there is a need to make links between the levels. Each piece of information on its own is useful and tells us something about the toxicity of compounds, but together the information has the potential to indicate how the toxicant is affecting the organisms.

The regulatory aspects of ecotoxicology are covered by a series of toxicity tests on a number of species, which are normally based on mortality, or single endpoint toxicity tests. The most direct way of assessing the impacts of pollutants on organisms is to measure life cycle parameters such as reproduction, growth and lifespan, which have high ecological relevance; however, the drawback to this is

that it can be time and resource consuming. Focussing on single end-points in toxicity testing has the weakness that there is no single parameter that is consistently the most sensitive trait across a range of chemicals or to a range of species or even different life stages (Forbes and Calow, 1999). In the studies presented in this thesis, the most sensitive trait measured by NOEC levels varied between size at maturity and time to reproduction for cadmium, maximum size and brood size for fluoranthene, maximum size and time to reproduction for atrazine, and under aldicarb exposure final body size and brood size were most sensitive. An important consideration in using sensitivity of maximum size and size at maturity is that these were calculated from a growth model, and as such were not explicitly measured. Of particular note are the hormetic effects observed at low doses in both cadmium and aldicarb in lifespan and in reproductive output with aldicarb.

This illustrates the difficulty of making generalised rules concerning the effects of toxicants on life history; and so a better approach is to perform life-cycle toxicity tests, which examine a number of sublethal responses to toxicants when an individual is exposed over its entire lifespan. This kind of data can also be modelled to predict effects on populations. Responses of individuals are mediated by changes in how the organism utilises its genome. Using molecular techniques these responses can be measured in order to gain a greater understanding of the underlying biology associated with chemical exposure and can potentially also be exploited for use as biomarkers in regulatory ecotoxicology. New molecular biology methods, alongside more traditional life cycle assessments in

ecotoxicology, will provide a fuller picture of the internal mechanisms of an organism.

One of the main aims of ecotoxicology is to identify indicators of environmental stress prior to the point where populations and communities are affected. Whilst sublethal responses of individuals are useful for this, the ability to examine changes brought about by toxicants at the molecular level may produce molecular biomarkers that are very early warnings of stress. The use of genomic methodologies in ecotoxicology is in its infancy, however, the potential for this has been recognised (Heckmann et al., 2006; Snape et al., 2004; Snell et al., 2004; Spurgeon et al., 2002).

7.2 Discussion

7.2.1 *C. elegans* as an ecotoxicology test species

C. elegans, a small nematode worm that was first pioneered as a model organism to study the developmental cell lineage by Sydney Brenner in 1965 has since been at the forefront in neurobiology, whole genome sequencing, studying human diseases, and more recently used in microarray studies. Its potential for use as an ecotoxicological tool has been evaluated and to date has been used by a number of groups to assess its suitability as a toxicity test species. It has many of the qualities required in an ecotoxicological test organism, in that it is small, inexpensive, and easy to work with. It has the potential to be used in liquid, soil, sediment, and agar toxicity tests, as it is able to survive in all environments. It has a short lifespan which greatly enhances its use in full life cycle toxicity

testing, as described in this thesis. The use of transgenic *C. elegans* as environmental bio-monitors have also been evaluated, however the sensitivity of the transgenic nematodes to toxicants is much higher than non-transgenics, limiting their usefulness. They have however been recommended as toxicity test organisms for toxicity assessment of pharmaceutical compounds (Dengg and Van Meel, 2004). Whilst *C. elegans* is not the most sensitive nematode in response to toxicants (Kammenga et al., 1994) I believe the sheer amount of information known about its biology and genome greatly enhances the quality of this organism as a tool to study the ecological impacts of toxicants. Due to the evolutionary conserved nature of stress responses it is likely that responses elicited in *C. elegans* will be utilized to understand similar processes and pathways in other organisms, including humans.

7.2.2 Temperature effects on life history and toxicity

Temperature affected the timings and values of life history parameters. Higher temperature caused the entire life-cycle of the nematode to proceed at a faster rate, explained by the knowledge that increases in temperature in ectotherms results in an increase in metabolism, and thus the speed at which animals grow and develop. The resulting effect on an increase in metabolic rate was a shortening of the lifespan, which can be explained by the oxidative theory of aging. This theory states that aging is caused by the accumulation of reactive oxygen species (ROS), which are a by-product of normal metabolic and respiratory activity. The higher the metabolic rate the faster the rate of aging.

The results from the experiments in Chapter 3 indicate that there is an interaction between temperature and toxicity of the metal cadmium, which has been indicated previously in studies using *Daphnis magna* (Heugens et al., 2003) and *Gammarus pulex* (Bat et al., 2000). *C. elegans* was more sensitive to sublethal effects of cadmium at a higher temperature, but less sensitive than the lower temperature on lifespan effects. However, in all cases worms died in their post-reproductive senescent period. From a population growth perspective this result is most probably irrelevant as non-reproducing adults do not contribute to population growth rate.

There were indications of a trade-off between lifespan and reproduction occurring under cadmium exposure. The results presented in Chapter 3 suggest that at the lower temperature (15°C) more energy was allocated to reproduction than to maintenance, repair, and defence mechanisms, as a higher number of eggs (in proportion to control brood size) and lower lifespan was seen. At 25°C the opposite was seen, in that fewer eggs (in proportion to control brood size) were produced but a longer lifespan was seen under cadmium exposure, suggesting that less energy was allocated for reproduction and more to maintenance and in repairing damage caused by cadmium. This may present a case of cross-tolerance between heat stress and cadmium stress. It has previously been demonstrated that acclimating *C. elegans* to 25°C prior to exposing them to cadmium increased cadmium tolerance (Treinin et al., 2003). As explained earlier, higher temperatures result in higher metabolic rate, and thus higher ROS production. ROS require detoxification and so, if there are more ROS, a higher level of expression of detoxification mechanisms would be expected. Heat stress also

induces heat shock proteins (HSPs), which are also induced by cadmium alone. The higher level of detoxification mechanisms brought about by heat, to combat both ROS and high temperature damage to proteins, may lead to a greater protection of cells against the toxic effects of cadmium.

7.2.3 Life-history of *C. elegans* strains and toxic response

The aim of this was to firstly gain good detailed life history data on three geographically distinct wild type isolates of *C. elegans,* as there is a lack of basic data regarding life history of this species, and secondly to examine whether the toxic response to cadmium was affected by the genotype of the organism. The effect of genotype on toxicity responses has been reported previously, with the response of different genotypes of *Daphnia magna* to cadmium varied by an order of magnitude (Barata et al., 2002). This obviously would be of importance in evaluating the use of *C. elegans* as an ecotoxicological test organism. Regulatory testing labs commonly use a single genotype of a species, however if responses to toxicants vary according to genotype then risk assessment must also take this into account and evaluate the genetic make-up of populations in the environment.

Strains were selected on the basis of their geographical isolation, namely Bristol, (UK), Bergerac, (France) and Hawaii, (USA), and also on their relative use in *C. elegans* research. All three strains have been used in QTL mapping studies (Gutteling et al., 2006; Shook et al., 1996). Strains from Bristol and Hawaii have also recently been examined in response to cadmium stress (Alvarez et al., 2005). However, the purpose of that toxicity experiment was to examine how the differing reproductive strategies of these strains, self-fertilising hermaphrodite

Bristol versus the male-hermaphrodite mating of Hawaii, affected response to cadmium.

The results of this study showed that strain had an impact on sensitivity to cadmium and that between life history parameters there was not one strain that was consistently most sensitive. The results also indicated that the $EC_{50\text{-reproduction}}$ for each strain were within an order of magnitude of each other. Bergerac was found to have a much lower fitness than either N2 or Hawaii; producing approximately 200 fewer eggs than either other strain and having a much slower developmental and growth rate. Bergerac was also more sensitive to cadmium than either N2 or Hawaii, in that at a cadmium concentration (10 mg L^{-1}) where the other strains were still capable of producing on average 70-100 eggs each, it was unable to reproduce at all. The reduced fitness of Bergerac is attributed to the high copy number of transposable elements in its genome, about 500 copies of the jumping element Tc1. It is possible that these elements may affect the functioning of metallothionein, or other mutations in the metallothionein genes may exist, which reduce the efficiency of cadmium detoxification.

7.2.4 Responses to chemicals from different major classes

Four chemicals were assayed to examine the life history stress response of *C. elegans* to chemicals from different major chemical classes; metals (cadmium), non-specific acting organics (fluoranthene), and specific-acting pesticides (atrazine and aldicarb). Whilst cadmium had significant impacts on all life history parameters measured, atrazine and fluoranthene had no impact on the length of the reproductive period, nor did they alter lifespan. Cadmium was the most toxic

compound to *C. elegans,* followed by fluoranthene, then aldicarb with atrazine being the least toxic compound. Cadmium is a non-essential metal which can not be broken down by the nematode, unlike the organic compounds for which there are detoxification pathways available to deal with these compounds. For cadmium ions, detoxification is best achieved by binding to metallothioneins so they cannot bind and disrupt cellular processes, and to eliminate them through excretion.

Chemical perturbation did not produce similar responses in life-history for all compounds; instead the stress response was compound specific. The sensitivity of life history parameters was different between compounds; however reproduction was always the most sensitive parameter. The elasticity analysis in Chapter 5 for cadmium, fluoranthene and atrazine and Chapter 6 for aldicarb allow for a clearer interpretation than can be achieved by simply taking NOECs or EC50 values for parameters (as attempted in Chapter 4, Table 4.8).

The most striking difference in response to the chemicals was that of growth. For cadmium, a large decrease in both final body size and growth rate as concentration increased was observed. In atrazine, final body size was reduced as concentration increased, however, to a lesser extent than cadmium. Growth rate in fluoranthene exposed worms was reduced; however, there was no difference in final body size as concentration increased.

The toxic response to aldicarb was somewhat different to that seen with cadmium, fluoranthene and atrazine in that when significant effects were seen on

reproduction, time to maturity and growth, lifespan was also reduced alongside the latter 3 compounds. However under aldicarb exposure lifespan was increased at low concentrations and unaffected at concentrations where reproductive and growth effects were seen.

7.2.5 A modelling approach

Life-cycle toxicity experiments have proven to be more useful than single end point toxicity tests. However they still focus on effects on individuals and not on effects on populations, which is the aim of regulatory ecotoxicology, to protect populations. To test the effects of chemicals on populations of organisms is however undesirable and would be too costly for general use. To provide a way of assessing population consequences of toxicity, various life history parameters measured in individuals can be integrated to predict the growth rate of a population. Alterations to the life schedules of individuals caused by pollutants were modelled to provide a prediction of how population growth rate would be affected. In the case of aldicarb it was shown that population growth rate would actually be enhanced by low doses of this compound.

Growth, reproduction, and maintenance are inextricably linked to each other in the competition for resources within an individual. In an attempt to explain the differences in response to different chemicals, energy budget modelling was used. DEBtox aimed to infer the energetic toxicant mode of action for each chemical. From this modelling, the toxicant impact on energetics for both cadmium and atrazine was proposed to be on the assimilation of energy, for fluoranthene increased costs for growth and for reproduction, and for aldicarb increased

maintenance costs. To confirm the DEBtox mode of action results it would be interesting to examine both pharyngeal pumping rates and lipid deposits in *C. elegans*. If the toxic mode of action is that assimilation of energy is reduced it would be expected to observe reduced pharyngeal pumping and starvation effects in the worms, which would include reduced lipid content.

DEBtox modelling also provided no effect concentration (NEC) values for each compound for effects on survival and effects on growth/reproduction. In comparison with NOEC values the NEC's were far lower (by 3-4 orders of magnitude) for effects on growth/reproduction. The values of NOEC and NEC for effects on survival were closer, within one order of magnitude of each other, except for aldicarb which was 2 fold different.

7.2.6 Transcriptomics

The amount of information created from microarray experiments is staggering and scientists are still struggling to analyse and organise the data in ways that are meaningful. Microarrays can provide the means to identifying changes in single gene expression which could be used as early warning biomarkers of stress and also to identify response cascades to toxicants which may be general or chemical class specific.

7.2.7 Aldicarb effects on life-history

Of particular significance was the identification that the acetylcholinesterase inhibitor aldicarb increased lifespan at low doses and reduced growth whilst

having no effect on reproductive output; in fact there were actually small increases in reproductive output at dose 1 mg L^{-1}. In an attempt to explain the results seen with aldicarb, dynamic energy budget, trade-off and ageing theories were considered. A trade-off in energy allocation between growth and reproduction is apparent. This would also fit with the DEBtox energetic mode of action prediction of increased costs for maintenance. If we examine energy usage in terms of DEB and the k-rule, then an increase in costs for maintenance would result in less energy left for growth, which is apparent, however this did not affect the amount of energy allocated to reproduction.

The increase in lifespan at low doses of aldicarb is of particular interest. Death and aging of an individual is thought to be brought about in a number of ways which have led to the development of a number of theories of aging. The oxidative theory of aging, which proposes that ROS, generated during normal metabolism, damage a variety of macromolecules and the accumulation of these ROS causes aging and ultimately death (Masoro and Austad, 2001). The regulation of lifespan in *C. elegans* has also been determined to be under the control of a hormonal signalling pathway, the insulin/insulin-like growth factor 1 (IGF-1)–like endocrine system (Hsin and Kenyon, 1999; Murphy et al., 2003). In reality it is likely that a combination of these factors affects lifespan.

The increased lifespan elicited by exposure to low doses of aldicarb may have been caused by a number of factors. Aldicarb may reduce metabolic rate and thus reduce the rate that ROS accumulate, an increase in stress response pathways

could confer protection against ROS, or aldicarb's effect on synaptic transmission may affect the neuronal regulation of lifespan.

The proposal of reduced metabolic rate in the nematode would mean that fewer ROS are produced and so the rate of aging would be slower leading to a longer lifespan. It has been widely researched in *C. elegans* that restricted calorie intake can result in an extended lifespan (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). A compound that diminishes the assimilation of energy is likely to extend lifespan through caloric restriction. I propose that aldicarb reduces energy assimilation via a reduction in feeding rate (contradicting the results of the DEBtox analysis). The AChE inhibition activity of aldicarb may affect the ability of the nematode to pump its pharynx and thus eat, causing a reduction in the assimilation of energy and thus a decrease in metabolism. Although pumping rate has not been directly measured in response to AChE inhibition, either in this current study or in previous studies, it may be that chemicals which inhibit AChE, and thereby affect nerve function, could have some impact on feeding rates. Evidence for this comes from the finding that acetylcholine is required for normal pharyngeal pumping. This was ascertained from *cha-1* mutant worms which rarely pump their pharynx (Avery and Horvitz, 1990), these mutant worms were defective in choline acetyltransferase, which is required for the production of ACh. Further evidence to support this comes from observations on the effects of nicotine (a nicotinic ACh receptor agonist) and oxotremorine (a muscarinic ACh receptor agonist). Nicotine stimulated the frequency of pharyngeal contractions and oxotremorine lengthened the time of pharyngeal contraction in wild type nematodes (Avery and Horvitz, 1990). *C. elegans* pharyngeal muscle contracts

upon ACh release from the MC motor neurons and ACh is the critical neurotransmitter in fast pumping through nicotinic receptors and also in regulating pumping through the muscarinic receptors (McKay et al., 2004; Raizen et al., 1995; Steger and Avery, 2004). The inhibition of feeding could easily be assayed by examining pharyngeal pumping rate in aldicarb exposed worms.

A second possible influence on increased lifespan may be that aldicarb induces stress response pathways which also confer protection against the endogenous product of metabolism, ROS, which may result in age-related declines of physiological processes, thereby conferring lifespan extension. Transcriptomic data would be of use in identifying if aldicarb induced super oxide dismutases (SODs), which detoxify ROS. The nematodes used for microarrays in this thesis had been exposed to a higher dose of aldicarb than that which produced a lifespan extension, so it would be interesting to profile the gene expression of nematodes exposed to low dose aldicarb.

The final proposal is that the increase in lifespan was a result of the disruption of neuronal signalling that regulates lifespan. Lifespan extension caused by exposure to specific chemicals has been reported previously, in response to anticonvulsant medications (Evason et al., 2005). The results from this study indicated that these drugs stimulate synaptic transmission in the neuromuscular system. Ethosuximide increased mean lifespan of wildtype nematodes by 17%, from 16.7-19.6 days at 15°C. Mutations that stimulate synaptic transmission cause hypersensitivity to aldicarb-mediated paralysis (Miller et al., 1999). Overall, the genetic and pharmacologic studies of *C. elegans* provides substantial

support that neural activity limits lifespan by accelerating the aging of non-neuronal cells, as reviewed by Kornfeld (2006). It has been shown that expression of the *daf-2* gene (part of the IGF-signalling pathway) specifically in neurons is able to rescue the extended lifespan of a *daf-2(-)* mutant (Wolkow et al., 2000). This raises the possibility that the increase in lifespan at low aldicarb concentration can be attributed to the effect of the chemical on nerve synapses. Again microarray experiments on nematodes exposed to low dose aldicarb would be useful in proving or disproving this. The examination of response to aldicarb in mutant strains which are defective for parts of the insulin signalling pathway, or by RNAi could also prove useful.

7.3 Concluding remarks and future work

The current thesis has incorporated three scientific fields; ecology, toxicology and genomics in an attempt to integrate the effects of pollutants on the nematode *C. elegans*. Very few studies have attempted to simultaneously investigate effects of toxicants on the molecular and life cycle of individuals. This type of approach, along with the added benefits of population and energy budget modelling, will greatly enhance ecotoxicological studies and offers the potential to reveal the mechanisms underlying changes at the individual life history level, and ultimately the effects on populations.

The use of *C. elegans* in ecotoxicology would be worthwhile, especially in the field of ecotoxicogenomics. The use of this organism would help elucidate mechanisms of toxicity at the molecular level which may then be used to ascertain

modes of action on more ecologically relevant species. It may also offer the opportunity to identify harmful effects of pollutants in humans.

One area for further exploration would be the comparison of parameters used in the DEB model (growth, maintenance, reproduction, assimilation) to the over- and under-representation of genes whose function is in these areas to confirm or not the DEBtox model. Whilst gene ontology terms already group genes into reproduction, growth and maintenance categories, careful consideration as to the grouping of genes to the terms in DEB would be needed. The gene ontology terms; *reproduction*, *growth* and *maintenance* may not provide the genes which are required for analysis in terms of energy budgets. There are also terms such as development which would need to be integrated into the DEB categories.

A second area of interest, with the use of transcriptomics in ecotoxicology would be in the identification of genes which are induced or repressed in response to chemical stress. This raises the possibility of creating a chip/system where a number of these known stress responsive genes can be assayed simultaneously to investigate general stress and chemical class specific response. For example, metallothioneins would be induced by metals or cytochrome P450's induced to detoxify organic compounds. This would greatly enhance pollution monitoring.

Future work to enhance this project would be to validate the transcriptomic data by selecting a number of significantly up- or down-regulated genes of interest and house-keeping genes to perform quantitative real-time PCR (Q-PCR). It would also be useful to perform RNA interference (RNAi) with *C. elegans* on selected

genes under chemical exposure, as this may help to indicate essential genes used in the detoxification of a compound, or genes that may be involved in life-history traits. It would also be desirable to perform a microarray experiment on nematodes exposed to a low dose, 4 mg L^{-1}, of aldicarb to elucidate mechanisms for lifespan extension.

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Appendix A

Table 1.1: Reagents and consumables supply

Supplier	Reagents
ABgene (Surrey, UK)	0.2ml PCR tubes dNTPs (dATP, dCTP, dGTP, dTTP) MMuLV Reverse transcriptase / 5 x RT buffer
Alpha labs (Hampshire, UK)	Falcon tubes, Filter tips
Ambion (Austin, TX, USA)	Amino allyl dUTP
Amersham Biosciences UK Ltd (Buckinghamshire, UK)	Cy3 / Cy5 dyes CyScribe™ GFX™ Purification Kit RNA internal control
Bioline Ltd (London, UK)	Agarose
BDH Laboratory Supplies (Poole, UK)	NaOAC, SDS
Eppendorf UK Ltd (Cambridgeshire, UK)	UVettes
Fisher (Loughborough, UK)	12 well sterile tissue culture plates, Acetic acid, $CaCl_2$, Chloroform, Ethanol, Fluoranthene, Glycerol, Hydrochloric acid, Isopropanol, $MgSO_4$,

Supplier	Reagents
	NaCl, $NaHCO_3$
Greiner Bio-one (Stonehouse, UK)	1.5ml micro centrifuge tubes, 24 well sterile tissue culture plates, 25 ml sterile disposable pipettes, 50 ml sterile disposable PP-tubes, 60/90mm sterile vented Petri dishes, Sterile, disposable universal tubes, Pipette tips
Invitrogen (Paisley, UK)	10 x TAE
Millipore UK Ltd. (Watford, UK)	0.22μM nucleopore™ filters
MWG Biotech (Erbesberg, Germany)	Oligo dT priimer Oligonucleotide primers
Oxoid Ltd (Basingstoke, UK)	Bacto agar Bacto peptone
Promega Ltd (Southampton, UK)	DNA molecular weight markers
QIAGEN Ltd. (Crawley, UK)	Rneasy mini kit
Roche	Tris
Sigma Aldrich (St. Louis, USA)	Agarose, Aldicarb, Atrazine, $CaCl_2$, $CdCl_2$, Cholesterol, Ethanolamine, Ethidium bromide, Glass beads, HEPES, HPLC grade water, Hydroxylamine,

Supplier	Reagents
	Isoamyl alcohol, KOH, LB agar, LB broth, $MgSO_4$, NaOAC, NaOH, KH_2PO_4, S-Gal / LB agar blend, Sodium hypochlorite, SSC, TRI reagent™,
Star Lab UK Ltd (Milton Keynes, UK)	Pipette tips

Table 1.2: Solutions, media and reaction buffers

Solutions	Components
Agarose gel electrophoresis (Section 2.4.4)	
Electrophoresis loading dye	50% (v/v) Glycerol, 1% (w/v) Bromophenol blue, 1x TAE (sterilised)
	40 mM Tris-acetate, 1 mM Na_2-EDTA (pH 7.6)
C. elegans culturing (Section 2.3)	
NGM agar	1.7% bacto agar, 0.025% bacto peptone, 0.005% Cholesterol 50mM NaCl, 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM KH_2PO_4
M9 buffer	20mM KH_2PO_4, 50mM $NaHPO_4$, 90mM NaCl, 1mM $MgSO_4$
Reverse - transcriptase - Polymerase chain reaction (RT-PCR) (Section 2.5.4)	
5x MMLV-RT buffer	50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT

Solutions	Components
Purification of total RNA (Section 2.4.3)	
RNeasy Mini Kit Buffer RLT and RPE	Proprietary Formulation

Appendix B

Table 1.1: Gene list of statistically significant up-regulated genes. Genes are more than 1.4 fold up-regulated, obtained using a t-test with the Benjamin and Hochberg false discovery rate set at $p < 0.05$.

Gene name	Description	Fold change
F42G2.3	F-box domain	337.5
F38E1.2		248.7
T19B10.3	Beta-galactosidase	203.4
C50H11.10	7-transmembrane receptor	199.7
R193.2		193.4
C55A1.4	Uncharacterized protein	188.3
E04A4.4b		182.6
F32B4.2	Translocase of outer mitochondrial membrane complex, subunit TOM20	180.5
F43D9.1	Predicted membrane protein (patched superfamily	163.7
F55D12.2		148.7
F41B5.3	Cytochrome P450 CYP2 subfamily	148.4
C23H4.4	Carboxylesterase and related proteins	145.6
C37A2.3	Short-chain acyl-CoA dehydrogenase	145.1
R13D7.10	7-transmembrane receptor	142.7
Y44E3A.4	Adaptor protein CMS/SETA	138.9
F40F9.1b	N-methyl-D-aspartate receptor glutamate-binding subunit	135.8
F20H11.4	Protein tyrosine phosphatase	134.2
C53B7.5	7-transmembrane olfactory receptor	133.4
T12C9.6	Hormone receptors	126.9
F15A8.6	Carboxylesterase and related proteins	126.7
Y51H7C.5		123.5
F14E5.6	Lysosomal & prostatic acid phosphatases	123.4
C05D11.8	Uncharacterized conserved protein	122.6
ZC247.2		122.6
R09H10.4	Predicted membrane protein (patched superfamily	118.6
F43C9.4b		116.9
C30B5.3	Predicted RNA-binding protein (RRM superfamily	110.2
C25F6.7b		109.2
F59B1.1	7-transmembrane olfactory receptor	104.5
F09E8.5	Defense-related protein containing SCP domain	104.2
F32H2.9	Alpha tubulin	103.2
C42D4.4	7-transmembrane olfactory receptor	100.2
F40H7.8	7-transmembrane receptor	99.16
	No Significant Match	93.1
C18A3.4b	Predicted seven transmembrane receptor - rhodopsin family	92.85
F11H8.2	GABA receptor	92.49
	No Significant Match	92.27
F28D1.8		92.19
M02H5.10	Unnamed protein	92.16
Y5H2B.5	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	91.41
F55B11.5		89.66
T13F3.5	Uncharacterized protein	89.24
F31D4.7	Tandem pore domain K+ channel	88.99
	No Significant Match	88.86
K09C6.5	Unnamed protein	88.72
T02G6.5	Uncharacterized protein	87.03
F54E4.3		85.91

Gene name	Description	Fold change
	No Significant Match	84.52
F09C8.2	Uncharacterized conserved protein	83.71
C09B8.5		83.64
C18G1.9	Unnamed protein	81.79
C13G3.2		81.64
R08E5.1	SAM-dependent methyltransferases	80.51
F09C6.5	Serpin	80.03
F01F1.10c	Predicted glycosylhydrolase	79.94
T13H10.1	Protein tyrosine kinase	79.28
F59A6.3	Unnamed protein	78.81
F37D6.3		78.56
M153.2	Uncharacterized conserved protein	78.47
W02B12.12b		78.44
Y113G7A.4	Ca2+/Na+ exchanger NCX1 and related proteins	78.17
W08D2.1	Wnt family of developmental regulators	77.27
F27B3.5	Predicted E3 ubiquitin ligase	77.24
F53C3.12	Beta, beta-carotene 15,15'-dioxygenase and related enzymes	77.07
Y49F6C.5	Uncharacterized protein, contains BTB/POZ domain	76.53
F36H5.4	Predicted membrane protein	76.31
		76.18
C08B11.2	Histone deacetylase complex, catalytic component RPD3	75.92
C25B8.5	Unnamed protein	74.52
F23B12.9		74.36
H05L14.2		74.13
Y71G12B.27	Cyclin-dependent protein kinase CDC28, regulatory subunit CKS1, and related proteins	73.97
C29H12.6		73.65
Y43F8C.17		73.58
W01A11.7		73.01
K08C9.5	Uncharacterized protein	72.49
C54D1.6	Armadillo/beta-Catenin/plakoglobin	72.38
C09E7.2		72.23
C08F1.9		72.22
R04D3.1	Cytochrome P450 CYP2 subfamily	71.12
F26H11.5	Intracellular Cl- channel CLIC, contains GST domain	71.11
T10D4.9	Predicted olfactory G-protein coupled receptor	71.05
C50H2.6		70.84
F32G8.1	Chemoreceptor/7TM receptor	70.37
Y57G7A.11		70.27
B0222.3	Na+/Pi symporter	69.26
T22B3.1	Unnamed protein	69.03
C35A5.2	UDP-glucuronosyl and UDP-glucosyl transferase	69.01
Y51H7BR.7		67.19
C16C4.5	Uncharacterized protein, contains BTB/POZ domain	66.94
T06H11.1b		66.54
C52B9.1b		65.95
C27C7.4	Hormone receptors	65.79
R05D7.4	Predicted alpha/beta hydrolase	64.99
B0280.13		64.42
C08F1.8	Predicted membrane protein	64.36
Y37H2A.11	Unnamed protein	63.59
D1044.5		63.22
F35G12.6	Mab-21-like cell fate specification proteins	62.76
D2092.2	Protein phosphatase 4 regulatory subunit 2 related protein	62.4
	No Significant Match	61.74
T04H1.4	DNA repair protein RAD50, ABC-type ATPase/SMC superfamily	61.62
K11H12.9	Jun-N-terminal kinase (JNK	61.49
Y41D4B.18	Unnamed protein	61.06
Y65B4BR.3	Predicted membrane protein (patched superfamily	60.92

Gene name	Description	Fold change
DC2.6		60.56
C50E3.5		60.27
F09C6.4	Serpin	60.13
C54A12.4	Ras-related GTPase	59.91
C17F4.1	C-type lectin	59.72
F10D2.8		59.43
K08D10.10	7-transmembrane receptor	58.47
		58.13
D2085.1	Multifunctional pyrimidine synthesis protein CAD (includes carbamoyl-phophate synthetase, aspartate transcarbamylase, and glutamine amidotransferase	57.94
	No Significant Match	57.69
		57.41
C44C1.3	Ca2+ sensor (EF-Hand superfamily	57.28
C06E2.3	Ubiquitin-protein ligase	56.9
	No Significant Match	56.64
C10H11.3	UDP-glucuronosyl and UDP-glucosyl transferase	56.51
K07B1.6a		56.35
	No Significant Match	55.77
C45H4.16	Unnamed protein	55.63
C08E3.7	Uncharacterized protein	55.27
T26C12.3	Ras-related GTPase	55.26
B0454.5		54.92
C27H5.2a	Unnamed protein	52.77
C56G2.1b		52.07
C39D10.1	Predicted TR:Q18528 AAK39205.1	51.76
T10A3.1a		51.35
F26D10.11	Uncharacterized conserved protein	51.08
ZK970.1b		50.3
C16D9.4	Unnamed protein	50.24
C08G5.1	WSN domain	48.82
F28H6.6	Unnamed protein	47.63
F32D1.6		47.48
B0412.1a	Transcription regulator dachshund, contains SKI/SNO domain	46.78
W09C3.2	Unnamed protein	45.84
C30F8.4b		45.48
E03H4.13	Hormone receptors	45.23
T13A10.2	Predicted E3 ubiquitin ligase	44.07
F22B5.4	Unnamed protein	42.63
Y16B4A.1	HLH transcription factor EBF/Olf-1 and related DNA binding proteins	42.2
Y105C5A.5	Uncharacterized protein	41.86
F52C6.14	C2H2-type Zn-finger	41.8
C43F9.4		39.06
F47G4.8		38.98
K04B12.1	Plexins (functional semaphorin receptors	38.38
F23H11.7		37.96
R12E2.6	Unnamed protein	37.29
Y56A3A.33	3'-5' exonuclease	34.59
		34.52
F15D3.4	Unnamed protein	32.33
R08F11.5	7-transmembrane olfactory receptor	31.23
K09C4.6		30.66
F47D12.1a		30.18
F48B9.1		29.11
C14F11.3	Unnamed protein	27.31
F42G9.4	Partially_confirmed TR:Q20348 AAA91351.2	22.98
T26G10.5		22.2
C48D1.3	Choline transporter	18.76

Gene name	Description	Fold change
C45H4.12	7-transmembrane olfactory receptor	18.14
T10C6.13	Histones H3 and H4	4.644
T05H10.3	Permease of the major facilitator superfamily	4.481
		4.191
C47C12.6	Non voltage-gated ion channels (DEG/ENaC family	4.148
Y43F11A.4	Unnamed protein	3.749
D1073.1		3.345
ZC262.1	Partially_confirmed TN:AAK67243 AAK67243.1	3.228
C49C8.2		3.154
Y62H9A.8		3.082
C55A1.5	7-transmembrane olfactory receptor	3.02
F45F2.3	Histone H4	3.005
D1009.2b	HSP90 co-chaperone CPR7/Cyclophilin	2.848
T07C12.6	Sre G protein-coupled chemoreceptor	2.685
C50F7.4	GTP-specific succinyl-CoA synthetase, beta subunit	2.577
Y39B6A.29	Uncharacterized conserved protein	2.572
F15A4.4	Receptor-like protein, Srg family	2.545
F22B5.9	Phenylalanyl-tRNA synthetase beta subunit	2.539
C31A11.3	Unnamed protein	2.527
Y57G11C.15	Transport protein Sec61, alpha subunit	2.392
M79.2	Predicted phosphate acyltransferase, contains PlsC domain	2.385
F08G2.3	Histones H3 and H4	2.372
F34D10.6		2.306
Y67D8B.5		2.275
Y38C1AA.7	Unnamed protein	2.272
C43E11.6a	Protein phosphatase 1 binding protein spinophilin/neurabin II	2.26
C44B12.6	Unnamed protein	2.243
F45E1.6	Histones H3 and H4	2.242
ZK131.9	Histone H2B	2.219
C09G9.5		2.217
T28C6.7		2.191
	No Significant Match	2.171
F47B3.7	Protein tyrosine phosphatase	2.156
F55G1.3_1	Histone H2B	2.148
T02H6.3	Uncharacterized protein	2.11
Y22D7AR.10	Histone acetyltransferase (MYST family	2.102
F35C11.4	Unnamed protein	2.092
F31F7.3		2.092
R05A10.2		2.09
Y59A8B.10	RNA-binding protein NOVA1/PASILLA and related KH domain proteins	2.088
C08F1.10	Predicted membrane protein	2.072
T08B1.6	Long-chain acyl-CoA synthetases (AMP-forming	2.071
Y46G5A.6	RNA helicase BRR2, DEAD-box superfamily	2.069
F53F1.3	Aldo/keto reductase family proteins	2.049
C08B6.7	WD40 protein DMR-N9	2.045
W06D4.6	DNA repair protein, SNF2 family	2.044
		2.037
	No Significant Match	2.03
T08G5.10	Predicted metallothionein	2.023
F59B2.12		2.018
F46F2.1	Predicted E3 ubiquitin ligase	1.997
F32E10.2	Heterochromatin-associated protein HP1 and related CHROMO domain proteins	1.986
Y71G12B.12a	Protein involved in autophagy and nutrient starvation	1.985
F44B9.6		1.968
ZK637.4		1.957
F02A9.4b	Ribonuclease III domain proteins	1.956
F46F5.6	Unnamed protein	1.956

Gene name	Description	Fold change
R53.3b	Zn-finger	1.947
C03A7.10	Integral membrane O-acyltransferase	1.942
K02G10.8	Molecular chaperone (DnaJ superfamily	1.94
F40E10.1	Meprin A metalloprotease	1.938
Y40B10A.8	Hormone receptors	1.93
ZK1240.3	Predicted E3 ubiquitin ligase	1.928
K04G11.4	WD40 repeat-containing protein	1.927
Y40B1B.7	Predicted coiled-coil protein	1.916
C37E2.1	Isocitrate dehydrogenase, gamma subunit	1.913
F55F10.1	AAA ATPase containing von Willebrand factor type A (vWA	1.913
F31A3.4	Unnamed protein	1.912
F32D8.3		1.908
C13G3.1		1.902
F49F1.3	Partially_confirmed TR:Q9GZE7 AAF99970.1	1.897
C50D2.2	Amino acid transporters	1.895
Y49A3A.5	Cyclophilin type peptidyl-prolyl cis-trans isomerase	1.892
C07H6.5	ATP-dependent RNA helicase	1.892
T10B5.3	WD40 repeat-containing protein	1.887
C33F10.8	Protein tyrosine phosphatase	1.885
F56F11.4b	26S proteasome regulatory complex, ATPase RPT6	1.882
ZK616.10c		1.876
C50F7.3	PDZ domain	1.871
C04F1.3	Transcription factor, contains HOX domain	1.869
DC2.7c_1		1.868
H10E21.5	Predicted E3 ubiquitin ligase	1.868
ZK1290.3b	Confirmed	1.867
C16D9.8		1.853
Y49F6C.8		1.848
Y41G9A.1	TPR repeat-containing protein	1.846
Y59H11AM.1	Major sperm protein domain	1.839
T21G5.1	Protein tyrosine kinase	1.837
F37C12.7	Acyl-CoA synthetase	1.836
K01A6.4	Predicted TR:Q21073 CAA92963.1	1.836
C35E7.2a	Uncharacterized protein	1.824
F59F4.3		1.811
T25E12.5	Glycosyl transferase, family 8 - glycogenin	1.807
F56F4.2	Uncharacterized protein with conserved cysteine	1.806
		1.805
Y22D7AL.5	Mitochondrial chaperonin, Cpn60/Hsp60p	1.802
F55C7.2		1.801
T19C3.7	Unnamed protein	1.798
M01E11.4c		1.794
C14B1.1	Protein disulfide isomerase (prolyl 4-hydroxylase beta subunit	1.788
T08A9.3	Synaptic vesicle protein Synaptogyrin involved in regulation of Ca2+-dependent exocytosis	1.787
M01A8.1		1.785
Y48G8AR.2		1.783
C15H11.3	mRNA export factor TAP/MEX67	1.78
F17E9.2	Unnamed protein	1.78
R160.3		1.777
F26D2.7	7-transmembrane olfactory receptor	1.776
R06F6.7		1.773
ZK353.3		1.771
Y23B4A.1		1.767
K08E5.2b		1.758
T27F6.5	Prolyl-tRNA synthetase	1.757
Y52E8A.2	Predicted E3 ubiquitin ligase	1.754
Y37A1C.1a	Na+/K+ symporter	1.745
C10B5.1		1.744

Gene name	Description	Fold change
Y75B12B.5	Cyclophilin type peptidyl-prolyl cis-trans isomerase	1.742
C47A4.5	Predicted TR:Q9U3L6 CAB62795.1	1.741
F56F3.6	Unnamed protein	1.739
C25A1.6	H/ACA snoRNP complex, subunit NOP10	1.738
Y44A6D.3		1.735
H34C03.1		1.73
R05F9.3	Major sperm protein domain	1.728
C08A9.3	Unnamed protein	1.708
W04B5.2		1.707
Y56A3A.30		1.701
K08E7.2	Heat shock factor binding protein	1.699
F33H1.2	Glyceraldehyde 3-phosphate dehydrogenase	1.696
CD4.6	20S proteasome, regulatory subunit alpha type PSMA1/PRE5	1.689
F58A6.9		1.683
R02C2.2	Checkpoint kinase and related serine/threonine protein kinases	1.682
F01D5.10	Chondroitin 6-sulfotransferase and related sulfotransferases	1.68
T05A8.1	Unnamed protein	1.679
C05D11.11b_1	Glycine/serine hydroxymethyltransferase	1.678
R11G11.5	7-transmembrane olfactory receptor	1.676
F38A3.1	Collagens (type IV and type XIII	1.675
Y39A1A.8	Multitransmembrane protein	1.673
Y71H2AM.20b		1.665
ZK218.1	Secreted surface protein	1.658
F35D11.8	C-type lectin	1.658
M03D4.3		1.647
Y53G8AR.2b		1.646
C56E6.4		1.646
W06E11.4	Predicted exosome subunit	1.645
F40E3.3	Predicted TR:O01548 AAC24294.1	1.642
	No Significant Match	1.64
W02D9.8		1.636
ZK896.9	Predicted UDP-galactose transporter	1.628
C36C9.4	Unnamed protein	1.626
F59B8.1b	Unnamed protein	1.626
ZK938.1	Serine/threonine specific protein phosphatase PP1, catalytic subunit	1.624
Y49E10.15	Small nuclear ribonucleoprotein E	1.623
C53A5.13	Immunoglobulin C-2 Type/fibronectin type III domains	1.622
	No Significant Match	1.622
Y45G12C.5	7-transmembrane receptor	1.621
C04G2.8	Confirmed TR:Q17626 CAA94670.1	1.615
T11F9.14	Unnamed protein	1.613
Y76G2A.1_1	Alternative splicing factor SRp55/B52/SRp75 (RRM superfamily	1.612
		1.605
Y54G11A.10	Receptor targeting protein Lin-7	1.604
B0218.6	C-type lectin	1.603
F43C1.6	Mitochondrial/chloroplast ribosomal L21 protein	1.601
ZK20.4	Uncharacterized conserved protein (contains TPR repeat	1.6
	No Significant Match	1.589
ZK682.5	Leucine rich repeat	1.587
T20F5.6	Predicted E3 ubiquitin ligase	1.585
F26D10.3	Molecular chaperones HSP70/HSC70, HSP70 superfamily	1.582
T28F2.1	Predicted glycosyltransferase	1.581
F13A7.8	Predicted olfactory G-protein coupled receptor	1.578
F27C1.10		1.576
ZK770.3	Innexin-type channels	1.575
Y48G9A.2		1.564
F53A3.4	Partially_confirmed	1.564
F40A3.3b		1.563

Gene name	Description	Fold change
Y60A9.3	CCCH-type Zn-finger protein	1.562
Y59A8B.23	Ste20-like serine/threonine protein kinase	1.556
Y38E10A.24		1.555
ZK287.8b		1.552
Y43F8B.14	Helicase-like transcription factor HLTF/DNA helicase RAD5, DEAD-box superfamily	1.549
C23G10.3	40S ribosomal protein S3	1.548
C15C7.4		1.539
	No Significant Match	1.538
F38A5.2b	Uncharacterized conserved protein, contains TraB domain	1.533
Y60A3A.12	Serine/threonine protein kinase Chk2 and related proteins	1.53
K10D3.1	Glutamate-gated kainate-type ion channel receptor subunit GluR5 and related subunits	1.53
C52B9.2b	Predicted transcription factor	1.528
R74.7	SAM-dependent methyltransferase/cell division protein FtsJ	1.527
C02F5.9	20S proteasome, regulatory subunit beta type PSMB1/PRE7	1.525
F26E4.6	Cytochrome c oxidase, subunit VIIc/COX8	1.524
C53C11.4	S. muris microneme antigen Predicted TR:P91183 AAB42309.1	1.523
C01B10.7	Unnamed protein	1.522
C02G6.1	N-arginine dibasic convertase NRD1 and related Zn2+-dependent endopeptidases, insulinase superfamily	1.519
Y54E5A.8b	Unnamed protein	1.518
ZC434.3	Unnamed protein	1.518
F15A4.1	Sre G protein-coupled chemoreceptor	1.513
H14A12.4	TBX1 and related T-box transcription factors	1.511
C30H7.2	Thiol-disulfide isomerase and thioredoxin	1.511
Y69A2AR.28		1.507
F01G4.4		1.507
Y17G7B.6	Unnamed protein	1.505
F25B5.4c		1.503
W06D12.4	7-transmembrane olfactory receptor	1.501
R05H10.6	Cadherin repeats	1.499
C27H5.6	Unnamed protein	1.499
M7.1	Ubiquitin-protein ligase	1.49
F26F12.7	Predicted helicase	1.486
Y41E3.10	Elongation factor 1 beta/delta chain	1.475
Y73C8C.4	Uncharacterized protein	1.468
F53G12.5b	Predicted RNA binding protein, contains KH domain	1.467
C32E8.10h	Clathrin assembly protein AP180 and related proteins, contain ENTH domain	1.465
ZK1127.1	Unnamed protein	1.462
C25F6.4	Discoidin domain receptor DDR1	1.451
Y48G8AL.11	Peptide exporter, ABC superfamily	1.449
C09B9.1	Unnamed protein	1.442
Y57G11C.11	Methyltransferases	1.437
K02B12.5	Ankyrin repeat	1.432
T05H4.12	Mitochondrial F1F0-ATP synthase, subunit Cf6 (coupling factor 6	1.429
Y37D8A.11b	Dosage compensation regulatory complex/histone acetyltransferase complex, subunit MSL-3/MRG15/EAF3, and related CHROMO domain-containing proteins	1.427
F42G9.9a	Microtubule-associated protein TAU	1.415
C05E7.2	Unnamed protein	1.405

Table 1.2: Gene list of statistically significant down-regulated genes. Genes are more than 1.4 fold down-regulated, obtained using a t-test with the Benjamin and Hochberg false discovery rate set at $p < 0.05$

Gene name	Descrption	Fold change
C01B10.3	Inositol polyphosphate 5-phosphatase, type I	0.713
F20B6.7		0.71
K07F5.13c	Nuclear pore complex, p54 component (sc Nup57	0.697
F35G12.9	Anaphase-promoting complex (APC	0.693
H06H21.8b	Predicted small molecule kinase	0.691
C03A7.14	Uncharacterized protein	0.685
Y62F5A.1a	Uncharacterized conserved protein	0.683
ZK822.4	Predicted alpha-helical protein	0.681
H42K12.2		0.679
ZK1225.5	Splicing factor RNPS1, SR protein superfamily	0.677
K07E3.4b	Formyltetrahydrofolate synthetase	0.676
		0.676
M02D8.3		0.67
ZK593.6	Microtubule-associated anchor protein involved in autophagy and membrane trafficking	0.669
C35C5.9	Confirmed TR:Q93333 CAB01689.2	0.668
M01A10.1	K-homology type RNA binding proteins	0.667
ZK455.8a	Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily	0.666
T01C8.2	Unnamed protein	0.663
B0412.2	Transforming growth factor beta, bone morphogenetic protein and related proteins	0.657
T25G12.4	GTPase Rab6/YPT6/Ryh1, small G protein superfamily	0.652
Y54F10AL.2a	Nonsense-mediated mRNA decay protein	0.651
Y7A5A.4_35	Predicted transposase	0.65
C50H2.10		0.65
F49D11.8	Calponin	0.649
C05E11.7	Unnamed protein	0.649
Y38H6C.8	C-type lectin	0.643
Y105E8B.1e		0.641
Y48D7A.2		0.638
T11B7.2		0.634
Y39A3CL.4b	Uncharacterized conserved protein with TLDc domain	0.632
T23G4.4	Integral membrane O-acyltransferase	0.629
Y41C4A.13	Unnamed protein	0.624
T22D2.1	Ankyrin repeat protein	0.622
Y49C4A.8b		0.619
ZK154.4		0.619
C18H7.3	cuticular collagen Predicted TR:Q9GZF7 AAF98609.1	0.614
C25E10.12	Phosphoesterases	0.612
F49B2.3	Unnamed protein	0.607
C52G5.2	Uncharacterized conserved protein	0.605
F53H4.2	Unnamed protein	0.605
T07F10.4	Predicted membrane protein	0.602
Y45G12C.4	Predicted small molecule kinase	0.598
C18C4.10b	Kinesin light chain	0.596

Gene name	Descrption	Fold change
Y7A5A.4_16	Predicted transposase	0.596
R52.3	Uncharacterized protein, contains BTB/POZ domain	0.596
Y54E2A.1	7 transmembrane receptor	0.593
F26H11.4	Unnamed protein	0.591
C18A3.6b	GTPase Rab3, small G protein superfamily	0.586
C18D11.4	RRM domain	0.584
T04A8.3	Lectin C-type domain/CUB domain	0.582
M03A8.3_1		0.581
Y43F8C.12	Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily	0.58
F08H9.9	C-type lectin	0.576
ZK643.5		0.576
Y49E10.14	CCCH-type Zn-finger protein	0.576
Y59C2A.3	Unnamed protein	0.575
R06C7.10	Myosin class II heavy chain	0.575
F56D5.1	Collagens (type IV and type XIII	0.573
F17B5.2	Integral membrane O-acyltransferase	0.571
B0035.13	Predicted lipase	0.57
Y67A10A.2	Zinc metalloprotein	0.567
K10D2.1a	Histone transcription regulator HIRA, WD repeat superfamily	0.564
F07C6.4c		0.561
B0228.4c	Copine	0.561
Y102A5C.7	C-type lectin	0.558
T11G6.2	Permease of the major facilitator superfamily	0.555
Y41E3.11	Scaffold/matrix specific factor hnRNP-U/SAF-A, contains SPRY domain	0.554
H24O09.1	Unnamed protein	0.552
	No Significant Match	0.552
M03F8.1	Unnamed protein	0.551
F17A9.5	NADH:flavin oxidoreductase/12-oxophytodienoate reductase	0.544
Y7A5A.4_4	Predicted transposase	0.541
Y48G8AL.12	Unnamed protein	0.537
T23F2.3	Stress responsive protein	0.536
C54F6.14	Ferritin	0.534
F38A5.5	Unnamed protein	0.533
T22A3.5	Uncharacterized conserved protein, contains double-stranded RNA-binding motif and WW domain	0.533
Y46B2A.3	Uncharacterized protein	0.529
B0564.9	Leucine rich repeat proteins, some proteins contain F-box	0.519
Y73F8A.6		0.516
F58G11.4	Predicted transporter/transmembrane protein	0.515
C52E2.3	Extracellular protein with cysteine rich structures	0.515
F08C6.1b		0.514
T04C12.3		0.513
Y57A10A.30b	Translation initiation factor 4F, cap-binding subunit (eIF-4E	0.509
Y67D8A.3	Transcription factor Doublesex	0.509
		0.508
B0304.1c		0.504
F01G4.1	Chromatin remodeling complex SWI/SNF, component SWI2 and related ATPases (DNA/RNA helicase superfamily	0.502
T23B3.2	Stress responsive protein	0.501
R03C1.1		0.5
T10B10.8	Glycosyl transferase, family 8 - glycogenin	0.499
C49A9.2	Uncharacterized protein with ubiquitin fold	0.498
M04F3.4	Ca2+-binding protein, EF-Hand protein superfamily	0.495

Gene name	Descrption	Fold change
C18D4.3	Unnamed protein	0.49
F35C5.2	Sra family integral membrane protein	0.489
T24H10.7b		0.485
ZK6.10	Uncharacterized protein	0.481
EGAP2.1		0.481
F26G5.9	Predicted E3 ubiquitin ligase	0.481
C07G1.1	Trypsin	0.48
F49H12.5	Uncharacterized conserved protein	0.477
F53F1.4	Unnamed protein	0.476
F40A3.1		0.47
T12G3.1	Uncharacterized conserved protein, contains ZZ-type Zn-finger	0.47
		0.466
E02H9.6	Unnamed protein	0.463
Y7A5A.4_8	Predicted transposase	0.463
Y105C5A.6	Uncharacterized protein	0.461
C10H11.6	UDP-glucuronosyl and UDP-glucosyl transferase	0.459
Y39D8C.1	Lipid exporter ABCA1 and related proteins, ABC superfamily	0.459
F52C6.10	Uncharacterized protein, contains BTB/POZ domain	0.454
		0.45
ZC8.3	Predicted histone tail methylase containing SET domain	0.449
F52A8.4	Unnamed protein	0.449
Y7A5A.4_20	Predicted transposase	0.446
T22C1.7	Junctional membrane complex protein Junctophilin and related MORN repeat proteins	0.445
F08B12.3c		0.444
		0.438
Y57G11C.32		0.432
W05G11.3	Collagens (type IV and type XIII	0.431
C44F1.3	Galectin, galactose-binding lectin	0.429
	No Significant Match	0.428
Y57A10C.6	Peroxisomal 3-ketoacyl-CoA-thiolase P-44/SCP2	0.427
ZC449.6	C-3 sterol dehydrogenase/3-beta-hydroxysteroid dehydrogenase and related dehydrogenases	0.425
T28B4.3	Uncharacterized protein with conserved cysteine	0.422
F47D12.1e		0.421
W06B4.2	Putative N2,N2-dimethylguanosine tRNA methyltransferase	0.418
F56B3.10	Glutathione S-transferase	0.417
B0495.4	Sodium/hydrogen exchanger protein	0.414
F44A2.3	BPI/LBP/CETP family protein	0.413
T01D3.6b	Ficolin and related extracellular proteins	0.412
F37C12.10		0.41
C10G8.2	Serine proteinase inhibitor (KU family	0.41
	No Significant Match	0.41
Y39F10C.1	Uncharacterized protein	0.409
C34G6.7b		0.407
ZK250.9_1	DNA helicase PIF1/RRM3	0.401
M28.6	Predicted esterase	0.401
ZC262.2a	Uncharacterized conserved protein	0.401
T13H5.5	Mitochondrial ribosomal protein S18b	0.392
F46F5.5	Unnamed protein	0.392
T21B4.7	Predicted olfactory G-protein coupled receptor	0.387
ZK1010.5	Unnamed protein	0.386
F33H1.1b	RFX family transcription factor	0.386
C32D5.8b	Thioredoxin, nucleoredoxin and related proteins	0.384

Gene name	Descrption	Fold change
Y53H1B.2	Unnamed protein	0.381
Y46H3D.8	Secreted surface protein	0.381
W04G3.2	Unnamed protein	0.377
F49E8.6		0.373
T13H5.2	Phosphatidylinositol transfer protein SEC14 and related proteins	0.361
Y73E7A.8	Unnamed protein	0.36
K08E3.6	GTPase-activating protein	0.357
Y18D10A.3	Uncharacterized conserved protein	0.355
T05B4.5	7-transmembrane olfactory receptor	0.354
ZK1055.2	Glycosyltranferase	0.349
Y38F1A.9		0.345
F26A3.6		0.343
AC3.4	Uncharacterized protein	0.337
T07H8.3	7-transmembrane receptor	0.333
K08C7.6		0.33
Y67D2.6	DEAH-box RNA helicase	0.33
F09C3.1	Ras GTPase-activating protein family - IQGAP	0.326
Y61B8A.1	Predicted olfactory G-protein coupled receptor	0.325
F41E6.7		0.321
F49E12.2		0.317
F47E1.3	Zn-finger	0.317
T23C6.3	Unnamed protein	0.316
F54B8.11	Unnamed protein	0.315
E04D5.3	Cuticulin precursor	0.315
VF36H2L.1	Predicted membrane protein	0.313
C25D7.5	Uncharacterized conserved protein	0.31
Y73F8A.8	Uncharacterized protein	0.309
T21H8.2	Sra family integral membrane protein	0.309
Y53F4B.2	Long chain fatty acid elongase	0.305
C24H10.1	Unnamed protein	0.303
T24E12.5	Unnamed protein	0.302
Y55F3BR.6	Alpha crystallins	0.299
C14C6.3	Predicted glycosyltransferase	0.298
F22B7.9	Predicted methyltransferase	0.298
C16C4.12	Uncharacterized protein, contains BTB/POZ domain	0.297
M60.4b		0.293
		0.289
T06C12.2	7-transmembrane olfactory receptor	0.287
Y40H4A.2	Serine/threonine specific protein phosphatase PP1, catalytic subunit	0.284
C34C6.3	Unnamed protein	0.282
Y67A10A.5	Uncharacterized protein	0.282
W03G11.3	Alpha-L-fucosidase	0.277
K10H10.6	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases	0.273
	No Significant Match	0.272
T21D12.9c	Membrane glycoprotein LIG-1	0.271
K04C2.5		0.262
Y67D8C.2	Uncharacterized conserved protein	0.247
T23F1.6	Uncharacterized protein	0.246
Y50D4B.2	Uncharacterized membrane protein	0.238
Y102A11A.5		0.235
H24D24.2	7-transmembrane olfactory receptor	0.233
Y38C1AA.4		0.233

Gene name	Descrption	Fold change
F59D12.3	Chondroitin 6-sulfotransferase and related sulfotransferases	0.229
F54D1.1	RNA-binding protein Sam68 and related KH domain proteins	0.227
T21C12.1f	GABA receptor	0.226
W09G10.6	C-type lectin	0.224
Y53H1B.6	Cytosolic Ca2+-dependent cysteine protease (calpain	0.223
F48G7.9	Serine/threonine protein kinase	0.221
Y54F10BM.13	Dual specificity phosphatase	0.22
F09F7.6		0.22
ZK6.6	Uncharacterized conserved protein	0.22
Y75B8A.9a	Polypeptide N-acetylgalactosaminyltransferase	0.219
ZK6.2	Nuclear hormone receptor	0.213
F08H9.5	C-type lectin	0.211
Y7A9C.1	Similar to ARD GTP-binding proteins	0.206
M05B5.4	Lecithin:cholesterol acyltransferase (LCAT	0.201
C49F8.1		0.197
F29C12.5	Uncharacterized protein, contains BTB/POZ domain	0.194
B0024.8	Uncharacterized conserved protein	0.194
	No Significant Match	0.192
Y18D10A.21	Unnamed protein	0.18
F07A5.4		0.178
	No Significant Match	0.175
W06G6.8	7-transmembrane olfactory receptor	0.175
ZK909.5	Predicted transposase	0.172
Y49A10A.1	Uncharacterized conserved protein, contains DM10 domain	0.171
K12G11.3	Alcohol dehydrogenase, class V	0.17
Y102A5B.2	C-type lectin	0.16
T27E4.9_1	Alpha crystallins	0.159
C17C3.2_1	Unnamed protein	0.154
Y56A3A.9	Reverse transcriptase	0.154
	No Significant Match	0.153
T04A6.2		0.151
T07G12.5	Xanthine/uracil transporters	0.143
T28F3.6	Unnamed protein	0.131
ZK112.5		0.129
Y59H11AR.1		0.129
T22A3.4b		0.126
		0.124
Y95B8A.1	Meprin A metalloprotease	0.123
ZK402.3	Uncharacterized protein	0.118
Y73B6A.1	Serine/threonine kinase (haspin family	0.113
F58E6.7		0.112
C16B8.1	Receptor-like protein tyrosine kinase RYK/derailed	0.111
T23D5.9	7-transmembrane olfactory receptor	0.109
H20E11.2	Unnamed protein	0.105
W04G3.3	Unnamed protein	0.103
H12C20.5	G protein coupled protein	0.103
R01H2.4	Unnamed protein	0.0998
T22C1.3	Major facilitator superfamily permease - Cdc91p	0.0935
T26H2.6	7-transmembrane olfactory receptor	0.0874
	No Significant Match	0.0825
	No Significant Match	0.0804
F49E11.10	Defense-related protein containing SCP domain	0.0793
Y67A10A.6	Predicted molecular chaperone, contains DnaJ domain	0.0769
ZK1055.6b	Unnamed protein	0.0764
T04A11.7b		0.0761

Gene name	Descrption	Fold change
	No Significant Match	0.0737
Y39C12A.7	Sre G protein-coupled chemoreceptor	0.0716
T19A5.5	Hormone receptors	0.0715
F09G2.6		0.0705
Y67D2.5	Predicted phosphoglucosamine acetyltransferase	0.0665
T23G4.2	Unnamed protein	0.0584
F56D1.6	Unnamed protein	0.0567
T25B9.10	Inositol polyphosphate 5-phosphatase and related proteins	0.0557
F52D10.6		0.0545
ZK381.2	Extracellular protein with conserved cysteines	0.0518
T13F3.3	Hormone receptors	0.0443
F52E1.13d		0.0415
F44C4.2	Hormone receptors	0.0395
		0.0389
ZK1321.1		0.0389
Y97E10B.7	K+-dependent Na+:Ca2+ antiporter	0.0379
H05B21.2	Predicted olfactory G-protein coupled receptor	0.0359
T25B2.2		0.0312
T20D4.7	Thioredoxin, nucleoredoxin and related proteins	0.0299
Y43B11AR.2		0.0279
C05D10.4b	Unnamed protein	0.0241
B0507.5		0.0236
Y70C5C.2	C-type lectin	0.0179
F28G4.2	Uncharacterized protein	0.0176
F45F2.5	Uncharacterized conserved protein	0.0165
H25K10.1	Purple acid phosphatase	0.0113
		0.0111
R13D7.1		0.00852
C10E2.4		0.00744