The mechanisms behind stress

From populations to genes in nematodes

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Para Toni y mi familia

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Chapter 1

General Introduction

Stress biology

Organisms continuously interact with their environment, and many factors in the environment can be far from what each organism perceives as optimal. It is well known that an optimal set of conditions for a certain organism can be considered as adverse by another even related organism. The diversity of life history strategies that inhabit the earth is primarily the result of this conflict between organisms and their environment, which is perceived by the organism or its cells as “stress” (Lakhotia 2004). These stresses can range from toxic chemicals generated or present in the environment, to radiation, drought, osmotic conditions, or temperature among others factors. It seems that stress is an inevitable part of the life of all organisms, which in all situations can affect their fitness. Consequently, even the most primitive organisms have developed means to protect themselves and respond to adverse conditions.

The growth of the world’s human population and the development of human society have led to a parallel increase in the deterioration of the environment. There is an increasing awareness of environmental problems such as climatic change and chemical contamination, where growing fields of research invest great efforts in developing a better understanding of these processes in order to design more efficient assessment approaches. Within this thesis, the main questions address the mechanisms through which factors such as toxicants or temperature (common situations in the field) can cause stress, and the mechanisms of the responses to such stresses. Furthermore, these questions can be addressed at different organizational levels from genes, cells, tissues, individuals, communities to finally ecosystems. This thesis comprises a study of stress that goes from the population level to the gene level.

Abiotic stressors

Toxicants

The fact that currently more than 100,000 chemical compounds are used worldwide (Hansen *et al.* 1999) is a growing concern that has lead to the inherent need of assessing the effects that the presence of these compounds has on ecosystems. The large amount of existing chemicals has been the driving force of the predominance of fast and reproducible protocols for toxicity testing that provide risk assessors with simple numbers such as LC50s, EC50s and NOECs. Although the main objective of risk assessors is to protect populations, testing population level effects is too complicated for routine applications and is generally substituted by short-term testing with few selected species (Jager *et al.* 2004)(see e.g. Rundgren & Augustsson 1998; Kula & Larink 1998; Van Gestel & Doornekamp 1998). The most common approach for data analysis in these tests involves fitting an empirical model to the data collected at a fixed time point and using this regression to estimate an EC50 or LC50 (e.g. 48 h for an acute Daphnia test) (Marchini 2002).This type of descriptive approaches have disregarded to a great extent the importance of the underlying mechanisms of toxic action and response. They are therefore limited when it comes to the interpretation and extrapolation of results and thus may lead to systematic errors in risk management decisions (e.g. Laskowski 1995; Alda Álvarez *et al.* 2006). These considerations are redirecting more attention towards understanding the underlying mechanisms. Mechanistic approaches for studying stress are not only interesting from a scientific viewpoint but will also in time lead to better risk assessment by providing results that improve the interpretation of toxicity data and bear more relation with the protection goal itself (populations, ecosystems).

Temperature

Over the past 100 years, the global average temperature has increased by approximately 0.6 °C and is expected to continue rising rapidly (Houghton *et al.* 2001). Although species have adapted to climatic changes throughout their evolutionary history (Harris 1993), the concern now is the rapid rate of change (Schneider & Root 1998) which places many

organism under temperature induced stress. There is evidence that climate change is already affecting the behavior and distribution of species and the composition and structure of communities and ecosystems (Hughes 2000; McCarty 2001; Walther *et al*. 2002; Stenseth *et al.* 2002; Parmesan & Yohe 2003). Furthermore, this situation is expected to become more severe as changes in temperature levels predicted by the Intergovernmental Panel on Climate Change (IPCC) run as high as 6 °C by 2100 (Houghton *et al.* 2001). This highlights the growing importance of understanding the mechanisms of response to temperature that take place in an organism. Studies that provide insight into how organisms sense and respond to a temperature change are essential and will help to project into the future the possible effects of climate change and to design more efficient assessment tools.

Understanding the mechanisms of stress

The framework of this thesis involves studying the mechanisms of stressor modes of action and stress response of organisms. The study of the underlying mechanisms that take place in exposure to a stressor can be performed in a process-based manner by studying the fluxes of energy in an organism, which are related to physiological processes and their variation throughout the whole life cycle. This concept is based on the Dynamic Energy Budget (DEB) theory which describes the functioning of organisms based on a set of rules for metabolic organization (Kooijman 2000; Kooijman 2001), where exposure to a stressor is regarded as a change in energetic parameters. This insight led to the development of DEBtox (Kooijman & Bedaux 1996), a suite of models to analyze toxicity experiments in a process-based manner. In the DEB scheme, food is transformed into feces, and part of the energy is assimilated contributing to the reserves. These resources are distributed in a fixed fraction between somatic growth/maintenance and reproductive output/maturation. Within this scheme a stressor may exert an effect on different energetic parameters: decreasing assimilation, increasing maintenance costs, increasing costs for growth, increasing costs for reproduction or posing a direct hazard to the embryo (Jager *et al.* 2004). The dissection of the energetic mode of action of toxic compounds is interesting from a scientific point of view since it provides an insight into the mechanisms through which a chemical is exerting toxic effects and how these are being reflected on the different endpoints of an organism (survival, reproduction, growth).

In addition, understanding the details about the mode of action of toxicants is essential for the extrapolation to population level effects (Jager *et al.* 2004). In this way, the effects on the different endpoints are integrated into a single parameter, the population growth rate, which provides a more relevant measure of ecological impact than any individual endpoint (Forbes & Calow 1999). This approach can be used to model the effects of different types of stressors. The population level effects of different temperatures can also be analyzed in this manner, since temperature has predictable consequences in DEB theory (Jager *et al*. 2005).

Stress mechanisms and life cycles

Life history is a term given to the pattern of development, reproduction, and mortality exhibited by an organism (Crawley 1986). The specific life history strategy of an organism is of great importance in determining its performance under different environmental conditions. Different strategies imply investing energy in different ways. The patterns of allocation between the different traits can be understood within the framework of the life history theory. The central assumption is that organisms adjust trait characteristics in response to different environments in order to maximize fitness (Stearns 1992). Different groups of animals vary in their physiological allocation of energy depending on their life history strategy. The energy allocation rules can be related to life-history traits such as body size and reproductive output, and can be expressed in terms of energy budgets (Kooijman 2000). After exposure to a stressor, the allocation patterns depend on the mode of action of the stressor (Van Straalen & Hoffmann 2000) as well as on the life history strategy of the organism.

For practical reasons, toxicity testing is currently biased towards the use of asexual species with relatively short life cycles. Aquatic crustaceans *Daphnia magna* and *Ceriodapnhia spp* are parthenogenetic (Persoone & Janssen 1998), as is *Folsomia candida* (Van Gestel & Van Straalen 1994) and other commonly used species such as the rotifer *Brachionus plicatilis* (Carmona *et al.* 1995) and the oribatid mite *Platynothrus peltifer* (Van Gestel & Van Straalen 1994). These species are commonly selected with the aim of producing rapid, reproducible and cost effective tests. However, this does not account for the diversity of life strategies that we find in ecosystems. In soil micro-arthropod communities only two out of twelve strategies were found to be asexual (Siepel 1994). The reproductive strategy of an organism is of great

importance in determining its performance under different environmental conditions. For instance, in the case of changing ambient temperatures, Grieg (Grieg *et al.* 1998) reported that sexual populations of yeast adapted more rapidly to higher temperatures than did asexual ones. Also, the significance of sexual reproduction in the studies of population recovery following toxic stress (Forbes & Depledge 1992) shows the importance of considering different reproductive strategies, since significant differences can be found in the way and efficiency of their response to stress. These considerations highlight the interest of studying organisms with different life history strategies in order to construct a more versatile and unbiased picture of stress mechanisms and responses that occur in ecosystems.

The nematode model

Nematodes comprise an excellent model for studying stress mechanisms and responses in different life history strategy scenarios. Within the phylum Nematoda, most free-living nematodes can be easily reared in the laboratory and whole life-cycle observations present no difficulties (Kammenga *et al.* 1996). The diversity of life history strategies that can be found in nematodes covers a large part of the strategies that can be found in the animal kingdom, including parthenogenetic, hermaphroditic and sexually reproducing species. The diversity of life-spans presents absolute differences between short and long living species ranging from 3 days to more than 12 months, while still easily reared under the same small scale conditions. Within this thesis we use two species of bacteriovorus nematodes that comprise three different life history strategies as described below.

Acrobeloides nanus

A. nanus (Cephalobidae) is easily reared on agar with a bacterial lawn of *Escherichia coli* as a food source. It is a parthenogenetic species, where the ovum develops into a new individual without fertilization. The reproductive period lasts about 40 days (at 20° C) during which they produce a total progeny of 400-500 (Jager *et al.* 2005). The average life span is of 50-60 days, falling into the category of intermediate life span nematodes.

Caenorhabditis elegans

C. elegans (Rhabditidae) can also be easily reared on agar seeded with *E. coli*. This nematode is a facultative self-fertilizing hermaphrodite which can also reproduce sexually in the presence of males. Males occur at very low frequencies in natural populations (Ward & Carrel 1979), and hermaphroditic reproduction is therefore the most common strategy. This mode of reproduction implies that one individual produces both male and female gametes to self-fertilize. In *C. elegans*, this yields a total progeny of about 300 in a reproductive period that lasts 5.5 days (15° C) (Alda Álvarez *et al.* 2005). These nematodes present short life spans of about 20 days at 15° C.

C. elegans can also be used for sexual reproduction studies. A sexually reproducing population can be selected for by including males in the assays. This will lead to differences in the life history traits caused by the different life history strategy. For instance, it is known that the number of offspring produced by a sexually reproducing nematode can double that of a non-mated hermaphrodite (Kimble & Ward 1988).

Within this thesis we use different life history scenarios to perform life cycle studies which focus on unraveling the stress mechanisms and responses to different toxicants at the individual and population level, and subsequently incorporating the additional effect of different temperatures.

Stress mechanisms and genes. Gene-environment interactions

At the most fundamental level, the response to different environmental conditions will be generated by individual cells (Schlichting & Smith 2002) and thus by genes. Different environmental factors can interact with the genotype and in that way influence some of the characteristics of the phenotype. These interactions are known as genotype by environment interactions (GxE).

The differences observed in a phenotype can be explained at the genetic level by gene expression differences. To this effect, the advances of microarray technology for studying gene expression have been ground breaking, allowing the high-throughput profiling of multiple genes simultaneously. These genomics approaches are usually used for comparing

two states such as mutant versus wild type, healthy versus diseased, control versus exposed or different conditions or time points. The statistical and bioinformatical analyses of the expression profiles of many organisms have been used to reveal genes that are up or down regulated in specific situations of interest. As an example, this approach was used with human microarrays to discover inflammatory disease-related genes (Heller *et al*. 1997). Zinke studied genes that responded to starvation in *Drosophila* (Zinke *et al.* 2002). Kimura identified genes responding to high light-stress *(*Kimura *et al.* 2003*)* and Sørensen studied heat stress response in *Arabidopsis* (Sørensen *et al.* 2005). This organism was also used to identify genes involved in the response to drought, cold and high-salinity stresses (Seki *et al.* 2002). Hamadeh used rats to reveal gene expression profiles related to exposure to different compounds (Hamadeh *et al.* 2002).

A few years ago, a new concept termed "genetical genomics" was developed by Jansen and Nap (Jansen & Nap 2001) which promised to be a new breakthrough in the study of gene expression. This concept incorporates the power of Quantitative Trait Loci (QTL) analysis, which uses the genetic variation in a segregating population to map complex traits (Lander & Botstein 1989), to that of gene expression analyses. In this approach, the expression profile of an organism is treated as the quantitative trait of interest for QTL mapping. Genetical genomics can therefore provide additional insight into the function and interrelation of gene products and gene action (Jansen & Nap 2001), by mapping the expression of large numbers of genes.

Genetical genomics approaches have successfully been used to dissect transcriptional regulation in yeast, stem cells, flies and rats (Brem *et al.* 2002; Bystrykh *et al.* 2005; Chesler *et al*. 2005; Schadt *et al.* 2003). Hubner (Hubner *et al.* 2005) compared expression linkage patterns between two different tissues and recently the genetic interactions between polymorphisms affecting gene expression in yeast have been described (Brem *et al.* 2005). But so far the influence of an environmental change on the genetic linkage of expression profiles, i.e. the *cis* or *trans* QTL pattern, remains unexplored. Here we use a genetical genomics approach to study the effects of a temperature change on the transcriptional regulation of *C. elegans*.

Outline of the thesis

The first part of this thesis (Chapters 2, 3 and 4) addresses stress mechanisms and responses to different toxicants in life cycle studies involving various life history strategies. ***Chapter 2*** focuses on studying the dynamics of one of the most commonly used summary statistics in risk assessment: the ECx. We study the behavior in time of the ECx for different endpoints and how it is affected by the characteristics of two different compounds (carbendazim and pentachlorobenzene) and of two different life history strategies (hermaphroditic and sexually reproducing *C. elegans*). We discuss the problems involved in the current use of this summary statistic, and demonstrate that the interpretation of the results from toxicity tests can be improved through process-based modeling. In ***Chapter 3*** we use process-based modeling to study cadmium stress on two different life history strategies (hermaphroditic and sexually reproducing *C. elegans*) and dissect the differences between them. We analyze the effects on the different endpoints and integrate these into population level effects. ***Chapter 4*** focuses on identifying the physiological mode of action of three different compounds (cadmium, carbendazim and pentachlorobenzene) and how these affect population growth rates in different ways, with a new life history strategy (parthenogenetic *A. nanus*), which allows us to compare with the results on the previously studied life history strategies. In this chapter we also study the effects of different temperatures on the population growth rates in combination with increasing concentrations of the toxicants.

The next section of this thesis is dedicated to the study of temperature as the abiotic stress factor. ***Chapter 5*** addresses the mechanisms behind the response to a temperature change at the gene expression level. We use a genetical genomics approach for this purpose. The experiments are performed using a 79 recombinant inbred line (RIL) panel derived from a *C. elegans* N2 x CB4856 cross. Whole genome expression profiles are obtained for each RIL at two different temperatures (16° C and 24° C). We then use QTL mapping to detect expression linkage patterns across the genome for each temperature. First we perform a separate analysis for each temperature, and then use a combined analysis of the two temperature data sets to increase the power of the expression linkage analysis. We study the QTL effect in the two environments to uncover temperature sensitive QTL (QTLxT) which generally have opposite effects at either temperature, or a strong QTL effect at one temperature but almost no effect in

the other one. For this purpose we first use a genome wide approach and then a two-stage test strategy to increase the power of QTLxT detections. The function and biological meaning of the uncovered QTLxT are subsequently discussed. To finalize, ***Chapter 6*** presents a summary of the principle conclusions, their implications, and perspectives for future research on this field.

Chapter 2

Temporal dynamics of effect concentrations

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Abstract

In effect assessment the comparability and applicability of LCx and ECx values, which are calculated at single points in time during exposure, relies on the ability to perform a valid extrapolation to other time points of interest. The behavior of LCx in time has been extensively studied, and the behavior of ECx in time is expected to follow similar dynamics, as it is considered that the LCx is just a specific case of ECxs. However, most models have focused on validating the dynamics of LCx and hardly anything is known about the time dependence of ECx for other endpoints, or whether it is comparable to that of LCxs. We have created four scenarios where we study the dynamics of the ECx for different endpoints and how it is affected by the characteristics of two different compounds (carbendazim and pentachlorobenzene) and of two different life history strategies (hermaphroditic and sexually reproducing strains of *Caenorhabditis elegans*). The observed patterns of behavior in time of the ECx for body size and for reproduction showed unexpected dynamics that deviate considerably from that of the LCx. It was demonstrated that the temporal dynamics of ECx were very different for each particular endpoint. The shape of the ECx-time curves depends on the intrinsic characteristics of the endpoint of study, as well as on the characteristics of the compound and life history strategy of the organism. This makes extrapolation in time or between endpoints difficult and hampers the comparability of results based on this summary statistic. The interpretation of the results from toxicity tests can be improved through process-based modeling, as demonstrated on the current data set.

Introduction

Within the field of effect assessment there is a growing need for consistent and comparable summary statistics in order to unify the large variety of approaches that are currently in use. The most common approach for analyzing toxicity data involves fitting an empirical model to the data collected at a fixed time point (e.g. 48 h for an acute Daphnia test), and using this regression to estimate an ECx or LCx (e.g. EC50, LC50) (Marchini 2002). These parameters are time dependent and therefore require the selection of a specific exposure time for their calculation. In order to unify the toxicity testing process, exposure times have been standardized rather arbitrarily (OECD 1998) for some organisms. However, standardization is only relevant if the time course of effects is similar for all compounds, and furthermore, in effect assessment it may be necessary to extrapolate these calculated values to other time points of interest.

The assumption that ECx and LCx values decrease with increasing exposure time is partly based on the fact that effects depend on internal concentrations (Kooijman 1981; Péry *et al.* 2002) and that it takes time for the compound to penetrate into the organism. The exposure time during which this decrease is substantial depends on the characteristics of the compound, of the organism, and of the type of effect. For organisms with large body sizes and for compounds with high octanol-water coefficients this period is usually long. As an example, surfactants are generally quick to cause ultimate effects, whereas for other chemicals such as cadmium or dioxins, the LC50 will continue to decrease in time for a considerable part of the life cycle (Kooijman 1996). Since the temporal dynamics of toxic effects are compound specific, the time course of the summary statistic needs to be considered. The comparison of an LCx or ECx value for a fixed exposure time between chemicals that have different toxicokinetics would be extremely misleading. Hence, in order for these parameters to be comparable and informative, the temporal dynamics that they present must be considered.

Although not generally used for risk assessment purposes, the behavior of LCx over time has been extensively studied for different types of chemicals, and different modeling approaches have been developed. Several models use an empirical hyperbolic relation were LCx values can be expressed as a linear model of the inverse of time (Mayer *et al.* 1994; Van Wijk & Kraaij 1994; Carter & Hubert 1984). Kinetic-based models were developed by Chew

and Hamilton (Chew & Hamilton 1985) and Kooijman (Kooijman 1981), this latter one being subsequently replaced by the DEBtox model (Kooijman & Bedaux 1996). Bonnomet reviewed these models (Bonnomet *et al*. 2002), and proposed a mechanistic model based on the Dynamic Energy Budget (DEB) theory, modified to express LC50 as a function of time. Marchini (Marchini 2002) used two different models of LC50 *versus* time to describe the behavior of narcotic and reactive chemicals, whose theoretical bases have been discussed by Verhaar (Verhaar *et al*. 1999). These models show that the behavior of LCx generally follows a predictable decrease in time. The behavior of ECx in time is expected to follow similar dynamics, as it is considered that the LCx is just a specific case of ECxs. However, most models focus on survival to validate the dynamics of LCx and there is hardly any knowledge on the time dependence of the ECx for other endpoints, or whether it is comparable to that of LCxs. Studying the shape of the ECx-time curve of different endpoints that are commonly used in toxicity assays is essential for the interpretation of toxicity data and can provide a better understanding of whether this parameter is being used in an informative and comparable way.

For each different endpoint, the shape of the ECx-time curve, and therefore the dynamics of this parameter, will depend both on the characteristics of the compound and of the organism as is the case for LCx. However, the intrinsic characteristics of the endpoint of study will undoubtedly determine the dynamics of its ECx. To address this issue we have created four scenarios where we study the behavior of the ECx (for body size and reproduction) and LCx (for survival) in time and how it is affected by the characteristics of the compound, and by the life history strategy of the organism throughout the whole life cycle. Two compounds were selected for having different toxic mechanisms: carbendazim (DNA synthesis inhibitor (Clemons & Sisler 1971)) and pentachlorobenzene (narcotic (Lydy *et al*. 1999)). Two strains of the nematode *Caenorhabditis elegans* that present differences in their life history strategies were selected. The N2 strain reproduces hermaphroditically whereas the CB4856 strain has a high occurrence of males which allows for the selection of sexually reproducing individuals. The same exposure conditions were used in all the scenarios to minimize differences due to factors other than those of interest.

Generally, the data points for different endpoints, at different time points, are treated as independent sources of information (by fitting separate dose-response curves). However, all

measurements are taken from the same animals, and are therefore closely linked. To obtain a more integrated analysis of the toxicity data, we applied the DEBtox method (Kooijman & Bedaux 1996). DEBtox is based on the Dynamic Energy Budget (DEB) theory which describes the functioning of individual organisms based on a set of rules for metabolic organization (Kooijman 2000; Kooijman 2001). In this method, results for all endpoints can be fitted simultaneously in time to obtain an integrated picture of toxicity, as was demonstrated earlier for cadmium in *C. elegans* (Alda Álvarez *et al*. 2005).

Materials and Methods

Chemicals. Carbendazim was purchased from Riedel-de Haen, Seelze, Germany (99% pure). A stock solution of 0.3 mg carbendazim/ml ethanol was prepared and stored in the dark. Nominal concentrations used for the experiments ranged from 0.48 to 4 mg carbendazim/L agar. An ethanol blank was used as the control.

Pentachlorobenzene was purchased from Aldrich, Steinheim, Germany (98% pure). A stock solution of 10 mg pentachlorobenzene/ml ethanol was prepared. Nominal concentrations used for the experiments ranged from 50 to 130 mg pentachlorobenzene/L agar. An ethanol blank was used as the control.

Chemical analysis of the available fraction of toxicants in the agar was performed (Alterra, Wageningen University and Research Center) with HPLC for carbendazim (Fig.1) and GC for pentachlorobenzene (Fig.2) after 3, 5 and 7 days of preparation of the dishes to monitor the available fraction of the toxic compounds (e.g. binding of compound to the plastic walls or matrix of the agar). These measurements were used to ensure that the available fraction of toxicants increased with increasing nominal concentrations. In order to minimize the changes in the available fraction of the compounds throughout the experiments, dishes were used no longer than 4 days after preparation.

Hermaphroditic *C. elegans*. The N2 (Bristol) strain of *C. elegans* was obtained from laboratory cultures of Caenorhabditis Genetics Center (University of Minnesota). This strain was selected for life cycle testing involving hermaphroditic reproduction, due to the low occurrence of males in this strain. These populations were started with only non-mated

hermaphrodites and screened regularly to remove any occurring males. Populations where maintained at 15°C.

Sexually reproducing *C. elegans*. The CB4856 (Hawaii) strain of *C. elegans* was obtained from laboratory cultures of Caenorhabditis Genetics Center (University of Minnesota). This strain was selected for the sexual reproduction experiments due to the greater activity and occurrence of males. In this case populations were started with gravid females that had been fertilized by males, by means of identifying a copulatory plug that remains in the female genitalia after copulation (Barker 1995; Hodgkin & Doniach 1997), obtaining in this way a 1:1 ratio of females to males in the offspring. In these experiments each female was kept with one male at all times, and checked for a copulatory plug to ensure that sexual reproduction took place. Populations where maintained at 15°C.

Culturing. All nematodes were reared on NGM agar plates seeded with OP50 strain of *Escherichia coli* as a food source (Brenner 1974; Sulston & Hodgkin 1988). Stock cultures of OP50 were stored at -80°C and the bacterial cultures were grown in autoclaved LB medium (10 gr peptone, 10 gr yeast extract, 5 gr NaCl / L water) for 16 hours at 37° C and 150 rpm.

All experiments were started with a first synchronization in which gravid adults were transferred to 6cm Petri dishes and allowed to lay eggs for a period of 4 hours, after which they were removed. These eggs were allowed to hatch and develop into gravid adults with which a second synchronization was performed, this time on agar containing the corresponding concentration of toxicant. In this way we obtained the individuals for study, which were exposed throughout their whole life span (since egg stage) to the experimental treatments. Two separate experiments were carried out: one to record the reproductive output and survival of the individuals and a second one to obtain the body size measurements throughout their life span. For carbendazim, separate experiments were carried out for each life history trait in the hermaphrodite scenario.

Reproduction and survival. The newly hatched synchronized nematodes were transferred to individual wells (12 well plates from Greiner Bio-one) for observation. Each well contained 2ml of agar with the corresponding concentration of toxicant. The nematodes were transferred to a new well daily in order to count the number of offspring per individual. Reproduction was recorded in 7-12 individuals for each concentration and the cumulative number of offspring per individual was calculated. Death was scored when observing absence

of pumping and no movement response to touch. Survival data was recorded in 20-40 individuals per treatment to produce survival curves.

Body size. Newly hatched synchronized individuals were kept on 6 cm Petri dishes with 10 ml of agar containing the different concentrations of toxicant. A maximum of 15 individuals were kept on each dish (in the case of the sexual reproduction experiment it was 15 hermaphrodites and 15 males) to avoid overcrowding and competition. Pictures were taken of 8-10 individuals per treatment with a Cool Snap camera at intervals of 6 hours at the beginning of the growth curve, and longer intervals towards the end.

The pictures were digitalized with the Image Pro Express 4.0.1 software package to obtain length and area measurements of the nematodes at the different time points. Volumetric lengths (cubic root of body volume) where calculated with the following geometric formula of a cylinder with rounded ends (approximation of the shape of a nematode):

$$\text{Vol. length} = ((1/8 * \pi * A^2)/(l))^{1/3}$$

Where A is the area of the nematode and l is the maximum length of the nematode.

Modeling. The DEBtox method (Kooijman & Bedaux 1996) was used to analyze the life-history traits and the effects of the toxicants on these traits. In this approach, the internal concentration causes the effects; the first step in the model chain is therefore a one-compartment toxicokinetic model. The internal concentration affects the probability of death, as well as a parameter of the animal model (e.g. the maintenance costs or the assimilation of energy from food). The animal model is based on the Dynamic Energy Budget (DEB) theory (Kooijman 2001). The DEBtox approach has been adapted to deal with life cycle toxicity studies (Jager *et al*. 2004), and the specific details of the nematode life cycle (Jager *et al.* 2005). The performance of this model for nematodes was already demonstrated for the effects of cadmium on *C. elegans* for the two modes of reproduction (Alda Álvarez *et al*. 2005), and the same approach is followed here to analyze the datasets for carbendazim and pentachlorobenzene.

The model was implemented in Matlab® Version 7.0 (Release 14), and model fitting was based on maximum likelihood estimation. The model fits provide the toxic mode of action of

the compound, based on resource allocation, and estimates for parameters governing toxicokinetics, toxicity and basic physiology (see Table 1). Confidence intervals were generated using profile likelihoods (Meeker & Escobar 1995).

The model fits can be used to obtain ECx estimates as a function of time for body size, reproduction and survival (LCx) for any value of x. We selected a value of 10% for x to demonstrate the time-dependence of the summary statistic. For this purpose, a large grid of time points and concentrations was simulated using the model, and EC10s and LC10s were interpolated.

Results and Discussion

Chemical analysis. The chemical analysis of carbendazim gave similar measurements in the different time points, showing that the available fraction of this compound remained constant within the tested time range (Fig. 1). The results of the pentachlorobenzene measurements were less satisfactory, showing some variation of the available fraction in time (Fig. 2). However, the measurements confirmed the increase in available fraction with increasing nominal concentrations which serves our purpose for dose response analysis. In order to minimize the changes in the available fraction of the compounds throughout the experiments, dishes were used no longer than 4 days after preparation.

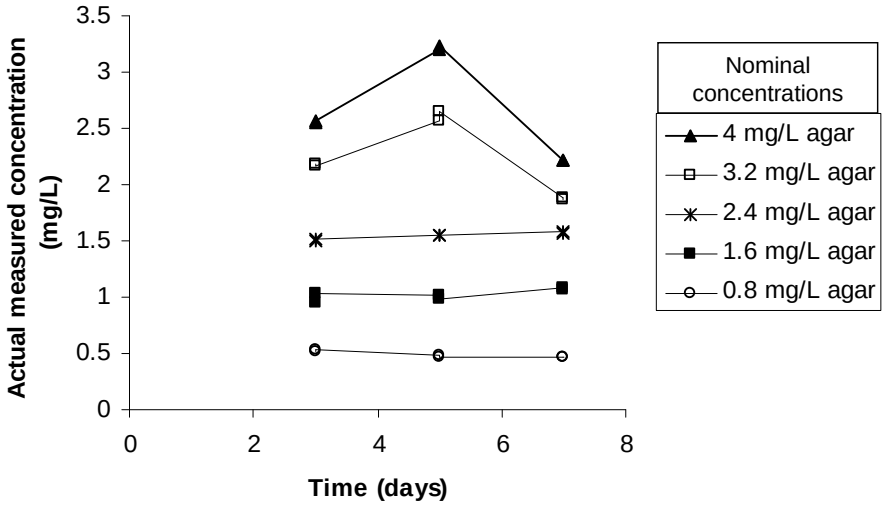

Figure 1. Chemical analysis of carbendazim in the soluble fraction of agar at different time points, showing actual versus nominal exposure concentrations.

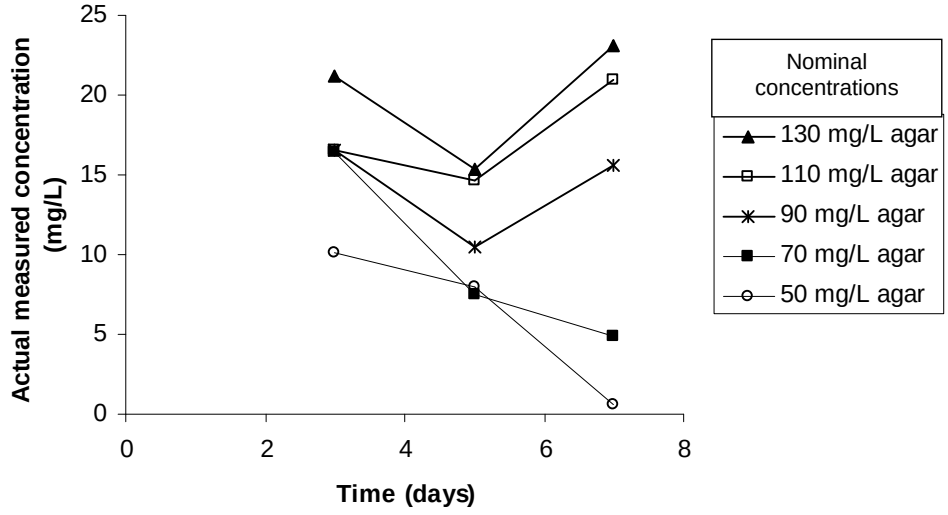

Figure 2. Chemical analysis of pentachlorobenzene in the soluble fraction of agar at different time points, showing actual versus nominal exposure concentrations.

Modelling life history traits with DEBtox. Model fits were obtained for the life history data sets, with few differences between the parameters for the N2 and CB4856 populations (Table 1). This shows that the basic life-cycle behavior of *C. elegans*, as well as its sensitivity to toxicants, does not strongly differ between these strains, as was shown in earlier experiments (Alda Álvarez *et al.* 2005). Note that the model was fitted to the data for all endpoints, all time points, and two strains together, resulting in a single set of parameter estimates for each compound.

Table 1. Parameters used in the model

	Carbendazim		Pentachlorobenzene	
	Hermaphrodites	**Sexual**	**Hermaphrodites**	**Sexual**
		Animal parameters		
Von Bertalanffy growth rate (d^{-1})	0.418 (0.384-0.441)		0.583 (0.554-0.620)	
Length at which ingestion is half of maximum (µm)	surv 29 (n.e.) repro 31.8 (31.6-32.4) grw 32.2 (32.0-32.6)	surv+repro 32.0 (32.1-32.7) grw 29.5 (29.2-30.0)	surv+repro 31.6 (31.6-31.7) grw 31.2 (31.1-31.2)	surv+repro 31.0 (30.9-31.1) grw 31.3 (31.3-31.4)
Maximum length (µm)	147 (146-151)		128 (127-129)	
Maximum reproduction rate (eggs/d)	231 (208-266)		261 (227-287)	
		Ageing parameters		
Maximum total number of eggs	268 (261-278)	∞ (n.e.)	238 (232-243)	∞ (n.e.)
Damage killing rate (d^{-1})	7.41 (5.27-10.4)·10^{-3}	32.5 (26.1-40.7) ·10^{-3}	7.31 (6.55-8.12) ·10^{-3}	22.3 (19.4-25.5) ·10^{-3}
Damage tolerance on reproduction [-]	∞ (n.e.)	2.22 (1.75-2.51)	∞ (n.e.)	4.09 (3.57-4.76)
		Toxicity parameters		
Mode of action	Assimilation		Costs for growth and costs for eggs	
Elimination rate (d^{-1})	0.0259 (0.0221-0.0288)		2.51 (1.37- ∞)	
NEC for survival (mg/L)	0.0706 (0.0566-0.0865)		∞ (n.e.)	
Killing rate (L/mg/d)	1.05 (0.763-1.29)		0 (n.e.)	
NEC for effects on growth/repro (mg/L)	0.00353 (0-0.00965)		0.0396 (0-1.96)	
Tolerance concentration (mg/L)	0.712 (0.537-0.915)		242 (215-258)	
Decrease of length at first reproduction due to chemical stress [-]	0.792 (0.405-1.53)	0 (n.e.)	0.487 (0.262-0.708)	0 (n.e.)
Decrease of total number of eggs due to chemical stress [-]	1.52 (1.05-1.93)	0 (n.e.)	1 (n.e.)	0 (n.e.)
Stress on costs for egg, relative to costs for growth [-]	n.a.	n.a.	2.27 (1.64-2.74)	

Fixed parameters: initial volumetric length 18.1 µm, volumetric length at puberty for hermaphrodites 83.4 µm, for sexual reproduction 69.9 µm, energy investment ratio 10, time before hatching 9 hours (Alda Álvarez *et al*. 2005). N.e. is not estimated, n.a. is not applicable, surv is survival, repro is reproduction, grw is growth, [-] is dimensionless. 95% confidence intervals in brackets.

We observed that the exposure to increasing concentrations of carbendazim led to a gradual decrease in reproduction which was visible in both strains (Fig.3). Growth also showed similar effects in both N2 and CB4856, with higher concentrations presenting lower growth curves, and survival was also decreased in response to this compound, with lower survival curves for the sexually reproducing individuals than for hermaphrodites. The negative effect of sexual reproduction on survival was reflected in the model as a higher damage killing rate (Table 1). This aging parameter reflects faster aging in the CB4856 strain than in the N2 strain, partly caused by mating costs (Alda Álvarez *et al.* 2005). The analysis also revealed the toxic mode of action based on the changes in energy allocation patterns that take place in response to the chemical. In the DEB scheme, food is transformed into feces, and part of the energy is assimilated contributing to the reserves. These resources are distributed in a fixed fraction between somatic growth/maintenance and reproductive output/maturation. Within this scheme, a chemical may exert a toxic effect on different energetic parameters: decreasing assimilation, increasing maintenance costs, increasing costs for growth, increasing costs for reproduction or posing a direct hazard to the embryo (Jager *et al*. 2004; Kooijman & Bedaux 1996). The life history traits were best described by the model when a decrease in assimilation was assumed as the mode of action for carbendazim, implying that either this compound affects the feeding rate of the organisms directly, or decreases the efficiency with which energy from food is assimilated.

Exposure to increasing concentrations of pentachlorobenzene also led to a gradual decrease in reproduction visible in both strains (Fig. 4). Growth exhibited a decrease which was more pronounced in the beginning and middle of the growth curves, and presented an apparent recovery towards the end of the life cycle. Survival, however, did not show any effects in exposure to pentachlorobenzene in either strain. None of the predicted modes of action (Kooijman & Bedaux 1996) was sufficient by itself to explain the toxic effects on growth and reproduction. The growth pattern clearly suggested costs for growth as the mode of action, because there is a clear effect on the growth rate but not on the ultimate body size. However, this mode of action does not explain the large effects on reproduction. We therefore propose that this compound acts through a combination of costs for growth and for reproduction, with no toxic effects on survival.

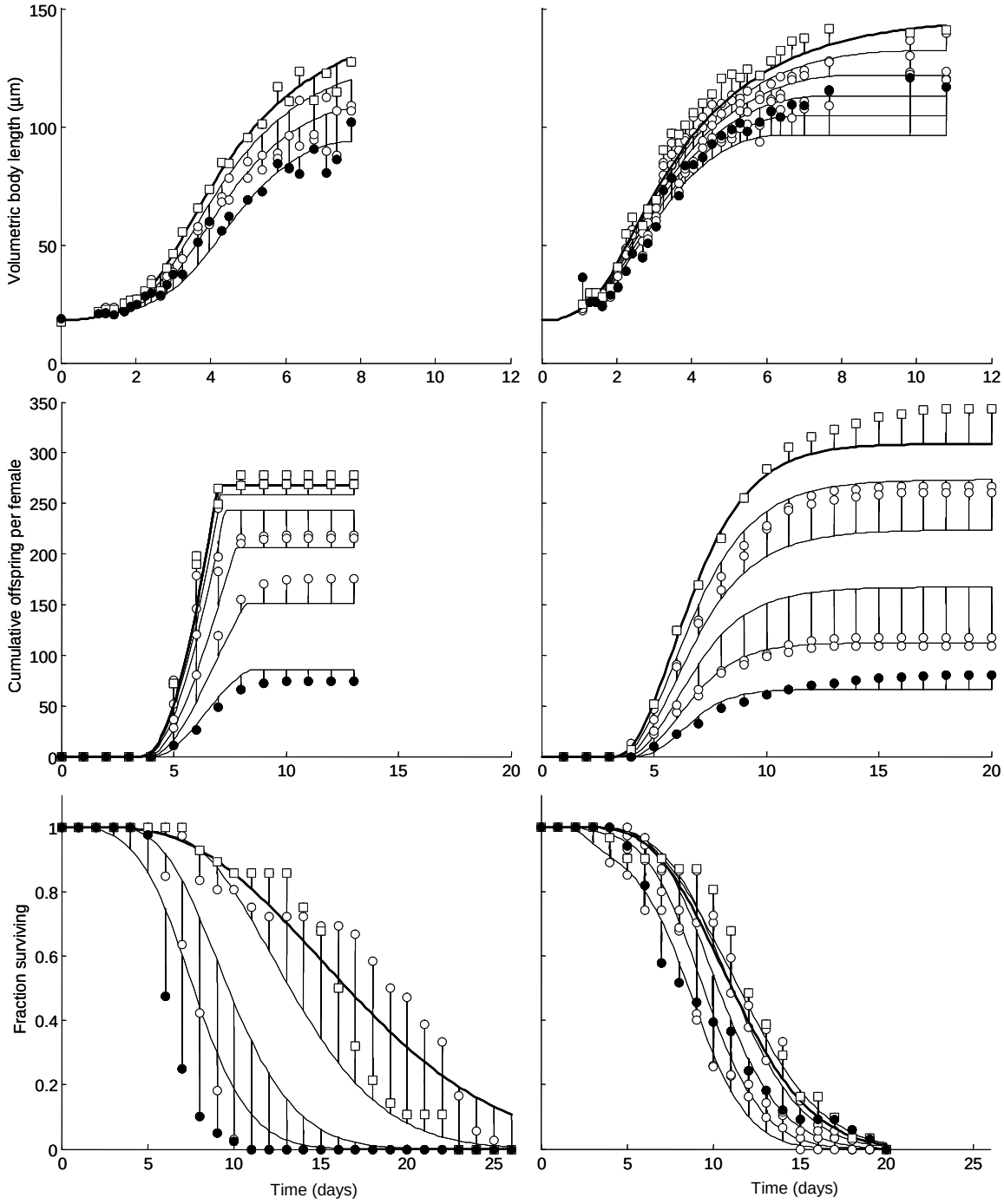

Figure 3. Data and simultaneous model fits for all endpoints of the life cycle study with *C. elegans* exposed to carbendazim, for hermaphrodites (left) and sexuals (right). These include survival, cumulative reproduction and growth. Simultaneous fitting was required because the data sets include common parameters. □ control, ○ concentrations 0.3, 0.6, 1.2 and 1.8 mg carbendazim/L ● 2.4 mg carbendazim/L.

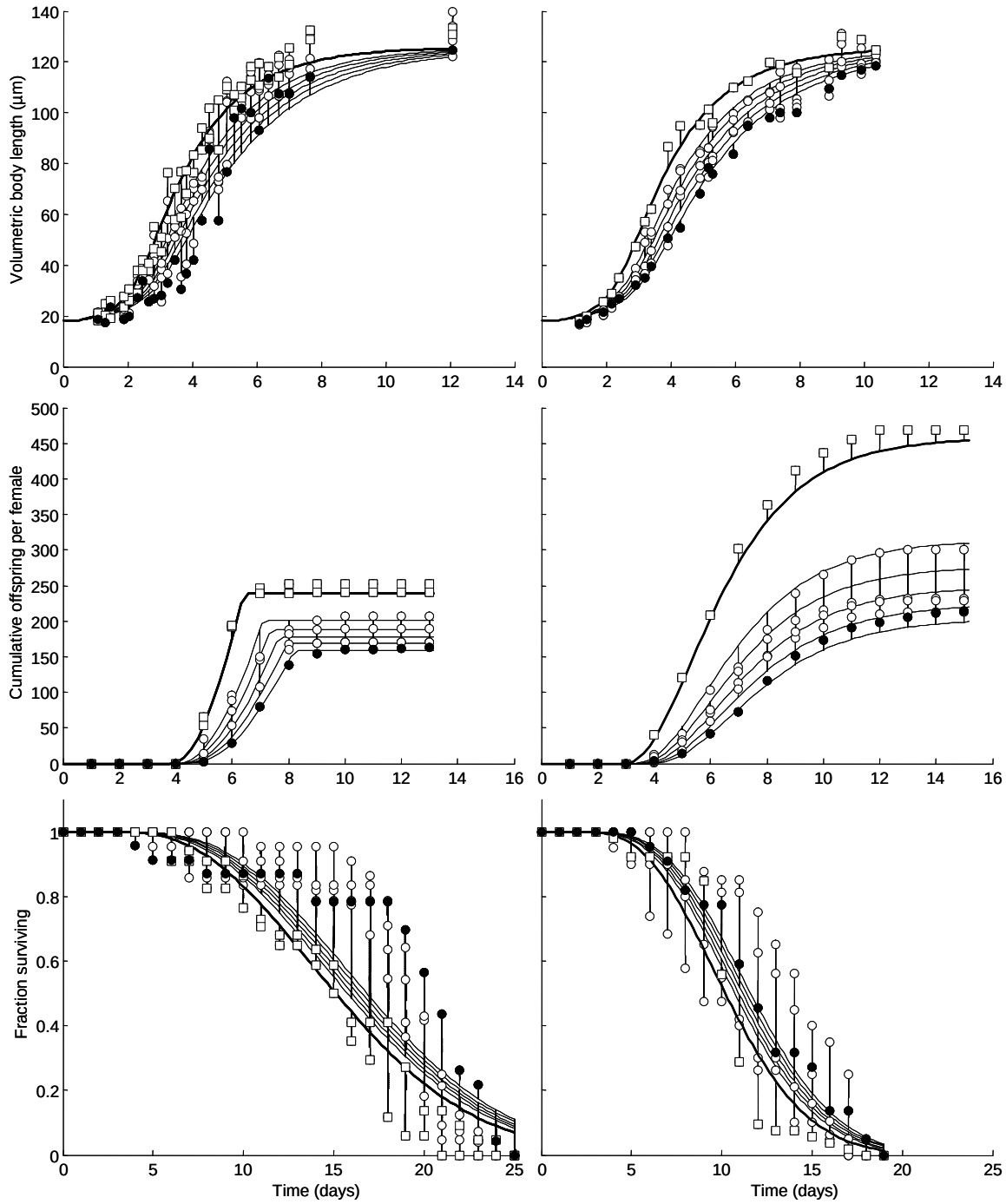

Figure 4. Data and simultaneous model fits for survival, cumulative reproduction, and growth in exposure to pentachlorobenzene, for hermaphrodites (left) and sexuals (right). Simultaneous fitting was performed because the data sets include common parameters. □ control, ○ concentrations 50, 70, 90 and 110 mg pentachlorobenzene/L ● 130 mg pentachlorobenzene/L.

These two compounds have essentially different toxic mechanisms, producing different patterns of sensitivity in the life history traits of *C. elegans*. The two strains also presented visible differences in their toxic response to the compounds, as was intended in the design of the four scenarios. The models for this life history data were then used to calculate the EC10 values in time.

Temporal dynamics of EC10. EC10 values were calculated for body size, reproduction and survival (LC10) in exposure to the selected chemicals throughout the life cycle of both strains (Fig.5). The observed patterns of behavior in time of the EC10 for body size and for reproduction deviated considerably from that of the LC10, which showed the expected decrease in time. The EC10s did not show a predictable decrease in time, but presented their own particular dynamics which differed to the dynamics of LC10s. This shows that in order to be able to interpret ECx values for a certain endpoint, it is necessary to study its specific dynamics for that particular endpoint, as it varies considerably from one endpoint to another.

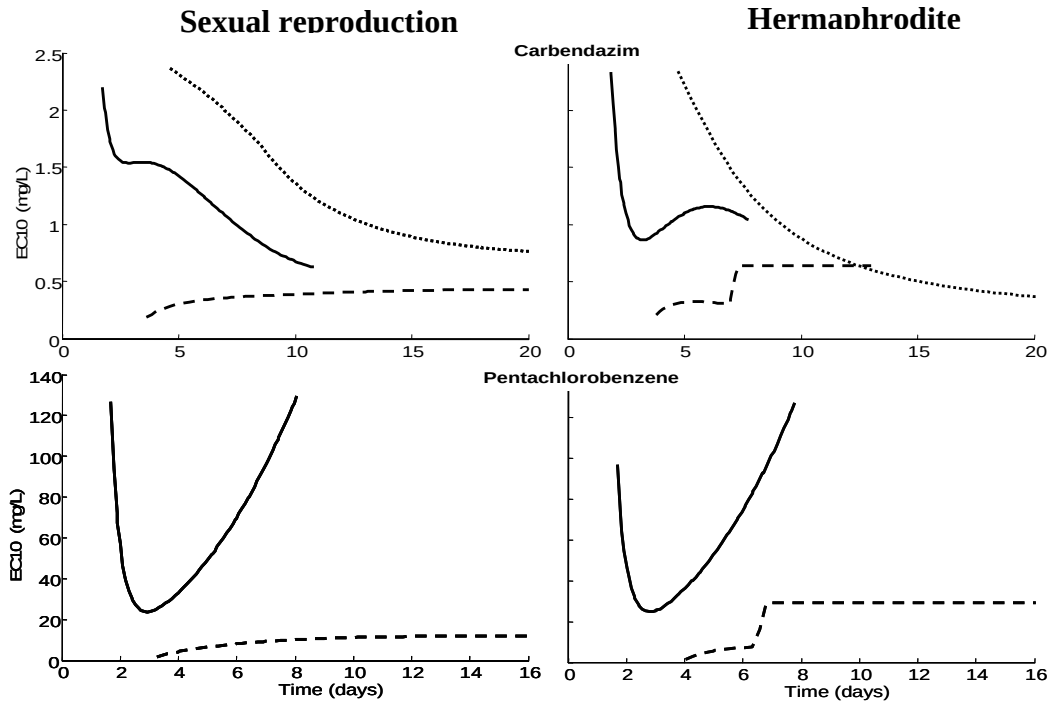

Figure 5. EC10 and LC10 for the different endpoints as a function of time for carbendazim (top) and pentachlorobenzene (bottom), for sexuals (left) and hermaphrodites (right). ●●●● survival (LC10), ——— body length, ---- cumulative reproduction. Survival is not shown for pentachlorobenzene as it does not seem to have a negative effect on this endpoint.

The dynamics of the EC10s for the different endpoints was found to be greatly influenced by the characteristics of the compound, and to a lesser extent by the reproductive strategy. If we first look at the differences caused by the characteristics of the organism we see that in the carbendazim scenarios the EC10 values for body size presented some differences between the two strains. There was an initial steady decrease which slowed down in day 2 for the CB4856 strain and in day 3 for the N2 strain. The following time points showed different patterns for both strains, where CB4856 continued to present decreasing values whereas N2 exhibited a slight increase of the EC10. This difference was probably caused by the fact that growth curves differed between the two strains. The length at which the ingestion rate was half the maximum value differed between experiments (see Table 1), and was probably not related to the mode of reproduction. These results showed that different growth patterns affect the EC10s in unexpected ways, mainly because body size is affecting toxicokinetics (Hendriks & Heikens 2001; Sijm & Van der Linde 1995). However, in the pentachlorobenzene scenarios, the EC10s for body size presented similar trends for both strains (the half-maximum ingestion length was similar in both strains). The behavior of the EC10 for reproduction exhibited the unexpected property of increasing in time, for both compounds and both modes of reproduction. This behavior therefore relates to specific properties of this endpoint. The increase can be explained by the fact that individuals that are exposed to the toxic conditions experience a delay in the reproductive peak with respect to the control, for these particular modes of action. Apart from this peculiarity that is inherent to the behavior of the EC10 for reproduction, the life history strategy lead to some differences in the patterns, related to the characteristics of the reproductive mode in each case. In hermaphrodites, egg production ceases when the storage of sperm cells is depleted (Ward & Carrel 1979), leading to a sudden halt of reproduction around day 7. In contrast, sexually reproducing *C. elegans* show a more gradual decline in egg production, presumably due to aging effects (oxidative damage) (Alda Álvarez *et al*. 2005). These differences in reproduction were reflected in the behavior of the EC10. The CB4856 strain showed a gradual increase in EC10 values which were smoothly stabilized, in contrast to the N2 strain which presented a gradual increase until day 7, at which a sudden increase in EC10 occurred, because the control individuals had stopped reproducing. This was followed by a sudden stabilization of the curve (at higher values than that of CB4856) indicating that the individuals exposed to the toxicants had also stopped

reproduction. These differences between strains were visible both in the carbendazim and pentachlorobenzene scenarios. Finally, the patterns for survival did not present any differences between strains in either of the scenarios, with the predictable smooth decrease in both strains for carbendazim, and an absence of response in pentachlorobenzene.

The differences caused by the characteristics of the compound were more pronounced than those caused by the mode of reproduction. In exposure to pentachlorobenzene the most apparent difference with respect to carbendazim were the absence of effect on survival (Fig. 5, EC10 for survival not plotted), clearly related to the toxic mechanism of this compound. The EC10 for body size in exposure to pentachlorobenzene exhibited a “U” shape as opposed to the trend described for the carbendazim scenarios. The initial decrease in this trend reflected the distancing of the growth curves at early time points (Fig. 4), where the control individuals had larger body sizes than those exposed to pentachlorobenzene. At later time points, the control individuals had completed their growth, but not so in the higher concentrations where growth still continued, shortening the distance between the growth curves again, and causing an increase in the EC10. This growth pattern is very specific for the mode of action “costs for growth”, where the toxicant affects the growth rate, but not the ultimate size. Reproduction, however, showed no differences with the previous compound. The CB4856 strain presented a smooth increase and the N2 strain exhibited the characteristic sharp step around day 7.

It is apparent that ECx dynamics are, first of all, endpoint specific. The dynamics of the ECx for growth and reproduction are essentially different, and clearly show different behavior to that of LCxs. The shape of the ECx-time curve therefore depends on the intrinsic characteristics of the endpoint of study and how it responds to the toxicant. For a particular endpoint, the specific toxic mechanism of the chemical has the largest influence on the dynamics of its ECx (especially for growth), which is also influenced, but to a lesser extent, by the life history strategy of the organism. These results show that the dynamics of ECx can be complex and that no simple factor exists for a general extrapolation in time or across endpoints. As long as this extrapolation is not possible, no single time point or single endpoint can be said to constitute 'the ECx'. This conclusion has severe consequences for the comparison of the sensitivities of organisms (e.g. when making species-sensitivity distributions), or for the comparison of the toxicity of different chemicals (e.g. in comparative

risk assessment, and QSAR development). Furthermore, it must be noted that this conclusion not only holds for the ECx and LCx, but also for the NOEC (which has even more disadvantages, (see e.g. Laskowski 1995)).

Clearly, any use of such summary statistic must therefore be interpreted with caution as it only gives a very limited indication of sensitivity to a compound. For this reason, other summary statistics are required that do not suffer from the problems related to the ECx. As an alternative, the no-effect concentration from DEBtox may be used, which is a time-independent statistic (Table 1). Furthermore, the intrinsic rate of population increase is also time independent and integrates the time course of effects on all endpoints into an ecologically relevant parameter (Forbes & Calow 1999). This statistic can also be generated using the DEBtox parameters (Jager *et al.* 2004; Alda Álvarez *et al*. 2005). The DEBtox analysis offers a deeper insight into the processes leading to toxic effects and allows for an identification of the toxic mode of action of the compound based on resource allocation, which is especially critical for the population level effects at limiting food densities (Kooijman & Metz 1984).

Given the predominance of ECxs, LCxs and NOECs in chemical risk assessment, it is important to acknowledge the limitations of these summary statistics, as they may result in systematic errors in risk management decisions.

Acknowledgements

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Chapter 3

Responses to stress of *Caenorhabditis elegans* populations with different reproductive strategies

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Abstract

Hermaphroditic and gonochoric reproduction are essentially different reproductive strategies which may lead to diverging population responses to adverse environmental conditions. Each strategy implies different physiological mechanisms, which affect life history traits and represent different ways of dealing with stress. We studied the performance of hermaphroditic vs. gonochoric strains in the nematode *Caenorhabditis elegans* exposed to cadmium stress at the individual and population level. Under control conditions, the gonochoric strain started reproduction earlier than the hermaphroditic strain at a smaller size. This was due to an earlier switch from sperm to oocyte production triggered by male sperm availability. Under cadmium stress hermaphrodites showed a decrease in the size at onset of reproduction, presumably as a strategy to maintain a high population growth rate. In contrast the body size of gonochoric nematodes was not affected. A process-based model (DEBtox) was used as a tool for analyzing life history data and calculating population growth rates. The model fitted the data well using physiologically relevant parameters such as ageing, survival or reproduction related parameters. The simultaneous fit of all life history traits was used to obtain populations growth rate estimates. The differences between the two *C. elegans* strains were reflected at the population level. Lower population growth rates, as calculated by DEBtox, were found in the gonochoric strain, largely determined by the proportion of males in the offspring. From the overall results we suggest that the differences found between both populations are due to the reproductive strategy. Under control conditions, CB strain (with gonochoric reproduction) does not favor population growth rates in the short term due to faster ageing and copulation costs on survival. Furthermore, in response to stress this strain also showed lower performance than the N2 hermaphroditic strain, mainly due to a higher sensitivity of survival to the stressor.

Introduction

The reproductive strategy of an organism is of great importance in determining its performance under different environmental conditions. For instance, Colegrave (Colegrave *et al.* 2002) investigated whether sexual recombination in *Chlamydomonas* generates more variation necessary to adapt to changing environments compared to asexual populations. Fitness of sexually reproducing populations exceeded that of asexual ones, and they concluded that sex accelerates adaptation to novel food environments. In the case of changing ambient temperatures, Grieg (Grieg *et al.* 1998) reported that sexual populations of yeast adapted more rapidly to higher temperatures than did asexual ones. The significance of sexual reproduction in the studies of population recovery following toxic stress (Forbes & Depledge 1992) shows the importance of considering different reproductive strategies, since significant differences can be found in the way and efficiency of their response to stress.

The advantages of sexual reproduction and more specifically gonochorism (separation of sexes in different individuals) over asexual reproduction have been sought primarily in relation to the production of variable offspring, with mutation clearance and partial escape from coevolving parasites as the most accepted prevailing theories (West *et al.* 1999). These theories suggest that gonochoric populations can respond more efficiently to changes in environmental conditions since they have higher genetic variance. An increased genetic variation allows for a larger opportunity to respond to selection than populations where there is no genetic exchange, such as those that have asexual or hermaphroditic (both sexes in one individual) reproduction (Maynard-Smith 1978). Gonochoric populations would therefore have the long-term advantage of a higher rate of adaptation although the short-term benefits are not so clear.

Gonochoric reproduction is a complicated and costly way to reproduce in comparison to asexual or hermaphroditic strategies. Extra costs for sex involve finding a mate and inducing it to cooperate (takes time and energy), competition for mates, possible increase of predation, and the extra investment of producing males in the offspring among others.

Gonochoric reproduction implies investing energy in different ways than in asexual reproduction. The patterns of allocation between the different traits can be understood within the framework of life history theory. One of the principles is that organisms are constrained

by trade-offs in energy allocation, and thus life history strategies have evolved (Van Straalen & Hoffmann 2000). Different groups of animals vary in their physiological allocation of energy depending on their life history strategy. The energy allocation rules can be related to life-history traits such as body size and reproductive output, and can be expressed in terms of energy budgets (Kooijman 2000). After exposure to toxicants the allocation patterns depend on the mode of action of the toxicant and the toxicokinetics (Van Straalen & Hoffmann 2000). Yet, the advantages of each of the two reproductive modes in their responses to adverse environmental conditions are unclear.

We studied the responses to stress in two populations that differ in reproductive strategy in the nematode *Caenorhabditis elegans* (Nematoda, Rhabditidae). *C. elegans* usually reproduces hermaphroditically but can switch to gonochoric reproduction in the presence of males. It has an average lifespan of about 15-20 days at 15°C, and produces 250-300 eggs during its lifetime. The hermaphroditic mode of reproduction implies that one individual produces both male and female gametes to self-fertilize. At the end of the third larval stage of hermaphrodites, the gametes begin to mature as sperm in the proximal end of the hermaphroditic gonad (Kimble & Ward 1988), and as maturation proceeds the type of gamete matured in the distal end switches to oocytes. Because almost all sperm are matured before the first oocytes mature, the individual possesses its full complement of sperm when fertilization starts (Hirsh, Oppenheim & Klass 1976; Kimble & Ward 1988). There is also a limit to the number of oocytes that can be produced by the hermaphrodite since there are a fixed number of germ cells available to mature into sperm and eggs. On maturity, a hermaphrodite releases a series of self-fertilized eggs until sperm is depleted and a few remaining unfertilized oocytes are laid. After this, egg release ceases (Ward & Carrel 1979). Males are able to fertilize hermaphrodites, and in this case the number of offspring produced can double that of a non-mated hermaphrodite due to the higher availability of sperm. This mode of reproduction does not seem to be the predominant one since males are usually infrequent, but when gonochoric reproduction does occur male sperm out-competes hermaphroditic sperm almost completely. This is due to the competitive superiority of male sperm, which is larger and has faster motility (Singson, Hill & L'Hernault 1999). The higher reproductive output produced by gonochoric individuals can be expected to have an effect on other traits. Previous studies with *C. elegans* already showed that mated hermaphrodites had a

shorter life span than unmated ones. Although this was initially thought to be an effect of the larger brood size that they produced, it was found to be an effect of copulation. The stress of copulation reduces hermaphrodite lifespan either directly or by increasing susceptibility to infection by the bacteria used as food. (Gems & Riddle 1996).

In order to be able to fully understand the underlying physiological mechanisms that lead to the observed effects at the population level, a description of the organism's response (survival, growth, reproduction) throughout its life is necessary. The Dynamic Energy Budget (DEB) theory describes the functioning of individual organisms based on a set of simple rules for metabolic organization (Kooijman 2000; Kooijman 2001), where exposure to toxicants is regarded as a change in energetic parameters. This theory makes use of the formalism of Individual-Based Population modeling to make the link from individual to population level. This insight inspired the development of DEBtox (Kooijman & Bedaux 1996), a suite of models to analyze toxicity experiments in a process-based manner. The DEBtox method has been extended recently to model effects at the population level by means of simultaneously fitting data from the different life-history traits (Jager *et al*. 2004). This approach was used in this paper to model the effect at the population level of hermaphroditic and gonochoric *C. elegans* individuals under toxic stress. Cadmium was chosen as a known chemical stressor expected to have effects on the life history traits of this nematode (Ura *et al*. 2002; Swain *et al*. 2004).

Materials and Methods

The N2 strain of *C. elegans* was selected as the most suitable for life cycle testing involving hermaphroditic reproduction, due to the low occurrence of males in this strain. These populations were started with only non-mated hermaphrodites and screened regularly to remove any occurring males. The closely related CB (CB4856) strain was selected for the gonochoric reproduction experiments, due to the greater activity and occurrence of males in this strain. In this case populations were started with gravid hermaphrodites that had been fertilized by males, by means of identifying a copulatory plug that remains on the hermaphrodites after copulation (Barker 1995; Hodgkin & Doniach 1997). The ratio of males to hermaphrodites in the offspring of these gravid hermaphrodites was therefore close to 1:1.

In these experiments each hermaphrodite was kept with one male at all times, and checked for a copulatory plug to ensure that gonochoric reproduction had taken place. The N2 and CB strain of were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota).

Cadmium chloride ($CdCl_2$) was purchased from Merck, Schuchardt, Germany (>99% pure). The nominal concentrations used were 0, 2, 6, 8, 10 and 12 mg $CdCl_2$/L agar (0, 10.9, 32.7, 43.6, 54.5 and 65.5 μM). Chemical analysis of the soluble fraction of the agar was performed with ICP-AES (Centraal Laboratorium, Wageningen University Researchcentrum) after 3, 6, 10, 13 and 19 days of preparation of the dishes to monitor any changes in the available fraction of $CdCl_2$ throughout the experiments (due to binding of compound to the plastic walls or matrix of the agar) (Fig.1). Precaution was taken against any changes in the cadmium concentrations by using the dishes no longer than 5 days after preparation, since a decrease in cadmium availability was observed after day 6. The relationship between nominal and measured-back concentrations is linear, allowing us to refer to the nominal concentrations for better clarity throughout this study without interfering with the observed patterns in the results.

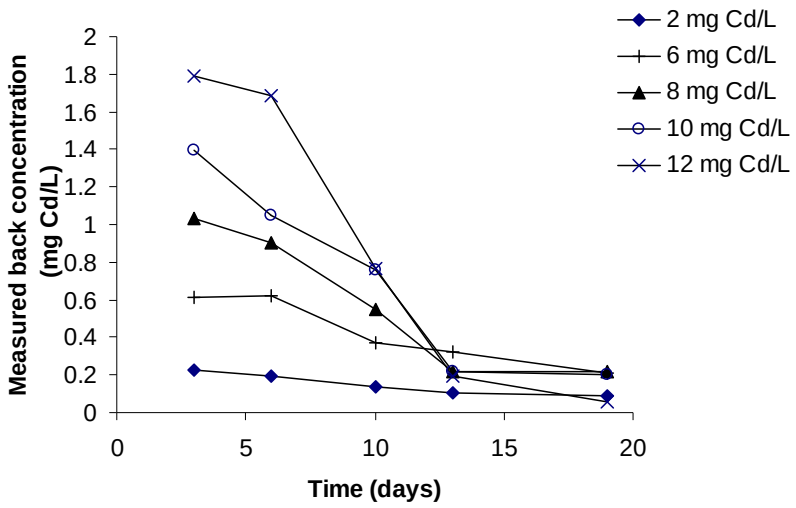

Figure 1. Chemical analysis of cadmium in soluble fraction of agar at different time points. ♦ nominal 2 mg Cd/L, + nominal 6 mg Cd/L, ▲ nominal 8 mg Cd/L, ○ nominal 10 mg Cd/L, x nominal 12 mg Cd/L. Analysis was performed with ICP-AES. A decrease in the measured concentrations is visible after the sixth day .

All nematodes were reared on NGM agar plates seeded with strain OP50 of *Escherichia coli* as a food source (Brenner 1974; Wood 1988). Stock cultures of OP50 were stored at -80°C and the bacterial cultures were grown in autoclaved LB medium (10 gr peptone, 10 gr yeast extract, 5 gr NaCl / l water) for 16 hours at 37° C and 150 rpm. Populations of these nematodes were maintained at 15°C.

All experiments were started by synchronizing populations. Gravid adults were transferred to Petri dishes (6 cm diameter) and allowed to lay eggs for a period of 4 hours, after which they were removed. After hatching the juveniles were allowed to develop into gravid adults after which a second synchronization was performed, this time on agar containing the corresponding concentration of toxicant. In this way we obtained the individuals for study, which were exposed throughout their whole life span (since egg stage) to the respective concentrations. Two separate experiments were carried out: one to record the reproductive output and survival of the individuals and a second one to obtain the body growth measurements throughout their life span.

Reproduction and survival. The newly hatched nematodes were transferred to individual wells (12 well plates from Greiner Bio-one) for observation. Each well contained 2ml of agar with the corresponding concentration of $CdCl_2$. The nematodes were daily transferred to a new well in order to count the number of offspring per individual. Reproduction was recorded for 7-12 individuals at each concentration. Survival was recorded when food pumping rate had ceased and the nematodes showed no movement after gently touching them with a small needle. Survival data was recorded in 20-40 individuals per treatment.

Growth. Separate growth experiments were performed for hermaphrodites and gonochoric individuals. In both cases, the newly hatched synchronized individuals were kept on Petri dishes (6 cm diameter) with 10ml agar containing the different concentrations of toxicant. A maximum of 15 individuals were kept on each dish (in the case of gonochorism 15 hermaphrodites and 15 males) to avoid competition. Pictures were taken of 8-10 individuals per treatment with a Cool Snap camera at intervals of 6 hours at the beginning of the growth curve, and longer intervals towards the end.

Additionally another experiment was performed to measure the size at onset of reproduction in 0, 6, 8 and 10 mg Cd/L for both reproductive strategies. Six individuals were synchronized

(within one hour) for each concentration and kept in individual wells. They were observed every 3 hours and pictures were taken when the first egg was laid.

The pictures were digitalized with the Image Pro Express 4.0.1 software package to obtain length and area measurements of the nematodes at the different time points.

Modelling. The DEBtox method to analyze the life-history traits and the effects of toxicants on these traits is described in more detail by Jager *et al*. (Jager *et al*. 2004 and Jager *et al* 2005). These models are based on the Dynamic Energy Budget (DEB) theory with some adaptations. A consequence of the DEB theory is that isomorphic animals grow according to a Von Bertalanffy curve as long as the environment remains constant. The rationale underlying this specific curve is that food uptake is generally proportional to a surface area, whereas maintenance costs are proportional to body volume (Kooijman, 2000). However, nematode growth curves are generally sigmoid when length is plotted against time (Byerly, Cassada & Russell 1976), showing a slower initial growth than predicted by the DEB model. An explanation for this phenomenon seems to be that the size of the buccal cavity (i.e. the tube-like mouth cavity, opening up into the esophagus or pharynx) limits the feeding rate, and thereby growth of the larval worms (Knight *et al*. 2002). Modifications of the growth curves are explained in more detail by Jager *et al*. (Jager *et al.*, 2005). These observations are also supported by the observed low pumping rates of newly hatched larvae, which gradually increased until the adult stage was reached. This shows that the buccal cavity was indeed limiting feeding (and therefore growth) in these early time points, whereas at a later stage, the nematodes were able to feed freely and the growth curves showed the expected pattern at this point. (Fig. 2).

Apart from the deviations due to the details of growth in the nematodes, some changes were also made to the model describing the effects of old age (senescence). Many aspects of nematode physiology will change with age, but in life-cycle toxicity studies, the primary concerns are the effects on survival and reproduction. Basically, the model by Van Leeuwen et al. (Van Leeuwen, Kelpin & Kooijman 2002) was followed. This model is based on the oxidative stress hypothesis: aerobic respiration produces free radicals that cause damage to cell components. This damage accumulates in the cell, leading to the aging phenotype. This approach was successfully applied to life-cycle studies with the springtail *Folsomia candida (*Jager 2004). We applied the same model to nematodes with a few modifications. Firstly, the

maintenance rate was not taken as a free model parameter, but was linked directly to the rate constant for growth (Kooijman & Bedaux 1996). Further, old-age effects were not related to damage density but to the absolute amount of oxidative damage in the organism's body, and no additional feedback mechanisms were assumed.

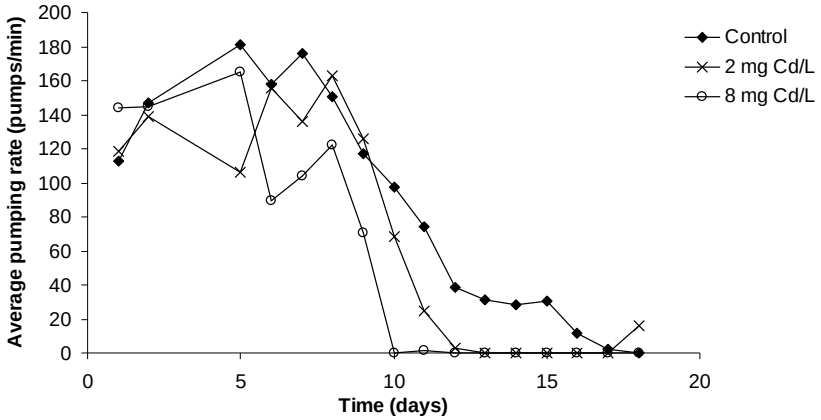

Figure 2. Average pumping rate throughout time in different concentrations of cadmium. ♦ control, x 2 mg Cd/L, ○ 8 mg Cd/L. Low pumping rates are observed in newly hatched larvae, which gradually increase until the adult stage is reached supporting observations made by Knight (Knight *et al.* 2002) where size of the buccal cavity limits the feeding rate at early time points and thereby growth of the larval worms. This leads to deviations in the expected Von Bertalanffy growth curve of nematodes.

A different mechanism for the old-age effects on reproduction was assumed for each strategy. In hermaphrodites, egg production ceases when the storage of sperm cells is depleted (Ward & Carrel 1979), leading to a sudden halt of reproduction. In contrast, gonochoristic *C. elegans* show a more gradual decline in egg production, presumably due to aging effects (oxidative damage) on the developing egg.

Finally, the observed toxic effects at the highest concentration were less than expected from the model. In order to accommodate this behavior, we assumed that the uptake of cadmium is saturating at higher external concentrations according to Michaelis-Menten kinetics. Similar

saturation was for example shown for cadmium in aquatic oligochaetes (Steen Redeker & Blust 2004) and in fish (Van Ginneken, Bervoets & Blust 2001).

The model fits for all traits and both reproductive modes were done simultaneously, which led to a total of eight data sets. These include survival, cumulative reproduction, and two growth data sets (one of which incorporates old age sizes) for each strategy (Fig. 3). Simultaneous fitting was required because the data sets include common parameters. It was assumed that most of the parameters do not differ between the hermaphroditic and gonochoric strains, such as the growth parameters and the intrinsic sensitivity to cadmium. However, the aging parameters needed to be given different values since gonochoric reproduction had a negative effect (copulation damage) on survival, which was accounted for as a higher damage killing rate, reflecting faster aging in these individuals than in the hermaphrodites. The aging parameters also differ because of the different mechanisms that govern the reproduction patterns at old ages (sperm shortage in hermaphrodites vs. oxidative damage in gonochoric individuals).

Difficulties were encountered when matching the data sets in the model fitting procedure because the experiments for growth were done separately from the determination of survival and reproduction. This led to slight variations in the initial part of the growth curve (i.e. the reproduction data may have resulted from animals with a slightly shifted growth pattern, compared to the body size data). This problem was solved by determining the body size at the onset of reproduction independently. In this way, the length at the start of reproduction became a fixed parameter.

The model was implemented in Matlab® Version 7.0 (Release 14), and model fitting was based on maximum likelihood estimation. Confidence intervals were generated using profile likelihoods (Meeker & Escobar 1995). The intrinsic rate of population increase was calculated according to the Euler-Lotka equation, as a function of exposure concentration (Jager *et al*. 2004). Further, the population effects at limiting food densities were predicted. This was possible as food limitation has predictable consequences in DEB theory (Jager *et al*. 2005); although care must be taken, as this calculation is an extrapolation beyond the information provided by the experimental data (e.g. it was assumed that the intrinsic sensitivity to cadmium is not affected by food level).

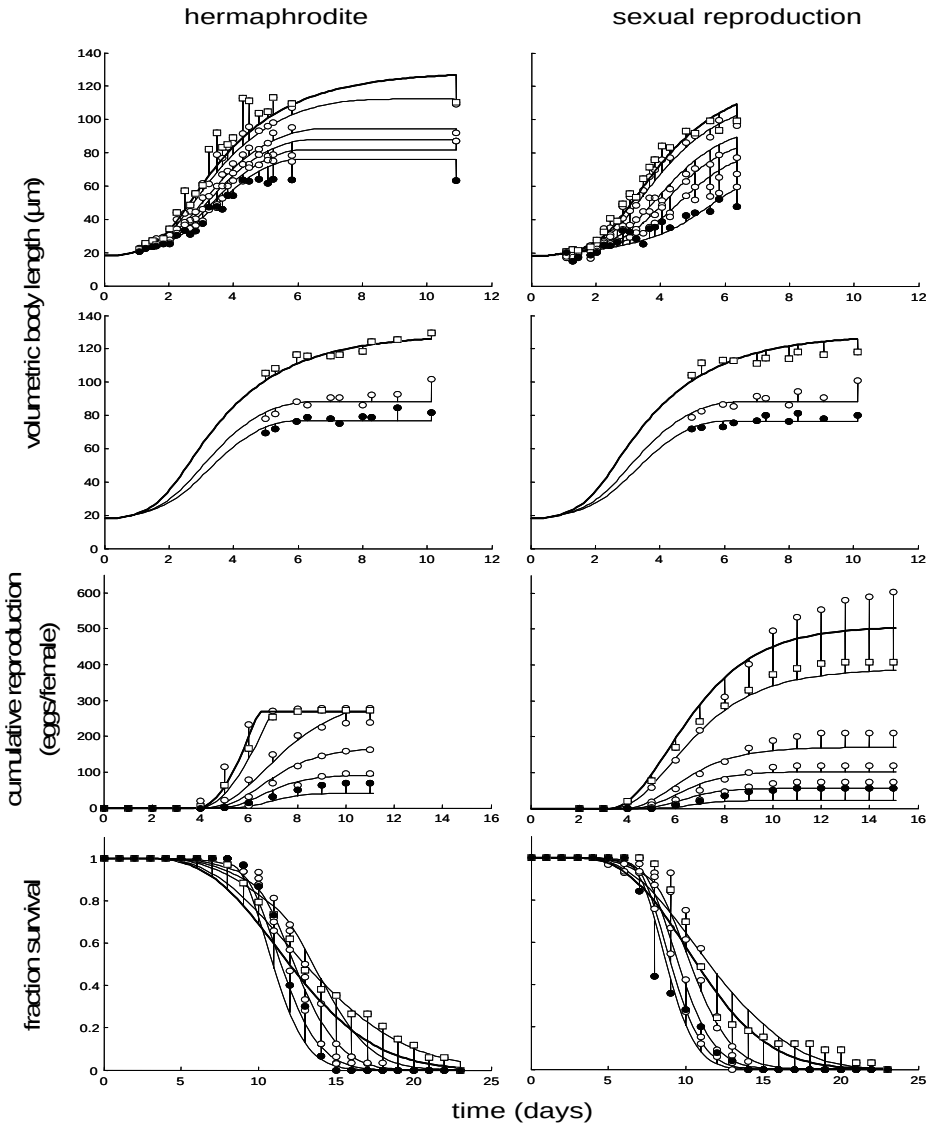

Figure 3. Data and simultaneous model fits for all endpoints of the life cycle study with *C. elegans* exposed to cadmium, for both reproductive strategies. □ control, ○ concentrations 2, 6, 8, and 10 mg Cd/L● 12 mg Cd/L (highest concentration). These include survival, cumulative reproduction, and two growth data sets for each strain. The top graphs show the data points for all concentrations until adult stage (day6), whereas the bottom graphs include the data points for 3 concentrations until the old-age stage (day 10) in order to calculated the point at which growth stops completely. Simultaneous fitting was required because the data sets include common parameters. The effects of cadmium on the different endpoints are visible as concentrations increase, as well as differences in reproduction and survival between the two strategies.

Results

Good model fits were obtained for the data sets, with few differences between the parameters for N2 and CB populations (Table 1). Fertilization by males was not affected by cadmium exposure (t-test, n_1= 7, n_2= 6, n_3= 7, t_1= 0.096, t_2= 2.205, t_3= 1.842, $p>0.05$); the male : hermaphrodite ratio in the F1 of the gonochoric (CB) experiments ranged from 0.45-0.51 in control conditions, 8 mg Cd/L and 12 mg Cd/L.

The decrease in reproduction at increasing cadmium levels was clearly visible in both strains. Both in control conditions and in the lowest concentration (2 mg Cd/L) CB yielded a higher reproductive output than N2, and in both strains the individuals exposed to 2 mg Cd/L reproduced more than the controls. However, apart from these two cases all other concentrations showed similar reproduction for both strains (Fig. 3).

Differences were found in the onset of reproduction between the two strains which was probably governed by the reproductive strategy. N2 started reproduction after 100 hours with an average volumetric length (cubic root of the volume) of 83.4 µm, whereas CB started reproduction earlier at 84 hours with an average volumetric length of 69.9 µm. The differences between the size at onset of reproduction were found to be significant at control conditions (t-test, n_1= 6, n_2= 6, t= 6.780, $p<0.05$). At increasing cadmium concentrations, size at onset of reproduction decreased in N2 whereas CB individuals showed little reduction in size (Fig. 4). This suggests a difference between toxicokinetics of cadmium between the two strains.

Cadmium affected survival with visible differences between the curves of the two strains. Lower survival was observed in CB under all conditions. This negative effect on survival was accounted for in the model as a higher damage killing rate (Table 1). This aging parameter reflected faster aging in CB individuals than in N2 individuals.

Table 1. Parameters used in the model

	N2 strain	**CB strain**
	Physiological parameters	
Von Bertalanffy growth rate (d^{-1})	0.488 (0.476-0.520)	
Initial length (µm)	18.1 a	
Length at first reproduction (µm)	83.4 a	69.9 a
Maximum length (µm)	130 (127-132)	
Maximum reproduction rate (eggs/d)	336 (297-370)	
Energy investment ratio (-)	10 a	
Time before hatching (d)	0.375 (9 hours) a	
Length where ingestion is half of maximum (µm)	30.0 (29.8-30.4) (growth 1)	31.1 (30.9-31.4) (growth 1)
	29.8 (28.9-30.6) (growth 2)	29.8 (29.1-30.6) (growth 2)
	31.0 (30.8-31.3) (repro/surv)	30.9 (30.7-31.2) (repro/surv)
	Ageing parameters	
Damage killing rate (d^{-1})	0.0185 (0.0146-0.0229)	0.0268 (0.0207-0.0338)
Damage tolerance on reproduction (-)	∞ a	3.04 (2.67-3.80)
Maximum total number of eggs (eggs)	269 (248-288)	∞ a
	Toxicological parameters	
Elimination rate (d^{-1})	0.0138 (0.0125-0.0161)	
Half-saturation conc. (mg/L)	22.9 (14.5-28.8)	
NEC for survival (mg/L)	0.559 (0.488-0.619)	0.265 (0.219-0.311)
Killing rate (L/mg/hr)	1.00 (0.674-1.51)	1.30 (0.935-1.67)
NEC for effects on assimilation (mg/L)	4.20e-6 (0-0.00637)	
Tolerance concentration (mg/L)	1.00 (0.751-1.08)	
Decrease in length at first repro due to Cd stress (-)	0.931 (0.665-1.24)	0 a

a indicates parameter is fixed, not estimated from the data. Confidence intervals obtained from DEB model in brackets.

The traits were best described by the model when a decrease in assimilation was assumed as the mode of action for cadmium, implying that either cadmium affects the feeding rate of the organisms directly, or by decreasing the efficiency with which energy from food is assimilated (Kooijman & Bedaux 1996). The last assumption was difficult to test experimentally but the first assumption was supported by the observation of pharyngeal pumping rates which were found to be significantly lower at increasing concentrations of cadmium (Fig. 2). These observations were also reported in earlier studies on feeding inhibition in *C. elegans* exposed to sublethal concentrations of cadmium (Jones & Candido 1999). Body size as well as reproduction were decreased by cadmium, presumably due to the effect on assimilation, without any differences in the physiological parameters of CB and N2 individuals apart from onset of reproduction (Table 1).

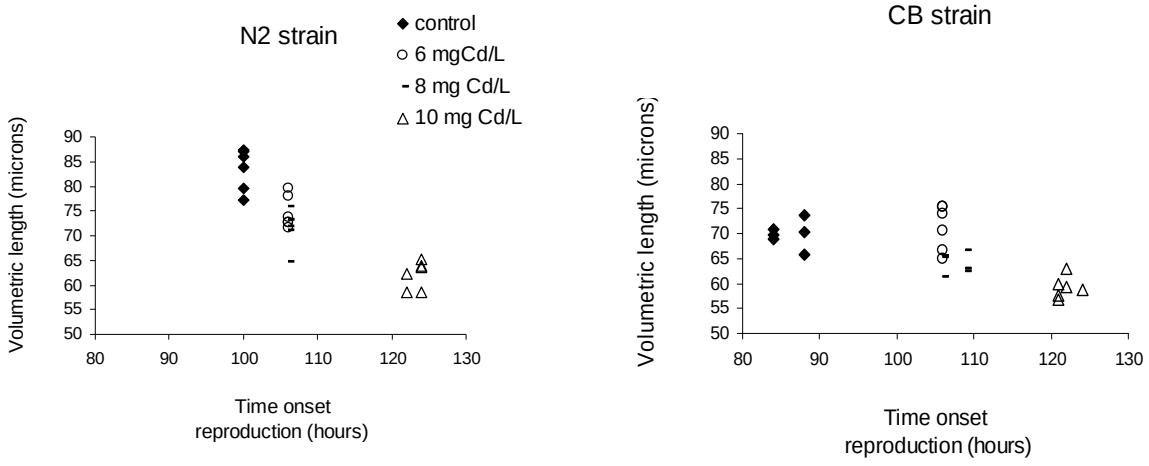

Figure 4. Size and time of reproduction onset in N2 and CB individuals with increasing concentrations of cadmium. ♦ control, ○ 6 mg Cd/L, ▬ 8 mg Cd/L, Δ 10 mg Cd/L. Differences in the onset of reproduction sizes were found to be significant between the two strategies (t-test, $p<0.05$). With increasing concentrations of cadmium, N2 individuals gradually decrease their size at onset of reproduction whereas CB individuals show little reduction in size. This suggests a difference in how cadmium stress was dealt with for onset of reproduction. N2 starts reproduction later and at a larger size than CB. This difference in timing is caused by the larger threshold size for reproduction of N2. This variation in threshold size is probably related to reproductive strategy, where the populations differ in the timing of the switch from sperm production to oocyte production during maturation.

Population growth rate was negatively affected by cadmium. Compared to N2, lower values were recorded for CB individuals in all treatments. (Fig. 5). Furthermore, CB individuals seemed more affected by the cadmium than N2 individuals. In control conditions, CB presented only a slightly lower population growth rate than N2, but as the concentration of cadmium increased this difference grew visibly. At limiting food levels, the model predicts that the population growth rate collapses at a certain concentration of toxicant, as individuals either do not reach the size threshold for reproduction or do not grow past the food-limited larval stage. The model also showed the increasing effects of cadmium for both strains when food level is reduced as could be seen from the decrease in the concentration at which growth rate collapses.

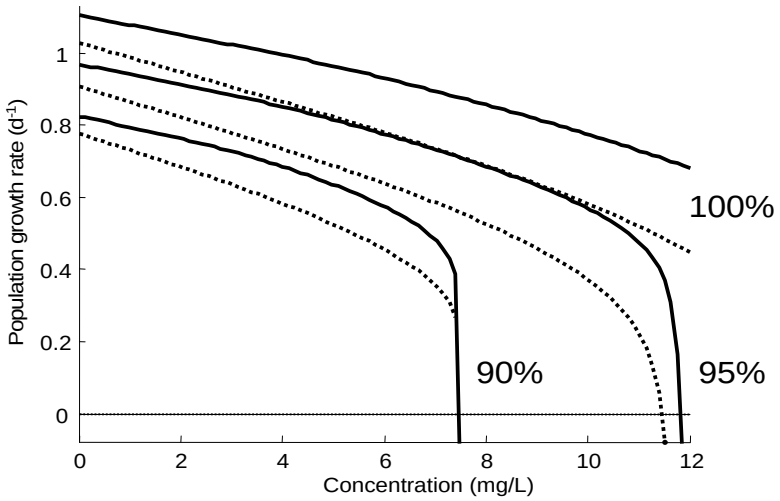

Figure 5. Modeled intrinsic rate of population increase for *C. elegans*, exposed to cadmium at different food levels (shown as percentage of the maximum ingestion rate). ── N2 strain, - - - CB strain. A decrease in the modeled population growth rates is observed with increasing concentrations of cadmium, also showing lower values for CB individuals in control conditions as well as in all of the cadmium concentrations. Furthermore, individuals from CB strain seem more affected by the cadmium stress than individuals from N2 strain. At limiting food levels, the model predicts that the population growth rate collapses at a certain concentration of toxicant, as individuals either do not reach the size threshold for reproduction or do not grow past the food-limited larval stage. The model also shows the increasing effects of cadmium for both strategies when food level is reduced.

Discussion

Onset of reproduction. N2 started reproduction later and at a larger size than CB (Fig. 4). The difference in timing is caused by the larger threshold size for reproduction of the N2 individuals. The variations in the threshold size are probably related to reproductive strategy, where the two populations differ in the timing of the switch from sperm production to oocyte production during maturation. This switch to oocyte maturation and ovulation is dependent on several gamete signaling events (McCarter *et al*. 1999), in which sperm play an important role. Their presence is necessary for triggering oocyte maturation and basal gonadal sheath activity (Singson 2001). A reduction in sperm production allows an earlier switch and therefore onset of reproduction can take place at a smaller size. Mating probably leads to this decrease in hermaphroditic sperm production and earlier switch to oocyte production. It provides an earlier source of mature sperm that can trigger the maturation of oocytes sooner than in non-mated hermaphrodites, where this does not occur until all sperm have matured.

In contrast to CB, the onset of reproduction in N2 occurs at a smaller size as cadmium concentration increases. This may reflect a strategy of N2 in response to lower growth rates (and not to the toxicant *per se*). It is conceivable that the number of sperm cells is such that it maximizes fitness (Cutter 2004). The production of more sperm cells does not necessarily increase the population growth rate because it simultaneously delays reproduction. In a stressed situation (such as Cd exposure), the outcome of this trade-off may be different, and less sperm production may maximize fitness. Thus, the threshold size in N2 is probably being reduced by a decrease in sperm production, triggering an earlier switch to oocyte production. CB individuals, however, already start reproduction at a small size in control conditions, with reduced sperm production in comparison to non-mated N2 individuals, and earlier maturation of oocytes. In CB individuals, the presence of male sperm allows hermaphroditic sperm production to stop early. This happens both in control conditions and in the different concentrations, giving way to similar sizes of reproduction onset. A further reduction in size might not be possible anymore in order for reproduction to take place.

Reproduction. Cadmium also affected reproduction (Fig. 3). In the control and at the lowest concentration (2 mg Cd/L) the decline in reproduction is governed by sperm limitation (sudden halt in reproduction due to lack of sperm) in hermaphroditic N2, and aging/oxidative

damage (gradual decrease of reproduction) in gonochoric CB. In this case, gonochorism produces higher lifetime reproductive output than hermaphroditism although it must be taken into account that half the offspring will be males. However, at higher cadmium concentrations the decline in reproduction is governed by toxicity rather than by the aforementioned mechanisms. Cadmium limits the resources available to the organisms for egg production and therefore comparable reproductive outputs in these cadmium concentrations (6, 8, 10 and 12 mg Cd/L) can be seen for both strains.

The reproductive output in the lowest concentration of cadmium (2 mg Cd/L) is higher than that of the control organisms. This apparent hormesis occurs for both N2 and CB individuals, and is only visible in the reproduction data. Hormesis has been defined as an adaptive response to low levels of stress that causes a disruption in homeostasis, resulting in improved fitness or modest overcompensation, for some physiological systems for a finite period (Calabrese and Baldwin 2001). The model does not incorporate this effect but responds to this data set by slightly increasing the curve for the control response.

Growth, aging, and survival. With increasing concentrations of cadmium, a reduction in the growth rate was also observed. The aging model assumes that a reduction in growth implies lower respiration rates and therefore less oxidative damage, which could lead to an increased longevity. This is one possible explanation for the effect, which is visible between day 5 and 10, where the survival curves of the exposed individuals rise above the control (Fig. 3). After these early time points the toxic effects of cadmium could be counteracting any increase in longevity caused by a reduced respiration, showing a decreasing percentage of survival in time with increasing concentrations.

In all cases, it can be seen that the survival of CB individuals is lower than that of N2 individuals due to the difference in the rate of aging (Table 1). This faster aging (higher killing rate) is partly caused by mating costs and the model of oxidative damage accumulation may not be entirely explanatory in this case. Survival in gonochoric CB individuals is more sensitive to cadmium than in N2 (lower No Effect Concentration, higher killing rate in CB) probably because the mating costs increase their sensitivity. They are, however, not more sensitive to cadmium for growth and reproduction through a decrease in assimilation, which indicates that effects on assimilation and effects on the hazard rate (for survival) may not be directly related.

The significance of gonochoric reproduction in C. elegans. The major differences found between the two strains were onset of reproduction (and how cadmium affects it), ageing, and sensitivity of survival to cadmium. When all endpoints are considered simultaneously, the analyses suggest that gonochoric reproduction in this species does not seem to favor population growth rates in the short term. It is a complicated and costly way to reproduce with copulation costs affecting the survival curves and a progeny composed of 50% of males, which also lowers the population growth rates. As performance of the gonochoric strain is expected to be lower compared to the hermaphroditic strain in the short term, it might not be surprising that there are lower frequencies of *C. elegans* males found in nature than of hermaphrodites.

For a long time, the evolution and significance of sex has been a major puzzle in evolutionary biology due to its two-fold costs (Maynard-Smith 1978) and to the challenge of unraveling the advantages that might outweigh the disadvantages of this mode of reproduction. One of the most widely accepted theories known as the “Red Queen” hypothesis suggests that continuous development is needed for an evolutionary system, just in order to maintain its fitness relative to the systems it is co-evolving with (Van Valen 1973). Another theory states that sex could act to encourage the removal of deleterious genes (Kondrashov 1988). The fact is that sex is an almost universal phenomenon and few groups of higher eukaryotes have persisted by asexual reproduction for a long period of time (Barton & Charlesworth 1998). However, it can be considered that gonochorism in *C. elegans* is probably not cost-effective under stable conditions, making asexual reproduction more effective in this case. In wild type *C. elegans* males are found at about 0.05% of the population, but this seems to increase under extreme conditions. Heat shocks of 30º C for 6 hours are commonly used in laboratory cultures to increase the rate of non-disjunction of the X chromosome in gametogenesis and therefore obtain a greater number of males (Sulston & Hodgkin 1988). A male pool could be providing this species with an emergency mechanism to confront the occurrence of adverse conditions, justifying what seems to be a less efficient and more costly reproductive strategy otherwise.

Acknowledgments

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Chapter 4

Assessing physiological modes of action of toxic stressors with the nematode *Acrobeloides nanus*

This chapter has been submitted as:

Alda Álvarez, O.; Jager, T.; Marco Redondo, E.; Kammenga, J.E. Assessing physiological modes of action of toxic stressors with the nematode *Acrobeloides nanus*. *Environmental Toxicology & Chemistry*. Submitted.

Abstract

Current classifications of compounds according to their modes of action are mostly derived from the structural characteristics of the compounds. The underlying assumption is that chemically similar compounds have similar modes of action, which is often but not necessarily the case. A deeper insight into the actual mechanisms of toxic action is important for risk assessment as it contributes to a more physiological interpretation of toxicity data. Here we study the physiological mode of action of three different compounds (cadmium, carbendazim and pentachlorobenzene) with an experimental data-based approach, using whole life cycle toxicity data from the nematode *Acrobeloides nanus.* We use a process-based model, based on the Dynamic Energy Budget theory, to study the fluxes of energy related to physiological processes and their variation throughout the life cycle. With this approach we unravel the physiological modes of action based on resource allocation and model the effects of the different modes of action at the population level. The mode of action of carbendazim was through a decrease in assimilation and an extra effect on reactive oxygen species (ROS) production. Cadmium increased the costs for growth with an extra effect on ROS production, and pentachlorobenzene decreased assimilation. We compared the results with previous studies on the nematode *Caenorhabditis elegans* and found that the modes of action of the three compounds differed to those found in *A. nanus*, showing that the life history characteristics of each organism have a clear influence on the resulting modes of action. This highlights the importance of the interactions between a chemical and the biological characteristics of the organism in the determination of resulting physiological modes of action.

Introduction

Current methods for classifying chemicals by their mode of action can generally be either experimental data-based or structure-based. Experimental data-based methods assess a chemical's mode of action by analyzing the data from toxicity tests, whereas structure-based approaches classify compounds based on their functional groups. A widely used structure-based classification was developed by Verhaar (Verhaar *et al*. 1999) who assigned chemicals to one of four classes: narcotics, polar narcotics, reactive chemicals, and specifically acting chemicals with receptor-mediated toxicity. This classification is derived from the structural characteristics of the compounds, with the underlying assumption that chemically similar compounds have similar modes of action, which is often but not necessarily the case. The activity of a toxicant in an organism depends on physical, chemical and biological factors among which interactions may also exist, making the determination of the mechanism of toxic action a difficult task (Bradbury 1994). The mechanism of action may vary depending on the species, compound and endpoint of study. Consequently, studies that provide a deeper insight into these mechanisms can become important tools for risk assessment contributing to a better interpretation of toxicity data.

Here we study the physiological mode of action of three different compounds: cadmium (heavy metal), carbendazim (DNA synthesis inhibitor (Clemons & Sisler 1971)) and pentachlorobenzene (narcotic (Lydy *et al.* 1999)). We take an experimental data-based approach that uses whole life cycle toxicity data from different endpoints. The study illustrates how physiological modes of action can be analyzed from a mechanistic perspective based on the chemical's effects on the organism's energy budget. This is done using a process-based model which studies the fluxes of energy, related to physiological processes, and their variation throughout the whole life cycle. This model is based on the Dynamic Energy Budget (DEB) theory, which describes the functioning of individual organisms based on a set of rules for metabolic organization (Kooijman 2000; Kooijman 2001). Exposure to toxicants is regarded as a change in energetic parameters, such as an increase in maintenance costs or a decrease in assimilation of energy from food. This insight inspired the development of DEBtox (Kooijman & Bedaux 1996), a suite of models to analyze toxicity experiments in a process-based manner. The analysis reveals the physiological mode of action based on the

changes in energy allocation patterns that take place in response to the chemical. In the DEB scheme, part of the food is assimilated contributing to the reserves, and the rest is transformed into feces. These resources are distributed in a fixed fraction between somatic growth/maintenance and reproductive output/maturation. Within this scheme (Fig. 1), a chemical may exert a toxic effect on different energetic parameters: decreasing assimilation, increasing maintenance costs, increasing costs for growth, increasing costs for reproduction or posing a direct hazard to the embryo (Kooijman & Bedaux 1996). The dissection of the physiological mode of action of toxic compounds is interesting from a scientific viewpoint since it provides an insight into the mechanisms through which a chemical is exerting toxic effects, and how these are reflected on the different endpoints (survival, reproduction, and growth). In addition, understanding the details about the mode of action of toxicants is essential for the extrapolation to population level effects.

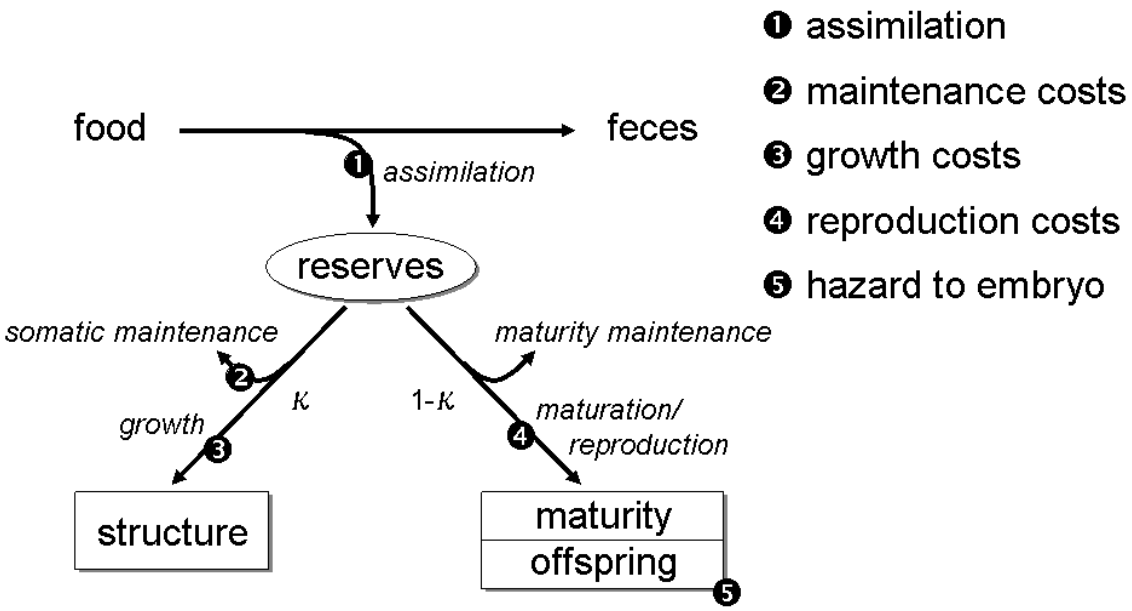

Figure 1. DEB scheme and target parameters where a compound can exert its toxic mode of action.

The DEBtox method has been extended recently to model effects at the population level by means of simultaneously fitting data from the different life-history traits (Jager *et al.* 2004). In this way, the effects on the different endpoints are integrated into a single parameter, the population growth rate, which provides a more relevant measure of ecological impact than any isolated endpoint (Forbes & Calow 1999). Furthermore, because other factors such as temperature or food limitation have predictable consequences in DEB theory (Jager *et al.* 2005), their effects on the population growth rates in combination to increasing concentrations of toxicant can be modeled.

DEBtox was used in this paper to simultaneously model the effects on survival, growth and reproduction of three different compounds (cadmium, pentachlorobenzene and carbendazim), in order to elucidate their physiological toxic mode of action, and to reveal how these modes of action affect the population growth rates in the nematode *Acrobeloides nanus.*

Materials and Methods

Chemicals. Pentachlorobenzene was purchased from Aldrich, Steinheim, Germany (98% pure). An initial solution of 10 mg pentachlorobenzene/ml ethanol was prepared. Nominal concentrations used for the experiments ranged from 50 to 130 mg pentachlorobenzene/L agar. An ethanol blank was used as the control.

Carbendazim was purchased from Riedel-de Haen, Seelze, Germany (99% pure). An initial solution of 300 mg carbendazim/L ethanol was prepared. Nominal concentrations used for the experiments ranged from 0.15 to 1.5 mg carbendazim/L agar. An ethanol blank was used as the control.

Cadmium chloride ($CdCl_2$) was purchased from Merck, Schuchardt, Germany (>99% pure). An initial solution of 1 mg $CdCl_2$/ml water was prepared. Nominal concentrations used ranged from 2 to 12 mg $CdCl_2$/L agar.

Acrobeloides nanus. Nematodes were extracted with an Oostenbrink elutriator (Oostenbrink 1960) from soil samples which were collected from an experimental field at Bovenbuurt, ca. 3 km NNE of Wageningen, The Netherlands. From the obtained water suspension, *Acrobeloides nanus* individuals were classified to the species level and placed on Petri dishes to start populations. Populations where maintained at 20°C.

Culturing. All nematodes were reared on NGM agar plates seeded with OP50 strain of *Escherichia coli* as a food source (Brenner 1974; Sulston & Hodgkin 1988). Stock cultures of OP50 were stored at -80°C and the bacterial cultures were grown in autoclaved LB medium (10 gr peptone, 10 gr yeast extract, 5 gr NaCl / l water) for 16 hours at 37° C and 150 rpm.

All experiments were started with a first synchronization in which gravid adults were transferred to 6cm Petri dishes and allowed to lay eggs for a period of 4-8 hours, after which they were removed. These eggs were allowed to hatch and develop into gravid adults, with which a second synchronization was performed, this time on agar containing the corresponding concentration of toxicant. In this way we obtained the individuals for study, which were exposed throughout their whole life span (since egg stage) to the experimental treatments.

In the case of the carbendazim, two experiments were carried out. One was performed as described above with concentrations from 0.15 to 0.75 mg carbendazim/L agar. A second one was carried out where all individuals were allowed to hatch in control conditions and then transferred to their respective concentrations (from 0.3 to 1.5 mg carbendazim/L agar). This is due to the fact that this compound dramatically inhibits hatching, so experiments performed since the egg stage can only be carried out with very low concentrations of this compound, giving way to very low sublethal effects. By allowing the individuals to hatch in control conditions and then transferring to the contaminated dishes, we were able to use higher concentrations and thus observe larger sublethal effects.

Reproduction and survival. The newly hatched nematodes were transferred to individual wells (12 well plates from Greiner Bio-one) for observation. Each well contained 2ml of agar with the corresponding concentration of toxicant. The nematodes were transferred to a new well daily in order to count the number of offspring per individual. Reproduction was recorded in 20-40 individuals for each concentration. Death was scored when observing absence of pharyngeal pumping and no movement response to touch. Survival data was recorded in 20-40 individuals per treatment.

Growth. Pictures were taken of 8-10 individuals per treatment with a Cool Snap camera at intervals of 6 hours at the beginning of the growth curve, and longer intervals towards the end. The pictures were digitalized with the Image Pro Express 4.0.1 software package to obtain length and area measurements of the nematodes at the different time points.

Modeling. The DEBtox method (Kooijman & Bedaux 1996) was used to analyze the life-history traits of *A. nanus* and the effects of the three different toxicants on these traits. In this approach, the internal concentration causes the effects; the first step in the model chain is therefore a one-compartment toxicokinetic model. The internal concentration affects the probability to die, as well as a parameter of the animal model (e.g. the maintenance costs or the assimilation of energy from food). The animal model is based on the Dynamic Energy Budget (DEB) theory (Kooijman 2001). The DEBtox approach has been adapted to deal with life cycle toxicity studies (Jager *et al.* 2004), and the specific details of the nematode life cycle (Jager *et al.* 2004). The performance of this model for nematodes was already demonstrated for the effects of cadmium on *C. elegans* (Alda Álvarez 2005), and the same approach is followed here to analyze the datasets for cadmium, carbendazim and pentachlorobenzene on *A. nanus*.

As with sexually reproducing *C. elegans* (Alda Álvarez 2005), *A. nanus* can apparently reduce its size at first reproduction when exposed to toxicants. An extra parameter is included to describe this behavior, although a more in-depth investigation is needed on the consequences for resource allocation.

The model was implemented in Matlab® Version 7.0 (Release 14), and model fitting was based on maximum likelihood estimation. The model fits reveal the toxic mode of action of the compound, based on resource allocation, and estimates for parameters governing toxicokinetics, toxicity and basic physiology (see Table 1). Confidence intervals were generated using profile likelihoods (see Meeker & Escobar 1995). The intrinsic rate of population increase was calculated according to the Euler-Lotka equation, as a function of exposure concentration (see Jager *et al.* 2004). This population growth rate is calculated both using the model predictions, as well as directly from the effects data themselves. For the latter, survival and reproduction are interpolated on a smaller time grid (using piecewise cubic Hermite interpolation in MatLab) and subsequently integrated in the Lotka-Euler equation. Simulations were made for the population growth rates under different temperatures. These predictions can be made assuming that temperature affects all rate constants according to the Arrhenius relationship (see Jager et al. 2005), and that the intrinsic sensitivity of the organisms is not affected by temperature. Although the first assumption appears to be quite

reasonable (Jager et al. 2005), the latter assumption is more questionable as it seems that temperature can affect the intrinsic sensitivity (Heugens *et al.* 2003).

Results

Model fits were obtained for the different life history traits of *A. nanus* in exposure to carbendazim (Fig. 2), cadmium (Fig. 3) and pentachlorobenzene (Fig. 4). Each scenario represents the toxic effects on survival, reproduction and growth throughout the life span of the organism.

In the case of carbendazim, the top panel presents the model fits for the first experiment where the individuals were exposed to the toxicant since the egg stage in concentrations ranging from 0.15 to 0.75 mg carbendazim/L agar. It was observed that even low concentrations of this compound severely affected egg hatching in *A. nanus*, making it difficult to obtain enough individuals for the experiment. Therefore, exposure to carbendazim since the egg stage could only be performed at low concentrations giving, as a result, very low sublethal effects on the observed life history traits. The model fits showed very small reductions in survival, reproduction and growth with increasing concentration of the compound (Fig. 2, top panel). In order to obtain more visible sublethal effects, the individuals were hatched in control conditions and then transferred to higher concentrations ranging from 0.3 to 1.5 mg carbendazim/L agar (Fig. 2, bottom panel). Survival in this case presented a small reduction in the lower concentrations, which then gave way to a sharp decrease in the three highest exposures. The reproduction curves show a substantial decrease, especially at early and intermediate time points, but exhibits an apparent recovery towards the end of the reproductive period where the exposed individuals reach a cumulative reproduction comparable to that observed in control conditions. Finally, the growth curves presented a clear reduction with increasing concentrations which was maintained over the entire duration of the experiment.

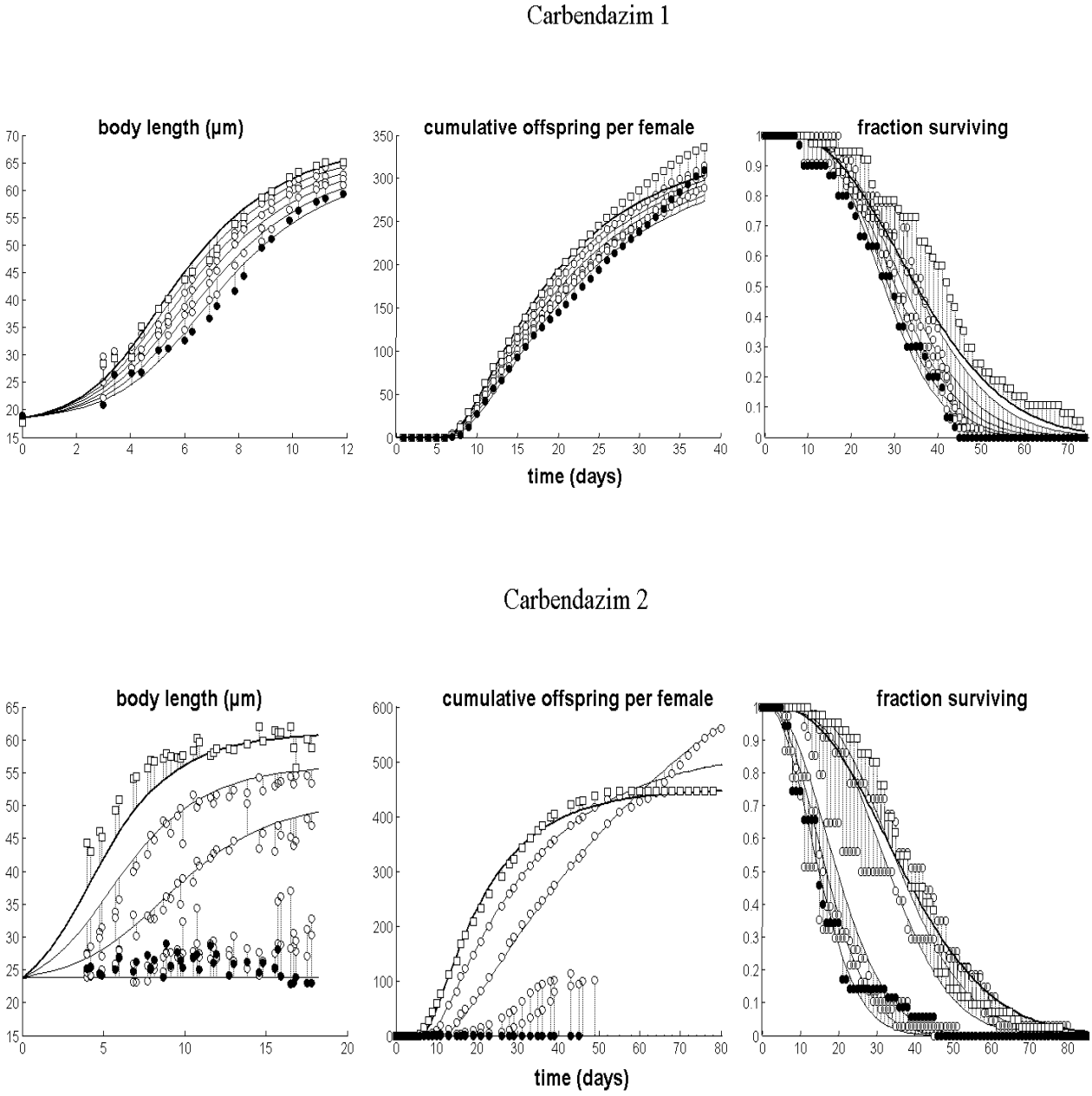

Figure 2. Data and simultaneous model fits for all endpoints of the life cycle study with *A. nanus* exposed to carbendazim. These include growth, cumulative reproduction and survival. The top panel corresponds to the first experiment, where individuals were hatched in carbendazim. □ control, ○ concentrations 0.15, 0.3, 0.45 and 0.6 mg carbendazim/L ● 0.75 mg carbendazim/L (highest concentration). The bottom panel corresponds to the second experiment where the individuals were hatched in control conditions. □ control, ○ concentrations 0.3, 0.6, 0.9 and 1.2 mg carbendazim/L ● 1.5 mg carbendazim/L (highest concentration).

In the cadmium scenario (Fig. 3) the survival curves of the two lowest concentrations presented a large reduction with respect to the control, and higher concentrations from this point on also caused a reduction, although to a lower degree. Reproduction presented a similar gradient of effects to that of survival, with the largest decrease observed in the two lowest concentrations. The growth curves in this case decreased gradually with increasing concentrations of cadmium in the early and intermediate time points, and exhibited an apparent recovery towards the end which reduced the differences in maximum size between the concentrations.

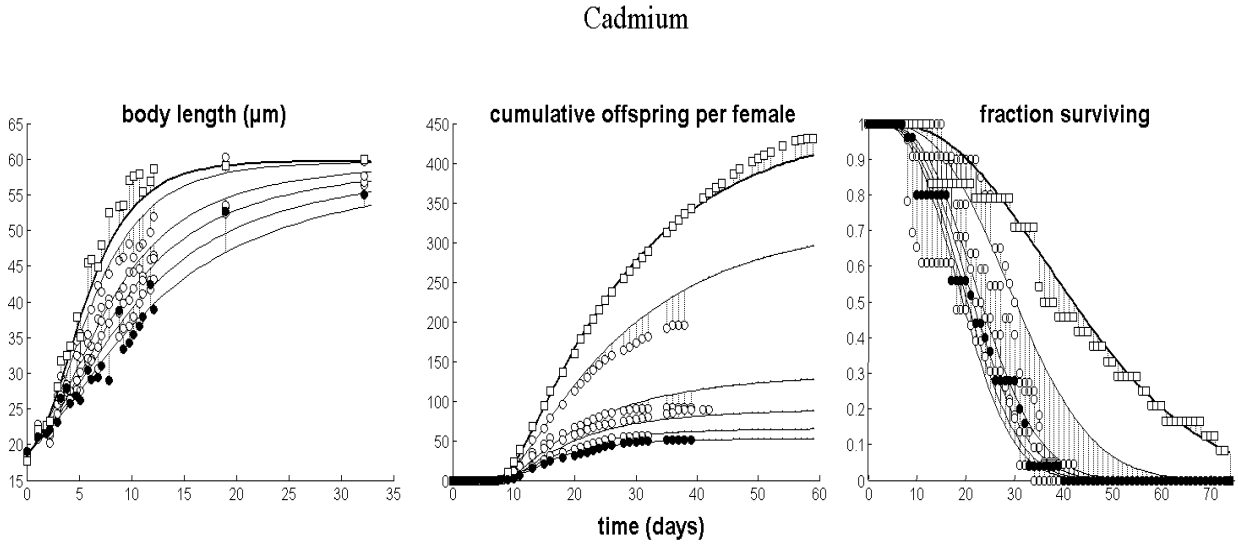

Figure 3. Data and simultaneous model fits for growth, cumulative reproduction and survival from the life cycle study with *A. nanus* exposed to cadmium. □ control, ○ concentrations 2, 6, 8 and 10 mg Cd/L ● 12 mg Cd/L (highest concentration).

The last scenario shows the effects of pentachlorobenzene (Fig. 4), where the survival curves presented a gradual decrease with increasing concentrations. Survival under control conditions deviated from the expected behavior, showing somewhat higher values than in the previous experiments. Reproduction and survival also presented a gradual decrease with increasing concentrations of this compound.

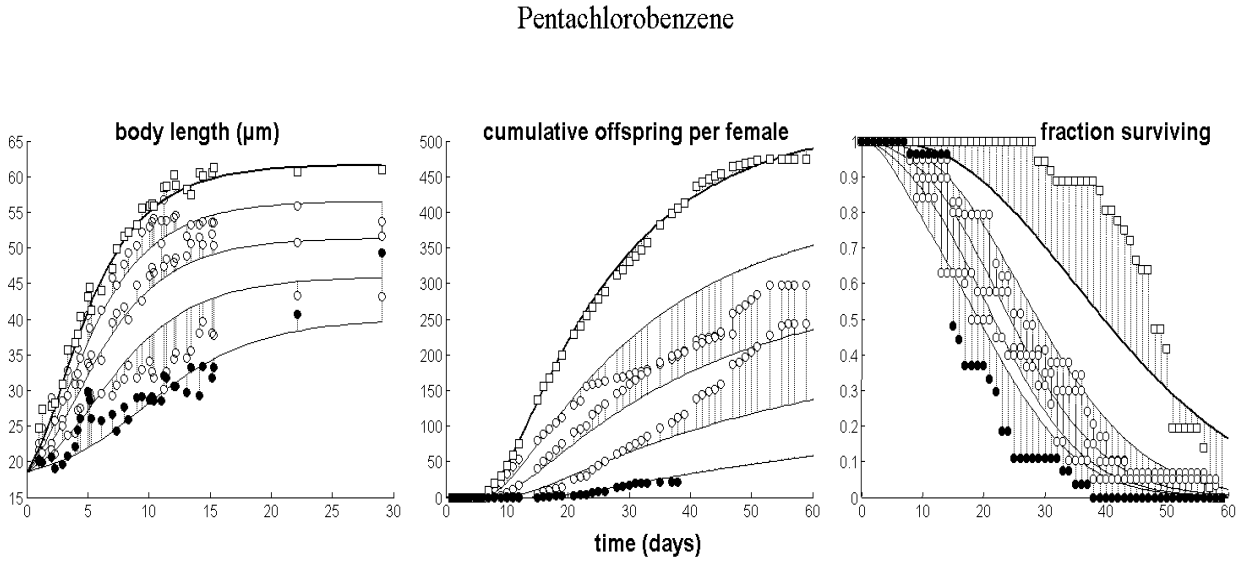

Figure 4. Data and simultaneous model fits for all endpoints (growth, cumulative reproduction and survival) of the life cycle study with *A. nanus* exposed to pentachlorobenzene. □ control, ○ concentrations 20, 40 and 60 mg PeCB/L ● 80 mg PeCB/L (highest concentration).

The model fits show how the life history traits present different response patterns to each of the selected toxicants. The response in each scenario is described by the model based on physiologically relevant parameters (Table 1), and assuming a mode of action that provides the best fit for the data (Table 2).

Table 1. Parameters used in the models. 95% likelihood based confidence intervals in brackets.

	Cadmium	**PeCB**	**Carbendazim**	
			Experiment 1	**Experiment 2**
		Animal parameters		
Von Bertalanffy growth rate (d^{-1})	0.314 (0.273-0.332)	0.271 (0.235-0.296)	0.388 (0.303-0.412)	0.421 (0.366-0.451)
Initial length (µm)	18.5 (n.e.)	18.5 (n.e.)	18.5 (n.e.)	22.9 (21.9-23.4)
Length at which ingestion is half of maximum (µm)	22.1 (21.7-22.4)	20.1 (19.3-20.5)	25.2 (24.8-25.2)	25.8 (24.4-26.6)
Length at start egg production (µm)	47.9 (46.8-48.7)	46.4 (44.9-47.5)	47.6 (46.0-48.8)	48.1 (45.7-49.5)
Maximum length (µm)	62.7 (61.8-64.1)	63.3 (62.1-65.1)	72.5 (69.6-74.4)	64.6 (63.8-66.1)
Maximum reproduction rate (eggs/d)	35.2 (32.7-37.1)	37.6 (34.4-39.8)	37.4 (35.6-39.1)	56.6 (50.1-60.9)
		Ageing parameters		
damage killing rate (10^{-3} d^{-1})	1.31 (0.859-1.87)	1.54 (1.11-2.17)	1.37 (1.02-1.75)	1.24 (0.903-1.63)
damage tolerance on reproduction [-]	18.0 (15.8-20.5)	15.9 (12.4-18.9)	13.9 (10.9-17.5)	16.6 (13.5-19.1)
		Toxicity parameters		
elimination rate (d^{-1})	0.0294 (0.0262-0.0321)	10 (n.e.)	10 (n.e.)	10 (n.e.)
NEC for survival, based on receptor occupation (-)	0.01 (n.e.)	0.01 (n.e.)	0.01 (n.e.)	0.01 (n.e.)
killing rate based on receptor occupation (d^{-1})	0.274 (0.0582-0.860))	0.179 (0.0816-0.645)	0.539 (0.179-0.832)	0.179 (0.0965-0.558)
Receptor knock-out rate (10^{-3} mg L^{-1} d^{-1})	10.3 (4.47-62.6)	0.617 (0.161-1.94)	10.3 (8.11-30.8)	28.3 (8.89-67.3)
NEC for effects on growth/repro (mg L^{-1})	$3.75 \cdot 10^{-5}$ (0-0.00423)	2.43 (0.514-4.69)	$1.70 \cdot 10^{-6}$ (0-0.0507)	$6.04 \cdot 10^{-6}$ (0-0.00392)
Tolerance concentration (mg L^{-1})	1.00 (0.187-1.91)	266 (237-286)	13.7 (12.2-15.8)	5.19 (4.40-5.87)
Decrease of length at first reproduction due to chemical stress [-]	0.192 (0.175-0.210)	0.586 (0.418-0.689)	3.24 (2.42-3.67)	0.855 (0.389-1.07)
Decrease of oxidative damage due to chemical stress [-]	n.a.	n.a.	0.622 (0-1.59)	3.82 (3.24-4.57)
Increase of oxidative damage related to costs for growth [-]	35.5 (31.7-39.5)	n.a.	n.a.	n.a.

Parameter estimates, n.a. is not applicable, n.e. is not estimated, [-] is dimensionless

Table 2. Predicted physiological mode of action of the selected compounds on *Acrobeloides nanus* and *Caenorhabditis elegans.*

Compound	**Predicted mode of action** ***Acrobeloides nanus***	**Predicted mode of action** ***Caenorhabditis elegans***
Carbendazim	Decrease in assimilation and extra effect on ROS production (slower aging)	Decrease in assimilation
Cadmium	Increase in costs for growth and extra effect on ROS production (faster aging)	Decrease in assimilation
Pentachlorobenzene	Decrease in assimilation	Increase in costs for growth and costs for reproduction with no toxic effects on survival

C. elegans modes of action are based on previous studies from Alda Álvarez (Alda Álvarez *et al.* 2005; Alda Álvarez *et al.* 2006)

For carbendazim and cadmium, a single mode of action was not sufficient to describe the time course of reproduction in the latter part of the experiment. The reproduction rate decreases with time due to ageing, presumably caused by the detrimental accumulation of oxidative damage (see Alda Álvarez *et al.* 2005). We therefore attribute the deviating behavior for these compounds to an interaction with the production of reactive oxygen species (ROS). In the case of carbendazim, the best model fits were obtained when the toxic mode of action was assumed to be a decrease in assimilation (see DEB scheme in Fig. 1) with a decrease in ROS production. For cadmium, the predicted mechanism was an increase in costs for growth with an increase in ROS production, whereas for pentachlorobenzene the fits revealed a decrease in assimilation as the mode of action of the compound.

The life history data for each of the three compounds was used to calculate the effects on the population growth rates for the different toxic concentrations (Fig. 5). The different shapes of the population growth rate curves for each compound reflect how each mode of action

impacts the population growth rate in a different way. The DEBtox model also predicts the effect of different temperatures (a common situation in the field) on the population growth rates for the different concentrations of toxicants. The modeled temperatures (25°C, 20°C and 15°C) give way to a visible reduction in the population growth rates as temperature decreases, which in combination with the increasing concentrations of toxicants lead to different patterns of effects.

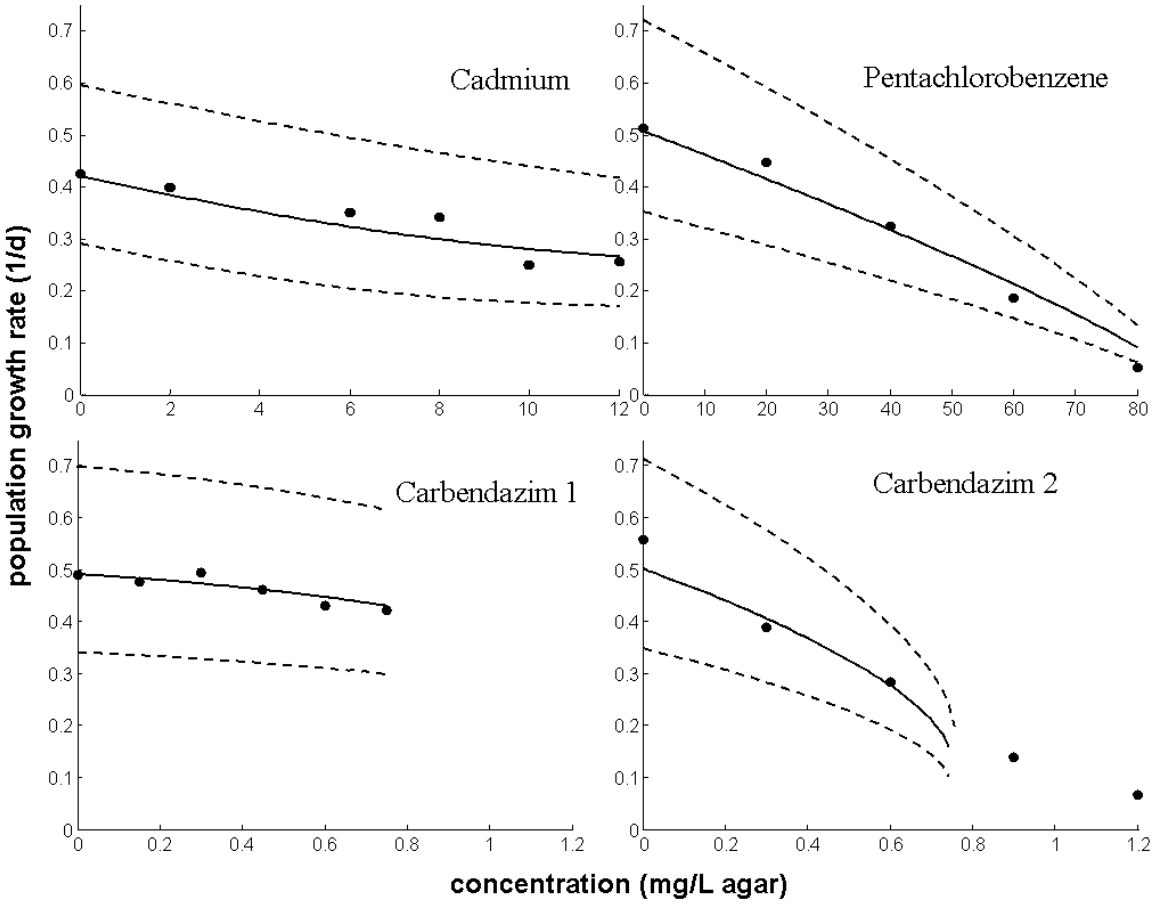

Figure 5. Modeled intrinsic rate of population increase for *A. nanus* exposed to the three compounds. Carbendazim 1 corresponds to the experiment where individuals were hatched in carbendazim, whereas Carbendazim 2 corresponds to individuals hatched in control conditions. ● population growth rates calculated directly from the life history data (at 20° C). Lines represent the DEBtox fits for the test temperature (20C, solid line), and predictions for other temperatures (25° C top and 15° C bottom broken line).

Discussion

Modes of action.

CARBENDAZIM. Two data sets were obtained for the carbendazim experiments (Fig. 2): one for individuals that actually hatched on carbendazim, and one for those that were hatched on uncontaminated agar, and subsequently transferred to contaminated medium. These two data sets were fitted separately because they clearly differ in several parameters (see Table 1).

Most interestingly, the worms hatched under uncontaminated conditions turned out to be more sensitive (a lower tolerance concentration) than the ones that successfully hatched under carbendazim exposure. At the three highest concentrations, the individuals even fail to grow and hardly reproduced (these concentrations were not included in the fit). It is unclear whether this difference results from the experimental procedure (i.e. hatching with or without toxicant), or whether these differences in sensitivity simply result from inter-experimental variation. Additionally, no difference in sensitivity was observed for mortality in the two experiments.

For this compound, it turned out to be complicated to identify an adequate mode of action. Both a decrease in assimilation as well as an increase in maintenance costs can be used to fit the data. We chose effects on assimilation since this yielded a slightly better fit, although further in-depth experimental work would be needed to settle this matter. This mode of action provides a good fit to the growth data, as well as the initial part of the reproduction curve. However, at later time steps, a peculiar pattern is observed in the second data set where animals exposed to carbendazim seem to age slower than expected. In the third concentration, ageing is almost completely stopped (Fig. 2 bottom panel) as judged from the straight line for the cumulative reproduction, even though exposure to the chemical causes mortality. A decrease in ageing related to a decrease in growth is expected (see Jager *et al.* 2004), but this effect on ageing is larger than predicted. We implemented this behavior by assuming that stress on assimilation is linked to a decrease in ROS production.

The relation between ROS production and aging has been extensively studied since the development of the Free Radical/Oxidative Stress Theory of Aging (Harmand 1956) where aerobic respiration produces free radicals that cause damage to cell components. This damage

accumulates in the cell, leading to the aging phenotype. In the case of carbendazim, the mode of action indicates a decrease in ROS production and thus a slower aging pattern. Although this explanation for the effects on aging that we observe with carbendazim remains speculative, it offers handles for further experimental testing to unravel the mechanisms at the molecular level.

CADMIUM. Figure 3 shows that the model provides a good fit to the data. The growth pattern clearly reflects the mode of action "costs for growth", presenting slower growth in increasing concentrations, but reaching a similar final body size as that of the controls. The rationale behind this mode of action is that the compound increases the energetic costs for making new tissue. This slows down the rate of growth, but not the ultimate size, which is reached when all of the assimilated resources are used for maintenance (neither assimilation nor maintenance costs per unit of body size is related to the costs for growth). The apparent recovery of the body size in Figure 3 is therefore simply a consequence of this mode of action, and is not related to a reversal of toxicity.

The effect of cadmium on body size has immediate repercussions for reproduction, reflected as a delay in the start of reproduction with increasing concentrations (although this effect is partly counteracted by a decrease in the size of first reproduction). Reproduction also starts at a slower rate since body size determines egg production. Nevertheless, this is not sufficient to explain the observed pattern of egg production in time. It appears that in the cadmium-exposed individuals, the rate of reproduction slows down in time more rapidly than expected. An adequate description of this pattern can be provided by assuming that cadmium stress additionally increases the ROS production related to growth leading to a faster aging pattern. Previous studies suggest that cadmium treatment causes lipid peroxidation (Manca *et al.* 1994) with consequential production of ROS (Zhong *et al.* 1990). Alternatively, it can replace iron from cellular binding sites to increase the free iron level and lead to iron-induced oxidations (Fenton-type reaction) (Wardeska *et al.* 1986), or increase ROS by reducing the cellular antioxidative capacity (Hussain *et al.* 1987). Indeed, an increase in hydrogen peroxide, superoxide anion, and hydroxyl radicals after cadmium exposure has been reported in several studies (Hassoun & Stohs 1996; O'Brien & Salacinski 1998; Oya *et al.* 1986).

PENTACHLOROBENZENE. Figure 4 shows that the model provides good fits for the growth data, but reproduction seems to be more variable in the exposed individuals (although

these deviations occur when most of the animals have already died). Survival data show a less satisfactory fit with a control pattern that is very different from that observed in the other two compounds, however, the reason for these deviations remains unclear.

The mode of action “assimilation” shows that exposure to pentachlorobenzene apparently decreases the energy intake into the organism (which also holds for carbendazim as previously mentioned). This can be achieved either by decreasing the feeding rate, or decreasing the efficiency with which the food is assimilated into the reserves (Kooijman & Bedaux 1996). The last assumption is difficult to test for, but previous experiments with the nematode *Caenorhabditis elegans* reported feeding inhibition in exposure to sublethal concentrations of cadmium (Jones & Candido 1999) which gave way to a decrease in assimilation when modeled with DEBtox (Alda Álvarez *et al*. 2005), supporting the first assumption as a possible explanation. This mode of action is characterised by a decreased growth rate and a lower ultimate body size. As stated earlier, growth stops when all of the assimilated energy is used for maintenance. A lower assimilation rate thus restricts the body size that can be achieved.

Population level effects. The intrinsic rate of population increase integrates the time course of effects on all endpoints into an ecologically relevant parameter (Forbes & Calow 1999), which bears a direct relation with the protection goals of environmental risk assessment (populations, ecosystems). Toxic compounds will exert their effect at different levels, but essentially we are interested in how these effects are reflected on the populations. The dissection of the mode of action of different compounds allows for the extrapolation into population level effects. Depending on the physiological mode of action of the compound, the effects on the population growth rates differ (Fig. 5). At this level, the effects of ROS production, and its interaction with the toxicant, have little relevance, since the early offspring contribute most to the population growth rate.

In the case of cadmium, an increase in the costs for growth as concentration increases leads to a gradual decrease of the population growth rate which seems to slow down in the higher concentrations. On the other hand, the decrease in assimilation caused by pentachlorobenzene and carbendazim (experiment 2) leads to a faster decrease of the population growth rates which tends to become more severe in the higher concentrations. In the first carbendazim experiment, where the individuals were hatched in exposure to the toxicant, there seems to be

a less sensitive response to the compound than that observed in the second experiment. A possible explanation is that either hatching in carbendazim selects the less sensitive individuals, or causes a change in the energy metabolism pathways.

When temperature effects are incorporated into the simulations, a substantial decrease in the population growth rates as temperature decreases is visible in the four scenarios. However, the interactions between temperature and toxicity differ in each case. In the case of cadmium, the differences between the population growth rates in each temperature are maintained throughout the concentrations of toxicant. However, with pentachlorobenzene and carbendazim the effects of temperature are smaller with increasing concentrations, as can be observed from the proximity of the curves (this is most clearly visible in the second carbendazim experiment). Such simulations are of interest since different temperature regimes that can be found in field conditions can severely affect the way populations respond to a toxic stress.

Interactions with life history characteristics. Previous studies using DEBtox have been carried out on another nematode species, *Caenorhabditis elegans*, which has a different life history strategy than that of *Acrobeloides nanus*. *A. nanus* is a parthenogenetic species, where the ovum develops into a new individual without fertilization. The reproductive period lasts about 40 days (at 20° C) during which they produce a total progeny of 400-500 (Jager *et al.* 2005). The average life span is of 50-60 days, falling into the category of intermediate life span nematodes. On the other hand**,** *C. elegans* is a facultative self-fertilizing hermaphrodite which can also reproduce sexually in the presence of males. Males occur at very low frequencies in natural populations (Ward & Carrel 1979), and hermaphroditic reproduction is therefore the most common. This mode of reproduction implies that one individual produces both male and female gametes to self-fertilize. In *C. elegans*, this yields a total progeny of about 300 in a reproductive period that lasts 5.5 days (15° C) (Alda Álvarez *et al*. 2005). These nematodes present short life spans of about 20 days at 15° C.

In *C. elegans*, cadmium was reported to act through a decrease in assimilation (Alda Álvarez *et al*. 2005) as was the case for carbendazim, and pentachlorobenzene presented a combination of costs for growth and costs for reproduction, with no toxic effects on survival (Alda Álvarez *et al.* 2006) (Table 2). These modes of action are therefore different to those that we find in *A. nanus*, except for the case of carbendazim which acts through a decrease in

assimilation in both species (although showing and extra effect on ROS production in *A. nanus*). This indicates that the life history characteristics of each organism have a clear influence on a chemical's physiological mode of action. This has important consequences for structure-based classifications of chemical modes of action which do not consider these interactions. As an example, pentachlorobenzene is generally classified as a narcotic but appears to exert its toxic effects on energy allocation through different mechanisms depending on the life history strategy of the organism. We have shown that different physiological modes of action impact population growth rates in different ways. It is therefore of great importance to understand the details of the mechanisms through which a compound exerts toxicity, as this will allow for more accurate predictions of the population level effects of different compounds and will therefore provide an important tool for risk assessment. Risk assessment approaches that rely on a strict chemical class perspective for the analysis of toxic stressors could benefit from evolving towards the incorporation of methodologies which are more consistent with actual resulting mechanisms of action.

Acknowledgments

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Chapter 5

Genetical genomics reveals gene network plasticity induced by environmental changes in *C. elegans*

This chapter is in preparation for submission as:

Li, Y.; Alda Álvarez, O.; Gutteling, E.W.; Fu, J.; Riksen, J.A.G.; Prins, P.; Breitling, R.; Jansen, R.C.; Kammenga, J.E. Genetical genomics reveals gene network plasticity induced by environmental changes in *C. elegans*.

Abstract

Recent genetical genomics studies have successfully provided intimate views on gene regulatory networks (Brem *et al*. 2002; Schadt *et al*. 2003; Stranger *et al*. 2005). Gene expression variations between genetically different individuals were mapped to the causal regulatory regions (termed expression quantitative trait loci or eQTL). Detecting these heritable variations in gene expression is important because they are the raw material for evolution (Whitehead & Crawford 2006). Although environmental change is one of the most important drivers of evolution, the manifestation of global gene regulatory networks in changing environments is still unknown. Here we show that a temperature change from 16°C to 24°C strongly affects the gene regulatory network in *C. elegans*. No less than 59% of 308 *trans*-regulated genes showed a significant eQTL-by-environment interaction. A prominent "trans-band" of co-regulated cell signaling genes appeared at 24°C. Only 8% of an estimated 188 *cis*-regulated genes showed interaction with the environment, indicating that these are less relevant for the plastic change of the network topology. Our results suggest extensive gene regulatory network plasticity in response to environmental impacts, which is central for ecological adaptation and reveal traces of evolution past. This exquisite sensitivity of gene regulation in changing environments is key to understanding the evolution of complex phenotypes, including maladaptive traits like life-style-dependent disease susceptibility.

Introduction

Expression QTLs (eQTLs) are polymorphic loci causing heritable differences in mRNA concentration and represent the raw material on which evolutionary forces act. These eQTLs therefore represent the qualitative and quantitative variations in the regulatory network – traces of evolution past (Whitehead & Crawford 2006). Selective advantages of a certain mRNA level will lead to enrichment of one of the forms of a gene (alleles) at the polymorphic regulatory locus underlying the expression difference. Thus a polymorphism that is observed between separate populations may itself be the result of a previous selection episode that favored one or the other allele. It has been shown that intraspecific evolution of variations in transcript abundance is generally dominated by intense stabilizing selection (Denver *et al*. 2005), which conserves or maintains a well-adapted fitness balance among the alleles that exist in a given population. The adaptive value of any eQTL allele should show its favorable effects selectively in certain environments without disrupting the existing adaptation to other conditions. This "genotype-by-environment" interaction of the eQTL is the prerequisite for adaptive evolution in a fluctuating environment (Levins 2004)**.** Recent studies have shown that over half of the regulatory connections in a gene expression network are unique for conditions such as cell cycle, sporulation, DNA damage, and stress response (Luscombe *et al.* 2004). We hypothesize that eQTLs will also vary among environmental conditions leading to quantitatively and qualitatively modified gene networks, termed here *gene network plasticity*. *C. elegans* lives under environmental conditions that vary drastically on a broad range of temporal and spatial scales. It is therefore particularly suited for a genetical genomics examination of major hypotheses resulting from the idea of gene regulatory network plasticity.

We used a set of 80 recombinant inbred (RI) lines generated from a cross of N2 and CB4856 (=HA8), representing two genetic and ecological extremes of *C. elegans*. The former is a solitary worm, isolated from mushroom compost in Bristol (average daily temperature range 6~14°C, strongly seasonal), the latter shows gregarious clumping behavior and was first collected in a pineapple field in Hawaii (daily temperature range 20~28°C, tropical) (Denver *et al.* 2003). Their genetic distance amounts to about one polymorphism per 873 base pairs (Wicks *et al*. 2001). We have exposed the recombinant inbred strains (RI) strains to 16°C and

24°C, temperatures which are known to strongly affect phenotypic characteristics such as body size, life span and reproduction (Gutteling *et al*. 2006). Gene expression patterns were assessed by oligonucleotide microarray hybridization (Genisphere) using a distant pair design (Fu & Jansen 2006). The genetic architecture of the 80 RI strains and the description of a dense single nucleotide polymorphism (SNP) map can be found in the work from Gutteling (Gutteling, 2004).

Schematic examples of eQTL, temperature and eQTL-by-temperature interaction effect are shown in Figure 1a-c, respectively. We used a two-step procedure to detect eQTL. Firstly we applied a separate eQTL analysis for the expression data at either temperature (Methods). With a genome-wide significance threshold of 4.25 (corresponding to an effective *p*-value of 0.001) there are 186 transcripts with significant eQTL effect at 16°C and 279 at 24°C respectively from separate analysis (42 of these are common for both temperatures), suggesting eQTLs vary significantly between environmental conditions. Secondly, using the eQTL positions from the separate analyses, we performed a sensitive search in a joint statistical analysis of data from both temperatures (see Materials and Methods for details).

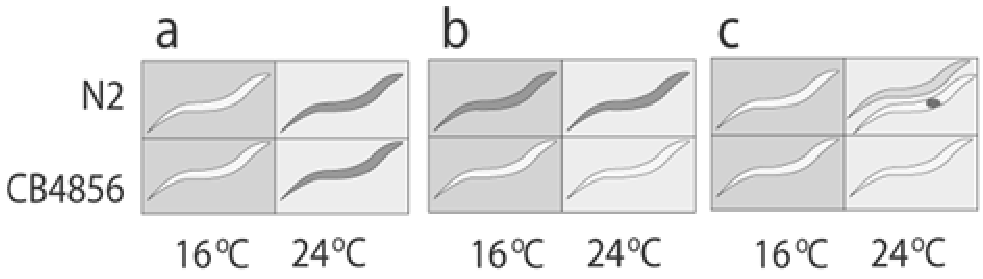

Figure 1. Illustration of temperature, eQTL and eQTL-by-temperature interaction effects. Genotype (N2 and CB4856) and temperature (16°C and 24°C) are two factors that might induce differential expression for transcripts. The colors of the animals correspond to the different gene expression levels. A Transcript with differential expression induced by temperature. The transcript is over-expressed at 24°C independent of the genotype. B Transcript with strong eQTL effect. At both temperatures worms with N2 genotype at a locus of interest show higher expression. C Transcript with eQTL-by-temperature interaction effect. At 16°C, transcripts show low expression in both genotypes. At 24°C, only one allele (e.g. N2, as shown here) shows a strong induction of gene expression. If this up-regulation is restricted to a specific tissue (the lower worm), it will be diluted in the total body when average of expression is measured (the upper worm). Other possible patterns of eQTL-by-temperature interaction can easily be conceived based on this example.

We found 308 transcripts showing a significant trans-eQTL effect (effective $p<0.001$), and 182 of these (59%) showed a significant eQTL-by-temperature interaction (or eQTL*T) effect (Figure 2), indicating that a drastic change in environment leads to a major rearrangement of the regulatory network.

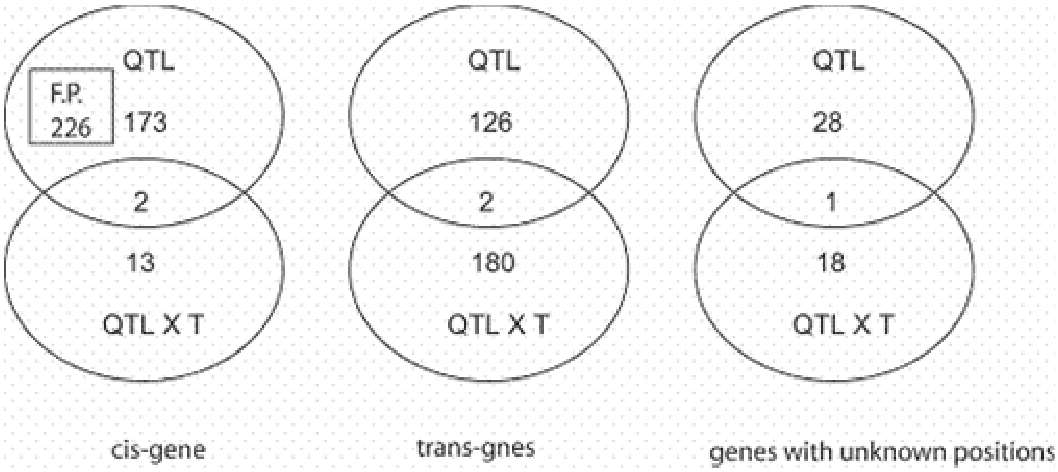

Figure 2. Venn diagram of joint analysis result. The figures indicate the number of transcripts detected with significant cis- and trans-eQTL or interaction effect ($p<0.001$ with FDR of 0.08 after multiple testing correction.) in a full ANOVA model (see Materials and Methods for details).

That the temperature shift is indeed as drastic as expected is confirmed by the major differential gene expression observed between the two temperatures (Figure 3a). We note that compared to the temperature-induced differential expression, the difference of expression level between two genotypes is usually relatively small (Figure 3b). Correspondingly, the interaction effect between genotype and temperature is also relatively small. This justifies the use of our powerful two-stage statistical analysis outlined above (Figure 3c).

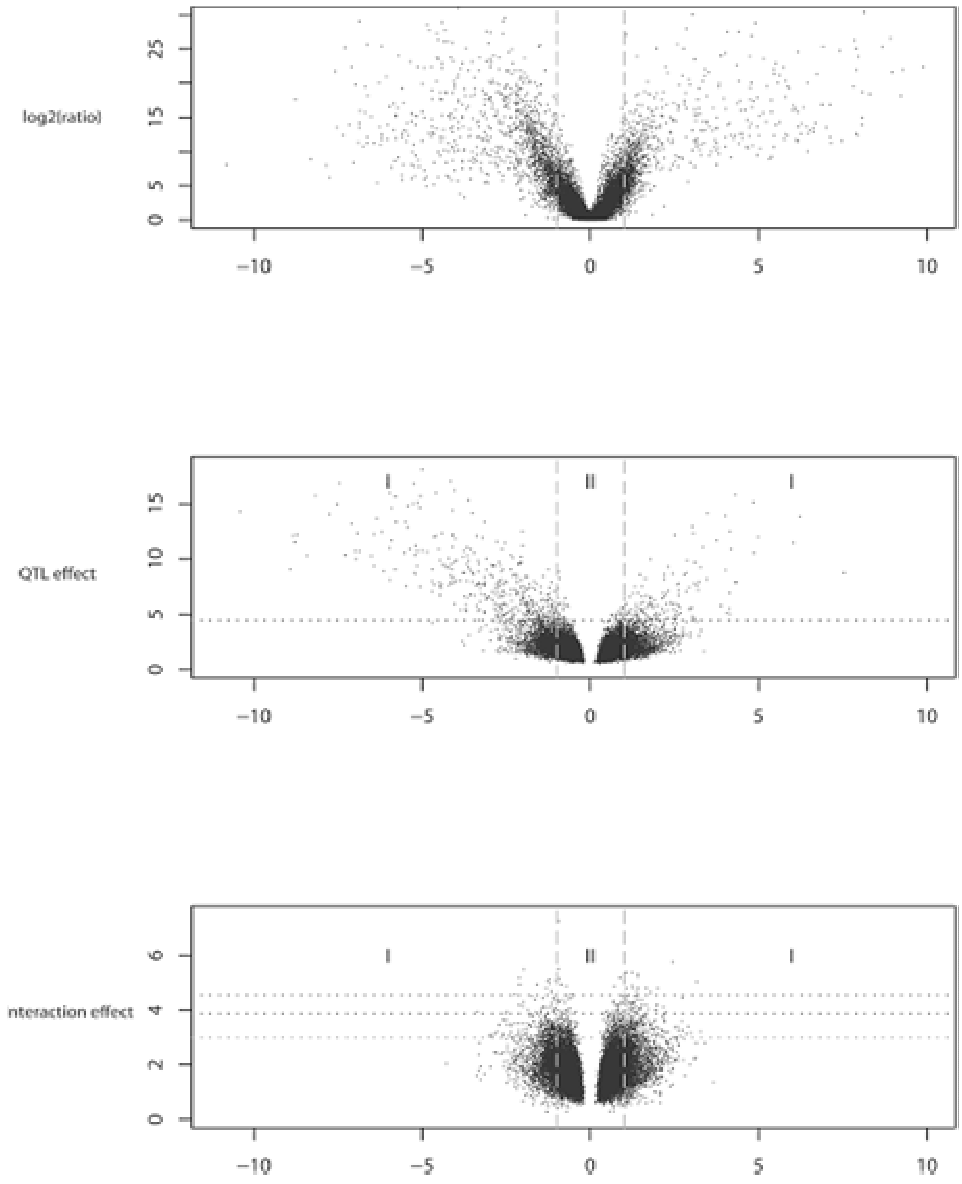

Figure 3. a: Volcano plot for all transcripts where p-value of wilcoxon test is plotted against logged ratio of intensity at both temperatures. **b, c**: Volcano plots for QTL and Interaction effect. The QTL (b) and interaction (b) effects for each gene are plotted on the x axes and –log10P values for QTL and interaction from full model are plotted on the y axes. The vertical dashed lines correspond to 2-fold change in expression. Genes with interaction effect that are considered both statistically significant and biologically large are located in region I. Genes with statistically significant but small eQTL effect are in region II. The horizontal dotted line represents the significance thresholds for QTL (b) or interaction(c) effect. **b**: The green dotted line corresponds to threshold of 4.5 for single-locus QTL search, while blue line indicates threshold of 4.41 for two-loci QTL search; c, the black horizontal line represents the genomewide threshold (4.56) for interaction. The green dotted line corresponds to threshold of 2.98 for single-locus QTL*T search, while blue line indicates threshold of 3.86 for two-loci QTL*T search.

The most prominent case of genotype-by-environment interaction in our dataset is that a group of 66 genes map to the same genomic region and 64 of them have strong eQTLs only at 24°C (Figure 4). Such a temperature-specific "trans-band" (TB) seems extremely unlikely, both statistically (p«0.001, hypergeometric test) and biologically, because it has been demonstrated recently that natural selection generally eliminates most mutations in loci that affect many downstream gene expression levels (Denver *et al.* 2005). In order to test that the TB is not an artifact we applied a permutation test (see Materials and Methods). The results show that the TB does have a strong and significant genetic component (p«0.0001). In addition to three miRNA genes in this region (cel-mir-48, cel-mir-241 and cel-mir-257), potential master regulators for the transband genes are listed in Appendix A. Additional analysis of the partial correlation coefficients (see Materials and Methods) shows that the TB genes are only partly controlled by the master regulator at the *cis* position. This suggests that these genes are involved in the same pathway and controlled by a number of shared upstream factors. In fact, the TB genes form a conspicuous biological unit according to a gene ontology analysis (Maere *et al*. 2005), with enrichments in signal transduction (p = 0.03 after multiple testing correction) and cell communication (p = 0.04, after multiple testing correction). The expression patterns of TB genes are also significantly correlated in a new independent data set (Kim *et al.* 2001) (Kimbig) as compared with randomly selected genes (one-way Kolmogorov-Smirnov test, p«0.001). It is particularly interesting to see that the group of 66 TB members contains one gene for an FMRFamide-related neuropeptide (*flp-9*) and four for G-protein coupled receptors (C17H11.1, C48C5.1, C24B5.1, K10C8.2), all of them uncharacterized (Fisher's exact test, p = 0.02). Expression variations of neuropeptides of the FMRFamide-related group (*flp-1* (Nelson *et al*. 1998); *flp-18* and *-21* (Rogers *et al*. 2003)) as well as single amino acid mutations of their G-protein coupled receptor (Denver *et al*. 2003) (*npr-1*) underlie important ecological and behavioral differences among *C. elegans* strains described so far. It is therefore tempting to speculate that the TB regulator occurred in two different alleles in the pedigree of the two parental populations (N2 and CB4856) because it controls an adaptive phenotypic difference in response to particular thermal conditions.

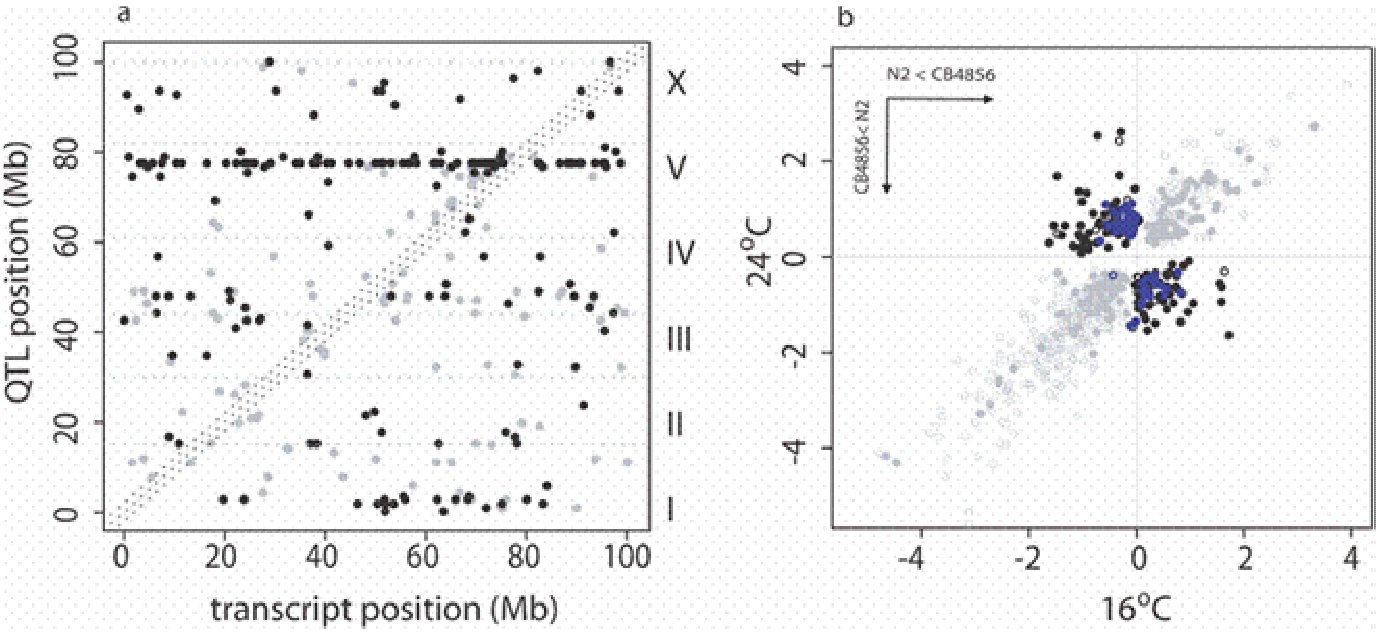

Figure 4. a: Comparisons of eQTL and transcript positions for *trans*-genes with significant eQTL and interaction effect. The grey dotted horizontal lines separate the genome into different chromosome. •: *trans*-acting transcripts with significant eQTL effect by joint analysis; •: *trans*-acting transcripts with significant eQTL*T effect by joint analysis. Among the transcripts with significant eQTL effect at both temperatures, a majority (72%) is *cis*-acting (not included in the plot), while most of the transcripts (85%) with interaction effect are *trans*-acting. A horizontal trans-band was observed at 77.56Mb (chromosome *V*) by joint analysis.
b: Comparison of eQTL effect for transcripts at two temperatures; open circles indicate *cis*-acting transcripts; grey • and black • circles are used for genes with significant eQTL and with significant eQTL*T effect, respectively, but *trans*-genes in the 77.56Mb transband are colored blue.

In addition to the *trans*-acting eQTLs, which are the primary focus of the present paper, previous studies have also reported numerous *cis*-acting eQTLs. However, as shown in Figure 2, in our data there is a surprisingly high proportion of *cis*-acting genes that show a negative eQTL effect (p =1e-9, Fisher's exact test). One likely explanation is the confounding effect of SNPs on array hybridization. Under the assumption that true *cis*-regulated genes with positive- and negative-eQTL effect should occur in equal proportions, we estimate that there are about 226 false positives among the *cis*-acting genes. (402 *cis*-regulated genes minus twice the number of 88 *cis*-regulated genes with positive eQTL effect) Following Hughes et al. (Hughes *et al*. 2001), we estimate that, on average, a single mismatch or indel in the 10 nucleotides most 5' in our 60mer probes would result in a significant detectable hybridization

difference (around 40% decreased signal). The parental strains, N2 (for which the arrays were designed) and CB4856, differ in their genome sequence up to one per 873 bp of aligned sequence. Thus we estimate the number of genes with one influential SNP is 238, which corresponds closely to the 226 false positives estimated above. This indicates that *cis* effects are not only less relevant for the gene network plasticity (topology of the gene regulatory network), but also very prone to hybridization artifacts.

The global gene network plasticity detected in the present study is even more striking when we consider that our approach is likely to underestimate the extent of genotype and environmental effects. This underestimation is due to the fact that these effects have been diluted by measuring the average abundance of transcripts from all cells of *C. elegans* (Figure1c); it is hard to detect a *large* eQTL or eQTL-by-environment effect if these effects actually are cell type specific. We noticed a surprisingly low proportion of regulatory connections seem to respond differentially to the major environmental change in the two genotypes. To check that this is not due to our stringent threshold, which might lead to false negatives, we estimated the detection power of interaction for various QTL effect sizes using simulation (see Materials and Methods). We detected 98% of interactions if the QTL effect is larger than 1 and has opposite signs at the two temperatures, which corresponds to the interaction effect of 2 (Materials and Methods). This suggests that our detection power is more than sufficient.

Our results demonstrate that the topology and tuning of gene networks is exquisitely sensitive to environmental conditions. To further study ecological adaptation and reveal traces of the evolution paste, future studies will have to take this gene network plasticity into account across different cell types, a wider range of environmental perturbations and a larger range of ecotypes.

Materials and Methods

Culturing. All nematodes were reared on NGM agar plates seeded with OP50 strain of *Escherichia coli* as food source. Stock cultures of OP50 were stored at -80°C and the bacterial cultures were grown in autoclaved LB medium (10 gr peptone, 10 gr yeast extract, 5 gr NaCl /

L water) for 16 hours at 37°C and 150 rpm. Populations were started with only non-mated hermaphrodites and screened regularly to remove any occurring males.

Synchronization. Experiments were carried out with nematodes belonging to the L3 life stage. In order to determine the entry into this stage at 16° C and 24° C, the size of the gonads and vulva were monitored. At 72 hours of age, nematodes kept at 16° C were at the L3 stage, whereas 40 hours of age determined this life stage at 24° C. Populations of each of the RILs were bleached (0.5 M NaOH, 1% hypochlorite) in order to collect synchronized eggs, which were then inoculated into fresh dishes. 4 replicate dishes of synchronized eggs for each RIL were kept in each of the two temperatures until L3 was reached. The nematodes were then collected and frozen in liquid nitrogen.

Probe construction and hybridization. The parental N2 and CB4856 strains differ in their genome sequence up to one per 873 bp of aligned sequence (Wicks *et al*. 2001). Koch *et al* reported that 85% of the SNPs were found in non-coding DNA (Koch *et al*. 2000). In an attempt to minimize hybridization difference based on SNPs, 60mer oligonucleotide microarrays were used in this study. The frozen nematode samples were ground and RNA was extracted using the Trizol method, and cleaned up with RNeasy Micro kit (Qiagen). RNA concentration and quality was measured with Nano Drop. cDNA was obtained using Array 900 HS kit (Genisphere) and Superscript II (Invitrogen). The cDNA samples were hybridized to 60-mere oligo arrays (Washington University) using the Array 900 HS protocol (Genisphere).

Pairwise design. We adopted a novel distant pair design for the microarray experiments, which was proposed specially for genetic studies on gene expression (Fu & Jansen 2006). In this design, 80 RILs are hybridized directly in 40 arrays.

Data Analysis. The expression data of two temperatures were first analyzed separately by the following ANOVA model (Fu & Jansen 2006):

$$y_i = \mu + Q_i + e_i$$

where y_i is the gene's log ratio at the *i*th microarray; μ is the mean; Q_i is $-\beta$, 0, β, for arrays comparing *A*/B, *A*/*A* or *B*/*B*, and *B*/*A*, respectively; β is the effect of differential allele expression between *A* and *B* at a regulatory locus (or nearby marker) under study; letters *A* and *B* correspond to N2 and CB4856, respectively; and e_{ij} is the residual error (see (Fu & Jansen 2006) for details).

Then expression data of two temperatures are combined together and analyzed by a full ANOVA model including T and eQTL*T effects:

$$y_{ij} = \mu + \mathrm{Q}_i + \mathrm{T}_j + (\mathrm{QT})_{ij} + e_{ij}$$

where y_{ij} is the gene's log ratio at the *i*th microarray ($i = 1,\ldots n$) and *j*th temperature; T_j is the temperature effect for *j*th temperature; $(\mathrm{QT})_{ij}$ is the interaction effect between the *i*th eQTL genotype and *j*th temperature, and e_{ij} is the residual error. To increase the power of detecting interaction, we not only did a genome-wide linkage analysis, but also reduced the multiple testing issue by focusing on those three marker positions that show a maximum QTL in either the full model or one of the two single temperature models. Interaction was assessed at these three positions using appropriately relaxed significance thresholds determined by simulation (SI Methods). The same strategy was applied for detecting significant eQTL effect.

Determination of genome-wide significance thresholds. To calculate the genome-wide threshold for separate analysis, we performed the following five steps. (1) We simulated trait data by randomly sampling from a standard normal distribution (with zero mean and unit variance) for 1000 times under the null-hypothesis of no QTL. We did this for 16°C and 24°C. (2) We carried out a single marker analysis for all 1000 runs mimicking 16°C, and then for the 1000 run mimicking 24°C. (3) At each marker, we obtained the corresponding $-\log_{10} p$. (4) We took the maximum over all markers, and stored this value. (5) These values were ordered from low to high over all 1000 runs and their $100(1-\alpha)$ percentile was the estimated critical value (genome-wide threshold).

For the joint analysis the threshold can be obtained in a similar way. After simulating the trait data under the null hypothesis of no QTL for two temperatures, the joint analysis was applied to the combined data of 16°C and 24°C. Then the genome-wide threshold for QTL and interaction was obtained at significant *p* value of 0.001. With the same simulated data, we calculated the $-\log_{10} p$ of interaction effect at *SL* position or *TL* positions and stored these values respectively. At the significance level of 0.001, the thresholds for single locus and two-locus analysis can be obtained. The same strategy was applied for QTL effect.

In our analysis, we set the genome-wide α to be 0.001 at 16°C, 24°C, as well as in the joint analysis. This implies that – with 20,490 transcripts – we expect only 0.001 x 20,490 ≈ 20.5 false positives. The threshold of 4.25 was obtained for the separate analyses at both

temperatures. For the joint analysis, the genome-wide threshold for QTL and is 4.50 and single-locus threshold is 4.41. For interaction effect, the genomewide threshold is 4.56 while the single-locus threshold and two-locus threshold are 2.98 and 3.88 respectively.

Two-stage search for interaction To increase the power of detecting interaction, we not only did a genomewide linkage analysis, but also reduced the multiple testing issue by focusing on those three marker positions that show a maximum QTL in either the full model or one of the two single temperature models. At the strongest QTL genome position *SL* (single locus), the corresponding QTL*T effect for each transcript was judged to be significant or not. As we expect the interaction for one gene happens at the positions with QTL at either T, we focus on the strongest QTL(obtained by separate analysis) genome positions for each transcript at 16° C and 24° C, which named *TL*(two loci, one locus per temperature). At the *TL*, we judged the QTL*T effect obtained by joint analysis are significant or not. The threshold were obtained by simulation (SI Methods). A gene is claimed to have significant interaction effect if it passes the threshold at one of three positions (*SL* and *TL*). The same strategy was applied for detecting significant QTL effect.

Co-expression of transband genes in Kimbig data set. The experiments in the Kimbig data set compare RNA between mutant and wild-type strains or between worms grown under different conditions. The data set consists of expression of 19738 genes in 553 experiments. 56 out of 66 of our transband (TB) genes are found in the Kimbig dataset. We calculated all pairwise Pearson correlation coefficients among these 56 genes. Then we randomly chose the same number genes from Kimbig dataset 10,000 times and calculated the correlation coefficients of each pair of them. The resulting distribution is compared with that among the original TB genes by a one-way Kolmogorov-Smirnov test.(p value « 10^{-10})

Permutation test for the transband. We used the real gene expressions of transband genes (i.e. the structure of correlation is kept unchanged), but reassigned different genomes to the different TB randomly to disturb the association between trait and genotype. From 10,000 permutations, the maximum genome-wide number of QTL for each permutation is stored and 99.9 percentile corresponding to 6 was obtained. The results show that the TB does have a strong and significant genetic component (p«0.0001).

***Cis*-factor test for transband.** Pearson correlation coefficients (zero order) were first calculated for the trait data of transband genes at 24°C. Then first order partial correlation

coefficients conditioning on the genotype of the transband position (marker 97th) were calculated according to the following formula:

$$r_{x,y|z} = \frac{r_{xy} - r_{xz} r_{yz}}{\sqrt{(1-r_{xz}^2)(1-r_{yz}^2)}}$$

where r_{xy}, r_{xz} and r_{yz} are the Pearson correlation coefficient of gene expression between x and y, x and z, and y and z, respectively. We simulated random trait data for the same number of genes in TB and calculated corresponding zero and first order correlation coefficients.

Power of detection for interaction by full model Compared with the total number of transcripts, only about 0.8% of 23,000 genes had a detectable interaction effect, i.e. a surprisingly low proportion of regulatory connections seem to respond differentially to the major environmental change in the two genotypes. To check that this is not due to our stringent threshold, which might lead to false negatives, we estimated the detection power of interaction for various QTL effect sizes using simulation. We simulated the expression data for 1000 genes with QTL effect size of B but opposite sign at two temperatures. Then the strategy of searching for interaction effect used in real data was applied for the simulated data. The detected proportion of genes with significant interaction effect indicates the power of our two-stage search method. With varying B between 0 and 5 with interval 0.25, power of detection for interaction can be estimated. We detected 98% of interactions if the QTL effect is larger than 1 and has opposite signs at the two temperatures, which corresponds to the interaction effect of 2 (SI methods). This suggests that our detection power is more than sufficient.

Master regulator for transband searching There are 66 genes with significant interaction effect at 77.56 Mb (chromosome V). It is likely there is a *cis*-acting master regulator at the transband. We first averaged the interaction profiles for the transband genes and then took 1.5 drop-off to obtain genome region (75.91~79.33Mb) as searching region. There are totally 819 potential candidates with physical location in this region in our dataset. We divided them into different groups according to their QTL and interaction effect and their annotation (see Appendix A). The top candidates might be the *cis*-acting genes with significant interaction effect, e.g. Y75B12B.3 and *cis*-acting genes with significant QTL effect, e.g. nhr-54 and nhr-116 involved in transcription factor activity.

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Appendix A

	gene WITH annotation		gene WITHOUT annotation	
genes WITH cis_QTL at transband	Num=18	F36D3.2 F09C6.9 C29F3.6 T06E6.3 W08G11.4 F08E10.3 F11A5.12 F21H7.4 K06B4.4 F26D2.10 T10H4.11 Y102A5B.3 T09F5.9 Y102A5C.17 Y102A5C.16 T27C5.7 F57A10.2 R12G8.2	Num=25	C47E8.8 W06A7.5 T09F5.11 C29F3.7 T26H5.4 K06B4.3 Y6E2A.8 T06C12.10 F35E8.11 F44G3.10 F21H7.1 T13F3.6 F57E7.1 Y102A5C.1 Y102A5C.3 ZK218.4 ZK218.8 T19C9.8 F47H4.9 F47H4.10 F47H4.2 Y20C6A.1 C38D9.9 Y6G8.2 F59A1.8
gene with cis-Interaction effect at transband	/			Y75B12B.3
gene with both cis-QTL and cis-Interaction effect at transband	/			T09F5.10
gene without statistically significant QTL or Interaction effect at transband	Num=451	Y49A3A.5 F56A12.1 F56A12.2 C15H11.1 C15H11.2 C15H11.3 C15H11.6	Num=323	Y49A3A.3 Y49A3A.4 F23B12.9 F23B12.3 F23B12.4 F08H9.2 F08H9.3

C15H11.5
C15H11.4
C15H11.7
C15H11.8
C15H11.9
F23B12.1
F23B12.5
F23B12.6
F23B12.7
F23B12.8
F08H9.1
F08H9.6
F08H9.5
F08H9.7
F08H9.8
C53A5.2
C53A5.1
C53A5.3
C53A5.4
C53A5.5
C53A5.6
C53A5.9
C53A5.11
Y40H4A.2
R11H6.1
R11H6.2
R11H6.3
R11H6.5
F43D2.1
C54G10.2
C54G10.3
C54G10.4a
C54G10.4b
C47E8.3
C47E8.5
C47E8.4
C47E8.7
R08A2.3
R08A2.2
Y50E8A.2
Y50E8A.4a
Y50E8A.4b
Y50E8A.5
Y50E8A.6
Y50E8A.7
Y50E8A.16
C48G7.3
F08H9.4
R02D5.7
R02D5.3
R02D5.4
R02D5.1
R02D5.6
C53A5.13
C53A5.8
C53A5.10
R11H6.4
F43D2.4
F43D2.3
F43D2.2
C54G10.1
C47E8.1
C47E8.6
R08A2.5
R08A2.4
R08A2.1
Y50E8A.1
Y50E8A.3
Y50E8A.8
Y50E8A.15
Y50E8A.14
Y50E8A.9
Y50E8A.10
Y50E8A.11
Y50E8A.12
C48G7.1
W06A7.2
W06A7.4
ZC412.4
ZC412.3
ZC412.5
ZC412.6
ZC412.9
H12D21.2
H12D21.5
H12D21.6
H12D21.10
H12D21.9
W09D12.2
F02D8.1
F02D8.2
F02D8.3
T01C3.1
T01C3.2

C48G7.2	T01C3.5
W06A7.3a	T01C3.8
W06A7.3b	T01C3.9
W06A7.3c	F14H8.2
ZC412.1	F14H8.5
ZC412.2	F14H8.4
H12D21.4	C25D7.4
H12D21.7	C25D7.5
H12D21.8	C25D7.9
W09D12.1	C25D7.8
C30G7.1	C25D7.10
F02D8.4	C01G10.11a
T01C3.3	C01G10.11b
T01C3.4	C01G10.8
T01C3.6	C01G10.6
T01C3.7	C01G10.5
T01C3.10	C01G10.4
F14H8.1	C01G10.3
F14H8.6	C01G10.2
C25D7.1	C01G10.13
C25D7.2	T01G5.4
C25D7.3	K08F9.2
C25D7.6	K08F9.4
C25D7.7	T09F5.2
C01G10.12	Y75B12B.1
C01G10.10	M01B2.4
C01G10.9	M01B2.3
C01G10.7	M01B2.8
C01G10.1	M01B2.10
C01G10.14	T10H4.4
T01G5.7	T26H8.3
T01G5.6	ZK1037.3
T01G5.1	ZK1037.6
T01G5.2	C29F3.3
T01G5.3	R08H2.8
K08F9.1	T06E6.11
K08F9.3	T06E6.10
T09F5.1	T06E6.13
T09F5.5	Y36E3A.1
T09F5.7	T23F1.5
T09F5.3	T23F1.6
T09F5.8	C06B8.2a
Y75B12B.2	C06B8.2b
Y75B12B.4	F21A3.3
Y75B12B.5	F21A3.4
Y75B12B.6	F28F8.4
Y75B12B.7	F28F8.5

C41G6.11	F28F8.7
C41G6.14	B0391.10
C41G6.10	B0391.9
C41G6.9	B0391.5
C41G6.6	C55A1.9
C41G6.7	C55A1.6
C41G6.8	C55A1.8
C41G6.1	F53E4.1
C41G6.3	K06B4.9
C41G6.15	Y6E2A.4
C41G6.5	Y6E2A.5
M01B2.6	Y6E2A.7
M01B2.1	T23D5.3
M01B2.9	F57A10.4
M01B2.7	T26E4.2
T10H4.3	T26E4.5
T10H4.5	T26E4.4
T10H4.6	T26E4.7
T10H4.8	T26E4.10
T10H4.9	T26E4.9
T10H4.10	F54B8.1
T10H4.2	F54B8.3
T10H4.12	F54B8.4
T26H8.2	F54B8.5
ZK1037.1	F54B8.8
ZK1037.4	F54B8.9
ZK1037.5	F54B8.10
ZK1037.7	F54B8.12
ZK1037.8	F54B8.11
ZK1037.9	K08G2.7
ZK1037.10	T06C12.4
C29F3.2	T06C12.12
C29F3.5	T06C12.14
C29F3.4	F35E8.1
C29F3.1	F35E8.2
R08H2.9	F35E8.6
R08H2.10	F35E8.7
R08H2.7	F35E8.8
R08H2.5	F35E8.9
R08H2.4	F35E8.10
R08H2.1	T05G11.2
R08H2.2	F36G9.3
R08H2.13	F36G9.7
R08H2.3	F36G9.6
T06E6.1	F36G9.5
T06E6.2b	F36G9.14
T06E6.4	F36G9.13

T06E6.5	F36G9.15
T06E6.7	C06C6.6
T06E6.6	C06C6.7
T06E6.8	C06C6.9
T06E6.9	C06C6.8
T26H5.3	T10C6.4
T23F1.3	T10C6.5
T23F1.4	T10C6.7
T23F1.7a	T10C6.9
T23F1.7b	T10C6.10
C06B8.1	F14H3.3
C06B8.3	F14H3.5
C06B8.4	F14H3.6
C06B8.6	F14H3.9
C06B8.7	F22B8.4
C06B8.8	F22B8.7
F21A3.1	F44G3.6
F21A3.2	F44G3.8
F21A3.5	F10A3.1
F21A3.6	F10A3.2
F28F8.1	F10A3.3
F28F8.2	F10A3.4
F28F8.3	F10A3.11
F28F8.6	K05D4.3
B0391.11	F11A5.6
B0391.4	F11A5.13
B0391.3	F21H7.2
C55A1.1	F21H7.12
C55A1.3	F21H7.3
C55A1.5	T13F3.4
K06B4.1	T13F3.5
K06B4.2	F28G4.2
K06B4.5	F28G4.4
K06B4.7	F28G4.3
K06B4.6	C31A11.3
K06B4.8	C31A11.4
K06B4.10	F57G8.7
K06B4.11	W08G11.1
K06B4.12	W08G11.3
Y6E2A.1	F13A7.7
Y6E2A.2	F13A7.9
Y6E2A.6	F13A7.11
T23D5.2	F26D2.13
T23D5.1	F26D2.14
T23D5.6	T08G3.6
T23D5.7	T08G3.8
T23D5.8	T08G3.11

T23D5.10	F57E7.2
T23D5.9	Y32B12A.4
T23D5.11	F36D3.1
F57A10.1	F36D3.3
F57A10.3	F36D3.8
F57A10.5	Y32B12B.1
T26E4.1	Y32B12B.2
T26E4.3	Y32B12B.3
T26E4.8	Y32B12B.4
T26E4.11	T03E6.8
T26E4.12	Y32B12C.3
T26E4.14	Y70C5A.2
T26E4.15	W06G6.2
F54B8.2	W06G6.13
F54B8.6	W06G6.6
K08G2.5	W06G6.7
T06C12.2	W06G6.8
T06C12.3	W06G6.9
T06C12.6	W06G6.10
T06C12.7	W06G6.11
T06C12.8	W06G6.12
T06C12.9	Y70C5B.1
T06C12.11	F14F8.8
T06C12.13	F14F8.3
T06C12.1	F14F8.4
F35E8.12	Y70C5C.5
T05G11.3	T25E12.11
T05G11.4	T25E12.6
T05G11.6	T25E12.4b
H24D24.2	W06H3.1
H24D24.1	T20B3.14
F36G9.1	T20B3.2
F36G9.2	T20B3.8
F36G9.9	F49A5.6
F36G9.8	F09C6.1
F36G9.11	F09C6.2
F36G9.12	F09C6.6
C06C6.5a	F09C6.10
C06C6.5b	Y102A5C.2
C06C6.4	Y102A5C.4
C06C6.3	Y102A5C.9
C06C6.2	Y102A5C.10
T10C6.2	Y102A5C.12
T10C6.1	Y102A5C.13
T10C6.3	Y102A5C.19
T10C6.6a	Y102A5C.23
T10C6.6b	Y102A5C.25

T10C6.11	Y102A5C.27
T10C6.13	Y102A5C.33
ZK285.1	F49H6.3
F14H3.2	F49H6.12
F14H3.4	F49H6.8
F14H3.7	F49H6.11
F14H3.8	F28B1.1
F14H3.10	F28B1.2
F14H3.11	F28B1.3
F14H3.1	F28B1.4
F14H3.12	F28B1.5
F22B8.1	T05E12.2
F22B8.3	T05E12.6
F22B8.5	ZK218.1
F22B8.6	ZK218.3
F44G3.2	ZK218.5
F44G3.5	ZK218.7
F44G3.11	ZK218.11
F44G3.1	Y102A5D.1
F44G3.9	F40D4.13
F44G3.7	F40D4.12
F10A3.9	F40D4.7
F10A3.8	F40D4.6
F10A3.7	Y68A4A.2
F10A3.6	Y68A4A.5
F10A3.5	T19C9.1
F10A3.12	T19C9.6
F10A3.13	Y68A4B.3
F10A3.15	K10G4.3
K05D4.4	K10G4.1
K05D4.2	K10G4.4
K05D4.6	K10G4.5
F11A5.1	Y61B8B.2
F11A5.2	F31E9.6
F11A5.3	F31E9.5
F11A5.4	F31E9.1
F11A5.5	F31E9.3
F11A5.7	F31E9.4
F11A5.8	F47H4.4
F11A5.9	F47H4.6
F11A5.10	F47H4.7
F21H7.5	F47H4.8
F21H7.7	F47H4.11
F21H7.11	Y20C6A.2
F21H7.9	Y20C6A.3
T13F3.2	T27C5.2
T13F3.3	T27C5.8

T13F3.1
F28G4.1
F28G4.5
C31A11.5
C31A11.6
C31A11.7
C31A11.9
F57G8.1
F57G8.8
F57G8.3
F57G8.4
F57G8.5
F13A7.3
F13A7.1
F13A7.8
F13A7.10
F13A7.2
F26D2.2
F26D2.4
F26D2.7
F26D2.9
F26D2.11
F26D2.1
F26D2.12
T08G3.4
T08G3.5
T08G3.2
T08G3.1
T08G3.3
T08G3.7
T08G3.10
F57E7.3
Y32B12A.1
Y32B12A.3
F36D3.6
F36D3.5
F36D3.4
F36D3.10
F36D3.9
Y32B12B.6
Y32B12B.5
T03E6.1
T03E6.3
T03E6.2
T03E6.4
T03E6.6
T03E6.5

F20E11.1
F20E11.5
F20E11.6
F08E10.2
K03D7.9
K03D7.8
K03D7.7
C18D4.1
C18D4.9
C18D4.4
C18D4.3
C18D4.6a
C18D4.6b
C18D4.8
C38D9.7
C38D9.6
C38D9.1
C38D9.4
Y6G8.1
F57G4.1
F57G4.2
F57G4.4
F57G4.5
F57G4.6
F57G4.9
F57G4.8
F59A1.7
F59A1.10
F59A1.11
F59A1.12
F59A1.6
Y26G10.1
W06D12.6
W06D12.1

T03E6.7
Y32B12C.1
Y32B12C.2
W06G6.3
F14F8.9
F14F8.1
F14F8.6
F14F8.7
F14F8.5
Y70C5C.1
Y70C5C.2
Y70C5C.3
Y70C5C.4
T25E12.10
T25E12.8
T25E12.9
T25E12.7
T25E12.5
T25E12.4a
W06H3.2
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T20B3.5
T20B3.4
T20B3.3
T20B3.7
T20B3.13
T20B3.12
Y102A5B.2
F49A5.2
F49A5.3
F49A5.4
F49A5.7
F49A5.8
F09C6.5
F09C6.4
F09C6.7
F09C6.8
Y102A5C.7
Y102A5C.8
Y102A5C.14
Y102A5C.15
Y102A5C.18
Y102A5C.21
Y102A5C.22
Y102A5C.24
Y102A5C.28

Y102A5C.29
Y102A5C.31
Y102A5C.32
F49H6.1
F49H6.2
F49H6.4
F49H6.5
F49H6.7
F28B1.6
T05E12.1
T05E12.3
T05E12.4
ZK218.6
F40D4.11
F40D4.8
F40D4.5
F40D4.1
F40D4.2
F40D4.3
Y68A4A.3
Y68A4A.6
Y68A4A.9
Y68A4A.7
T19C9.4
T19C9.3
T19C9.2
Y68A4B.2
Y68A4B.1
Y61B8A.1
Y61B8A.2
K10G4.2
Y61B8B.1
F31E9.2
F47H4.1
T27C5.1
T27C5.5
F20E11.2
F20E11.12
F20E11.10
F20E11.4
F20E11.7
F08E10.1
F08E10.6
F08E10.7
K03D7.6
K03D7.4
K03D7.2

K03D7.11
C18D4.2
C38D9.8
C38D9.2
C38D9.3
Y6G8.3
F59A1.9
F59A1.4
F59A1.3
F59A1.13
Y26G10.2
W06D12.7
W06D12.5
W06D12.4
W06D12.3
W06D12.2
R12G8.1
C47A10.1
C47A10.2
C47A10.3
C47A10.4

Chapter 6

Concluding remarks and future perspectives

A fundamental understanding of stress mechanisms is central to developing new rationales for dealing with environmental problems. Although much effort has been put into assessing the effects of the presence of different abiotic stressors in our ecosystems, there is still a need for a better understanding of the underlying mechanisms of stress and stress response. In the past years, studies on toxic stress have been predominantly focused on simplistic and descriptive models, driven by the need of producing fast and inexpensive laboratory tests for the evaluation of large numbers of compounds. The most common approach for the analysis of these tests involves producing descriptive parameters such as EC50s and LC50s; a methodology that has disregarded to a great extent the underlying mechanisms. In addition, these parameters are associated to certain limitations and simplifications. The study of the temporal dynamics of the ECx for different endpoints (Chapter 2) reveals that the behavior in time of this parameter is endpoint specific as well as influenced by the characteristics of the compound and organism of study. Surprisingly, the temporal dynamics of this widely used toxicity descriptor have hardly been touched upon in previous studies in spite of the relevance that this has for its applicability. Our observations suggest that any use of such summary statistic must be interpreted with caution as it only gives a very limited indication of sensitivity to a compound and the comparability between values obtained for different endpoints and time points is hampered. These studies lead us to conclude that the correct interpretation and comparability of toxicity data would greatly benefit from the incorporation of ECx or LCx *versus* time curves, or alternatively focusing on parameters that do not suffer from these problems (i.e. NEC, population growth rate).

Another issue that was already brought up by Stark (Stark *et al.* 2004) is the general assumption that values generated for one species can be directly comparable to those of other species. This is however not the case, even to the extent that they might not even be comparable *within* one species if it adopts a different life history strategy. The life history strategy of an organism plays an important role in determining its performance under different environmental conditions. Each strategy implies different physiological mechanisms, which affect life history traits and represent different ways of dealing with stress. This is clearly visible in *C. elegans*, which can switch from hermaphroditic to sexual reproduction in the presence of males (Chapter 3). This change in reproductive strategy affects the way these organisms respond to a toxic stress (in this case cadmium stress). We observed that the size at

onset of reproduction in response to increasing stress behaved differently depending on the mode of reproduction, and overall, sexually reproducing individuals appeared to be more sensitive to cadmium than hermaphrodites, mainly because the mating costs increased their sensitivity. The different responses of the two life history strategies were clearly reflected at the population level where sexually reproducing individuals yielded lower populations growth rates than that of hermaphrodites. The differences in the life history strategies of organisms even within one species can therefore lead to very diverse responses. This must be taken into account, as toxicity parameters generated for a particular life history scenario might not hold for another. Life history details are therefore important parameters to be considered and incorporated into toxicity testing in order to obtain a complete picture of the stress processes that occur in ecosystems.

These observations are meant to bring awareness to the fact that there is a need to improve or consider alternative methodologies for the evaluation of toxic stressors. As management issues are becoming more complex, attention is now shifting to the use of, first of all, ecologically relevant parameters that bear more relation to the protection goals (populations and ecosystems); and secondly, offer a deeper insight into the mechanisms that govern stress. In order to address these issues, in this thesis we use a process-based model, based on the Dynamic Energy Budget (DEB) theory, to study the fluxes of energy related to physiological processes and their variation throughout the whole life cycle. The model gives insight into the physiological modes of action of toxic stressors based on resource allocation, and models the effects of these different modes of action at the population level (Chapter 4). This type of mechanistic approaches allow for a more detailed dissection of the mechanisms through which a stressor exerts its effects and the processes that take place in such a situation, consequently allowing for a more robust and comparable assessment of effects together with valid extrapolations from single species to population levels. We find that the physiological mode of action of a toxic stressor varies depending on the species of study, highlighting the importance of the interactions between the compound and the life history characteristics of the organism. Such interactions are not considered in current classifications of compounds according to their modes of action, which rely mainly on the structural characteristics of the toxicants for their classification. Although mechanistic approaches such as those used throughout this thesis generate larger amounts of data that require more complex analysis, we

believe that they will greatly increase our understanding of the processes of stress, helping to improve the strategy and design of laboratory tests in order to achieve a scientifically based assessment of effects.

Zooming in to the most fundamental level, stress can be understood in terms of the processes that take place at the genetic level. With the development of microarray technology, our knowledge of gene expression and the processes that affect it is growing rapidly. Genes whose expression is affected by different environmental conditions such as heat, salinity, drought or cold have been identified. However, unraveling the interactions between the environment and the genetic control of gene expression comprises a challenge that has only just started to be explored. The combination of gene expression profiling and QTL analysis, known as "genetical genomics" (Jansen & Nap 2001), sets the ground for addressing this issue. Our study of *C. elegans* transcriptional regulation maps (transcriptomes) at two different temperatures reveals that there are indeed differences in the genetic control of gene expression even with a temperature shift of only 8° C (Chapter 5). Many of these interactions are missed with genome-wide testing and require more powerful approaches for their detection, such as the proposed two-stage test which reveals that these interactions are clearly widespread. 28% of the detected expression QTL were temperature sensitive. This newly observed phenomenon of "*expression linkage plasticity*" is in accordance with the definition of phenotypic plasticity i.e. the range of phenotypes which can be produced by a genotype when exposed to different environments (Piglucci 2005). As an example, Paterson (Paterson *et al.* 1991) already observed that only 14% of the QTL detected in tomato where present in all the environments whereas 52% where detected in only one environment. *Drosophila melanogaster* (Vieira *et al.* 2000) and *Arabidopsis thaliana* (Stratton 1998; Rauh *et al*. 2002; Loudet *et al.* 2003) also showed different QTL for complex traits in response to varying environmental conditions. These observations are also supported by the QTL studies of Gutteling on the life history traits of *C. elegans* at two different temperatures (Gutteling 2004). It should therefore not be so striking that we find expression linkage plasticity as well, as it is a representation of the same phenomenon but at the level of transcriptional regulation. However, apart from highlighting the importance of gene-environment interactions in the determination of complex traits, the acknowledgement that environmental conditions can

influence transcriptional regulation to such an extent is in itself somewhat disconcerting. Different expression QTL can be found depending on the environmental conditions suggesting that a considerable percentage of these QTL are only relevant for those particular conditions. This might have important implications in the studies of transcriptional regulation of genes underlying complex traits. Disease related traits where temperature (i.e. fever) or other characteristic environmental factors can play a major role are generally studied in a single environment. Although the ideal situation would be to visualize the transcriptomes in a range of plausible environments, this is most of the times inconceivable due to the amount of work that it implies. The task of unraveling candidate genes for phenotypes or pathways of interest can be more complex in the light of this variation in QTL patterns. However, we should not forget that a high percentage (72%) of the detected QTL seem to be robust against temperature changes (at least within the considered range), suggesting that although part of the transcriptome (mostly *trans* QTL) seems to be flexible, another part could act as a "backbone" (highly represented by *cis* QTL) that shows consistency in the different environments.

Also intriguing is the appearance at 24° C of the *trans* band in chromosome V, suggesting the presence of a temperature sensitive regulatory locus which modulates the expression of a large number of genes. Gene ontology studies of these *trans*-regulated transcripts revealed that neuron related genes were overrepresented compared to the non-*trans* band transcripts. This could suggest an increased activity in neuronal pathways that could relate to environmental detection, but further studies would be necessary to unravel the biological function of this *trans* band. Additionally, RNAi experiments could be performed for *cis* candidate genes in that location in order to try to uncover the master regulator of the band.

As previously mentioned, future experiments in several more temperatures could provide a visualization of the variation in QTL patterns in response to a larger gradient, giving more insight into the changes that are taking place in the transcriptome.

Although microarray technology itself, as well as the statistical and bioinformatical tools for its analysis have developed immensely in the past few years and are becoming more and more robust, currently much of the work is still considered to benefit from the validation with quantitative RT-PCR. In this case, the use of RT-PCR served to visualize the different

expression of genes selected from each of the four QTLxT scenarios, and in this way validate for the first time genetical genomics data with this technique.

Within this thesis, this study presents the first confirmation of a matter that has been lingering for many years; do environmental cues affect genetic linkage of gene expression? Undoubtedly, these results provide a wealth of information that opens many doors for future research on this field which has only just started to be explored.

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Summary

The increasing presence of abiotic stress factors in ecosystems over the past few decades has become an issue of major concern. The growing awareness of the detrimental effects that processes such as climatic change or chemical contamination can have on ecosystems and the species that inhabit them has been the driving force of research that focus on environmental risk assessment approaches. These approaches are often based on naive and descriptive models, disregarding to a great extent the underlying mechanisms of stress response. In this thesis I have addressed the need for a fundamental understanding of the stress response mechanisms in order to develop new rationales for risk assessment procedures.. I focus on the mechanisms underlying the effect of different abiotic stress factors such as those imposed by toxic compounds and temperature changes on various species of nematodes. Nematodes are a relevant study object to this respect because they play an important role in biological soil processes and they can easily be studied under laboratory conditions. The study cross cuts through a range of different organizational levels; from the level of populations which holds the greatest relation to the protection goal itself (a population or ecosystem), to the most basic level of gene expression where the initial stress response takes place.

Whole life cycle toxicity tests were performed using various toxic compounds with nematodes having different life history strategies. A mechanistic model based on the Dynamic Energy Budget (DEB) theory was used to analyze the data. In Chapter 2, the data from two strains of *Caenorhabditis elegans* with different reproductive strategies (hermaphroditic and sexual), in exposure to pentachlorobenzene and carbendazim, were used for the analysis of the temporal dynamics of a widely used summary statistic in effect assessment; the EC_x (where x is a time unit). The analysis revealed that the behavior in time of this parameter depended on the life cycle trait and the characteristics of the compound thus hampering the comparability between different traits, compounds and time points. This suggests that the correct interpretation and comparability of toxicity data generated with current ECx approaches would greatly benefit from the incorporation of ECx or LCx *versus* time curves, or alternatively focusing on parameters that do not suffer from these problems (i.e. NEC, population growth rate which are time invariant). In Chapter 3 I focused on the differences in the response of diverging life history strategies to a toxic stressor (in this case cadmium), and

investigated how these differences are reflected at the level of population growth rate. The results show that certain traits respond differently to stress depending on the life history strategy (hermaphroditic or sexually reproducing *C. elegans*). Overall, sexually reproducing individuals appeared to be more sensitive to cadmium than hermaphrodites. The different responses of the two life history strategies were clearly reflected at the population level where sexually reproducing individuals yielded lower populations growth rates than that of hermaphrodites. The differences in the life history strategies of organisms even within one species can therefore lead to very diverse responses implying that toxicity parameters generated for a particular life history scenario might not hold for another. In order to gain further insight into the mechanisms of these toxic stress scenarios, in Chapter 4 another nematode species (*Acrobeloides nanus*) was used to dissect the physiological mode of action of the three previously mentioned compounds, and to model their effects at the population level. The resulting physiological modes of action of these compounds were compared to those observed in *C. elegans* and it appeared that the modes differed in every case ??? which case??. This indicates that the life history characteristics in each case had a clear influence on the resulting physiological mode of action of the compounds and consequently lead to very different effects on the population growth rates. Classifications of compounds according to their mode of action, currently based mainly on their chemical structure, should also consider that interactions with the life history characteristics of the organism may lead to different mechanisms of toxic action.

In Chapter 5 I studied the effects of temperature as the abiotic stress factor at the gene expression level. We used a 79???80 recombinant inbred line (RIL) panel derived from a *C. elegans* N2 x CB4856 cross to extract RNA from each RIL grown at 16° and 24°C and subsequently hybridized it to whole genome microarrays (approximately 22,000 transcripts). We then used QTL mapping to detect expression linkage patterns across the genome for each temperature. Using different analysis approaches (separate and combined analysis of the temperatures) we found that 28% of the detected expression QTL were temperature sensitive and comprised mainly by *trans* QTL. These QTLxT interactions were in many cases below detection level in genome wide analyses. These results present the first validation of the phenomenon of "*expression linkage plasticity*" in response to different environmental

conditions, showing that the control of gene expression is sensitive to environmental cues such as temperature changes.

Samenvatting

Gedurende de afgelopen decennia is de zorg over de invloed van abiotische stressfactoren op ecosystemen sterk toegenomen. Dit heeft geleid tot een sterke toename van het onderzoek dat zich richt op de ecologische risicoevaluatie van klimaatverandering en chemische contaminanten. Het overgrote deel van dit onderzoek is evenwel beschrijvend van aard en geeft geen inzicht in de onderliggende biologische mechanismen van de stress respons. In dit proefschrift geef ik aan waarom het van groot belang is om deze mechanismen te kennen teneinde een gefundeerde onderbouwing te geven voor nieuwe instrumenten voor de ecologische risicoanalyse van chemische contaminanten en omgevingstemperatuur. Het onderzoek richt zich op de stress respons in verschillende soorten nematoden. Dit zijn kleine wormpjes die in grote aantallen in de bodem voorkomen en hier een belengrijke rol spelen in het decompositieproces. De gekozen bacterie-etende nematodensoorten zijn goed te bestuderen onder laboratoriumomstandigheden en zijn uitermate geschikt om effecten te bestuderen op het niveau van genexpressie tot populatie.

Eerst heb ik de effecten op de populatiegroeisnelheid bestudeerd van diverse relevante toxische stoffen op nematoden met verschillende levenscycli. Hierbij is gebruik gemaakt van een mechanistisch model op basis van Dynamische Energie Budgetten (DEB). In hoofdstuk 2 worden de dynamische effecten beschreven van pentachloorbenzeen en carbendazim op twee stammen van *Caenorhabditis elegans*, ieder met een verschillende levenscyclus (sexueel reproducerend en hermafroditisch). Uit de resultaten bleek dat de wijdverbreide en algemeen geaccepteerde maat voor toxisch effect (de ECx, dat is de concentratie van een stof die leidt tot x procent effect) een sterke, en onverwachte, temporele dynamiek vertoont. Deze dynamiek was afhankelijk van de toxicant, het type levenscyclus en de eigenschap waarvoor het effect gemeten is. Geconcludeerd kan worden dat de ECx waarden tussen verschillende soorten niet zonder meer met elkaar kunnen worden vergeleken. Geadviseerd wordt om in de toekomst de risicobeoordeling te baseren op de tijdsafhankelijheid van de ECx waarden of op tijdsonafhankelijke parameters. In hoofdstuk 3 bestudeer ik de effecten van cadmium op de populatiegroeisnelheid van dezelfde twee stammen. Hieruit bleek dat de populatiegroeisnelheid van sexueel reproducerende stammen gevoeliger was dan die van hermafroditische stammen. Vervolgens zijn de effecten van de voorgaande stoffen bestudeerd

op de populatiegroeisnelheid van *Acrobeloides nanus*. De belangrijkste conclusie was dat de effecten van de verschillende stoffen op de populatiegroeisnelheid afhankelijk is van de onderliggende fysiologische relaties tussen de verschillende onderdelen van de levencyclus (reproductie, groei, ontwikkeling en levensduur).

Naast toxische stoffen kunnen organismen sterk worden beïnvloed door de omgevingstemperatuur. In hoofdstuk 5 is de invloed van temperatuur op de genexpressie van C. elegans bestudeerd. Hierbij is gebruik gemaakt van een splitsende populatie (80 nakomelingen in de vorm van homozygote inteeltlijnen) afkomstig van een kruising van de ouderlijnen N2 x CB4856. Elke nakomelingslijn is blootgesteld aan 16°C en 24°C waarna vervolgens het geïsoleerde RNA gehybridizeerd is met bekende transcripts (ca. 20.000 in totaal) op een microarray. Vervolgens is er een Quantitative Trait Loci (QTL) analyse uitgevoerd op de transcripts om de regulerende QTL voor elk transcript te identificeren bij elke temperatuur. De temperatuurverandering bleek een grote invloed te hebben op het gen regulatie netwerk in C. elegans. Bijna 60% van alle trans-gereguleerde genen vertoonden een significant “QTL-by-temperature” effect. Bij 24°C werd een significante trans-band van 66 cell signaling genen gevonden. Slechts 8% van 188 cis-gereguleerde vertoonden een significante interactie met temperatuur. Deze resultaten laten zien dat het gen regulatie netwerk sterk afhankelijk is van de omgevingstemperatuur hetgeen van groot belang is voor de genetische adaptatie aan fluctuerende temperatuursomstandigheden.

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Curriculum Vitae

Olga Alda Álvarez was born on the 21st of May 1977 in Madrid. In 1991 she moved to Canada and did her highschool studies in Lorne Park Secondary School (Toronto), after which she obtained a College degree in Veterinary assistant. She then returned to Spain, this time to Alicante. There she studied Biology from 1995 to 1999 in the Universidad San Vicente del Raspeig, specializing in environmental sciences. In the two years that followed she spent part of her time working in the field of biological pest control. In the year 2002 she was appointed at the Wageningen University as a PhD student in the project “Development of a versatile software protocol for ecotoxicological risk assessment at the population level: the missing link between single-species tests and higher level test systems”. The results obtained from this research are partly reflected in this book.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (4 credits)
- Development of a versatile software protocol for ecotoxicological risk assessment at the population level (2002)

Post-Graduate Courses (5 credits)
- DEB tox course (2002)
- Functional genomics course (2003)
- Basic and advanced statistics (2003-2004)

Deficiency, Refresh, Brush-up and General Courses (3 credits)
- QTL course (2002)
- Isotope course (2003)
- Micro array course (2004)

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- The ecology of temperature (2005)

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- SETAC Prague (2004)
- Complex trait consortium (2005)
- MGED 8 Bergen (2005)
- Ecogenomics Kansas (2005)

Laboratory Training and Working Visits (3 credits)
- Micro array technology, Plant Research International (2004)
- NOMIRACLE work visit, Barcelona (2005)

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